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Coding of tsetse repellents by olfactory sensory neurons: towards the improvement and the development of novel tsetse repellents

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DECLARATION

I declare that this thesis/dissertation, which I hereby submit for the degree Doctor of Philosophy in Bioinformatics at the University of the Western Cape:

(i) is my original research work;

(ii) has not been presented before at this University or any other University/institution for a degree award of any kind; and

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Souleymane Diallo,

06th October 2020

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ABSTRACT

Tsetse flies are the biological vectors of human and animal trypanosomiasis and hence representant medical and veterinary importance. The sense of smell plays a significant role in tsetse and its ecological interaction, such as finding blood meal source, resting, and larvicidal sites and for mating. Tsetse olfactory behaviour can be exploited for their management; however, olfactory studies in tsetse flies are still fragmentary. Here in my PhD thesis, using scanning electron microscopy, electrophysiology, behaviour, bioinformatics and molecular biology techniques, I have investigated tsetse flies (*Glossina fuscipes fuscipes*) olfaction using behaviourally well studied odorants, tsetse repellent by comparing with attractant odour. Insect olfaction is mediated by olfactory sensory neurons (OSNs), located in olfactory sensilla, which are cuticular structures exposed to the environment through pore and create a platform for chemical communication. In the sensilla shaft the dendrite of OSNs are housed, which are protected by called the sensillum lymph produced by support cells and contains a variety of olfactory proteins, including the odorant binding protein (OBP) and chemosensory proteins (CSP). While on the dendrite of OSNs are expressed olfactory receptors. In my PhD, studies I tried to decipher the sense of smell in tsetse fly. In the second chapter, I demonstrated that *G. f. fuscipes* is equipped with diverse olfactory sensilla, that various from basiconic, trichoid and coeloconic. I also demonstrated, there is shape, length, number difference between sensilla types and sexual dimorphism. There is a major difference between male and female, while male has the unique basiconic sensilla, club shaped found in the pits, which is absent from female pits. In my third chapter, I investigated the odorant receptors which are expressed on the dendrite of the olfactory sensory neurons (OSNs). *G. f. fuscipes* has 42 ORs, which were not functionally characterised. I used behaviourally well studied odorants, tsetse repellents, composed of four components blend. I demonstrated that tsetse repellent is also a strong antifeedant for both *G. pallidipes* and *G. f. fuscipes* using feeding bioassays as compared to the attractant odour, adding the value of tsetse repellent. However, the attractant odour enhanced the feeding index. Using DREAM (deorphanization of receptors based on expression alterations of mRNA levels). I found that in *G. f. fuscipes*, following a short in vivo exposure to the individual tsetse repellent component as well as an attractant volatile chemical, OSNs that respond to these compounds altered their mRNA expression in two opposite direction, significant downregulation and upregulation in their number of transcripts corresponding to the OR that they expressed and interacted with odorant. Also, I found that the odorants with opposite valence already segregate distinctly at the cellular and

molecular target at the periphery, which is the reception of odorants by OSNs, which is the basis of sophisticated olfactory behaviour. Deorphanization of ORs in none model insect is a challenge, here by combining DREAM with molecular dynamics, as docking score, physiology and homology modelling with Drosophila a well-studied model insects, I was able to predict putative receptors of the tsetse repellent components and an attractant odour. However, many ORs were neutral, showing they were not activated by the odorants, demonstrating the selectivity of the technique as well as the receptors.

In my fourth chapter, I investigated the OBPs structures and their interaction with odorants molecules. I demonstrated that OBPs are expressed both in the antenna, as well as in other tissues, such as legs. I also demonstrated that there are variations in the expression of OBPs between tissues as well as sexes. I also demonstrated that odorants induced a fast alteration in OBP mRNA expression, some odorants induced a decrease in the transcription of genes corresponding to the activated OBP and others increased the expression by many fold in OBPs in live insect, others were neutral after 5 hours of exposure. Moreover, with subsequent behavioural data showed that the behavioural response of *G. f. fuscipes* toward 1-octen-3-ol decreased significantly when 1-octen-3-ol putative OBPs were silenced with feeding of double-stranded RNA (dsRNA). In summary, our finding whereby odorant exposure affects the OBPs mRNA, their physiochemical properties and the silencing of these OBPs affected the behavioural response demonstrate that the OBPs are involved in odour detection that affect the percept of the given odorant. The expression of OBPs in olfactory tissues, antenna and their interaction with odorant and their effect on behavioural response when silenced shows their direct involvement in odour detection and reception. Furthermore, their expression in other tissues such as legs indicates they might also have role in other physiological functions, such as taste.

THESIS TITLE

Coding of tsetse repellents by olfactory sensory neurons: towards the improvement and the development of novel tsetse repellents

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Dedication

I dedicate this thesis to my family, who were the source of my energy every morning every hour, and my support in the distant depths of science.

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LIST OF ABBREVIATIONS

AAT	African Animal Trypanosomiasis
ABPX	Antennal Binding Protein X
AL	Antennal Lobe
ASP	Antennal Specific Protein
cDNA	Complementary Deoxyribonucleic Acid
CHARMM	Chemistry at HARvard Macromolecular Mechanics
Cas9	Crisper-associated protein 9
CRISPER/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats
CSP	Chemosensory Protein
DEET	N,N-Diethyl-meta-toluamide
DFT	Density Functional Theory
DoOR	Database of Odorant Responses
DNA	Deoxyribonucleic Acid
DREAM	Deorphanization of Receptors based on Expression Alterations in mRNA levels
DW	Double Walled
FFT	Fast Fourier Transform
UAS	Upstream Activation Sequence
GDP	Gross Domestic Product
GPCR	G protein-coupled receptors
GROMACS	GROningen MAchine for Chemical Simulations
GROMOS	GROningen MOlecular Simulation
HAT	Human African Trypanosomiasis
HEK	Human Embryonic Kidney
ICIPE	International Centre of Insect Physiology and Ecology

IRs	Ionotropic Receptors
LINCS	LINCS
MCDOCK	Monte Carlo Docking
MD	Molecular Dynamics
MM-PBSA	Molecular Mechanics Poisson–Boltzmann Surface
mRNA	Messenger Ribonucleic Acid
MUSCLE	MUltiple Sequence Comparison by Log-Expectation
NMR	Nuclear Magnetic Resonance
NPT	constant pressure
NVT	constant volume
OBPs	Odorant Binding Proteins
ODEs	Odorant Degrading Enzymes
ORs	Odorant Receptors
OSN	Olfactory Sensory Neuron
PAAT	Programme Against African Trypanosomiasis
PCR	Polymerase Chain Reaction
PHYRE	Protein Homology/analogY Recognition Engine
RT-qPCR	Reverse Transcriptase – quantitative Polymerase Chain Reaction
RMSD	Root-Mean-Square Deviation
SAT	Sequential Aerial Treatment
SIT	Sterile Insect Technique
SM	Shape Matching
SW	Single Walled
T&T	Tsetse and Trypanosomiasis
TEVC	Two-Electrode Voltage Clamp
WHO	World Health Organization
WRC	Waterbuck Repellent Compounds

LIST OF PUBLICATIONS

1. Cellular and Molecular Targets of Waterbuck Repellent Blend Odours in Antennae of *Glossina fuscipes fuscipes* Newstead, 1910; doi:10.3389/fncel.2020.00137.

2. Structural insights into the functional analysis of *Glossina fuscipes fuscipes* (Newstead, 1910) odorants binding proteins. Under-review

3. Morphological characterization of antennal sensilla in *Glossina fuscipes fuscipes* (Newstead, 1910). In preparation.

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Chapter One:

Introduction and literature review

1.1 Introduction

Chemoreception is a sensory process by which organisms detect and respond to external chemical stimuli (Hodgson, 1958; Pelosi, 1996; Pelosi *et al.*, 2006). Hence, chemoreception relies on chemical odorants that act as signals to induce a behavioural response through olfaction. Olfaction is a sensory modality involving the detection of volatile molecules presents in immediate environment of the organism (Pelosi *et al.*, 2014). In general, chemosensory processing mainly involves peripheral (reception of semiochemicals) and central processing (complex signal conversion and processing including translation of olfactory signals to behaviour). Currently, remarkable progress has been made to elucidate the structure and the function of both peripheral and central chemosensory processing mostly in *Drosophila melanogaster* (Dobritsa *et al.*, 2003; E. A. Hallem, Ho and Carlson, 2004; Hallem and Carlson, 2006; Wang *et al.*, 2010). In olfaction, the mechanism of chemoreception starts by trapping chemical molecules dispersed in the air by olfactory sensilla housing olfactory sensory neurons (OSNs) (Shanbhag, Müller and Steinbrecht, 1999, 2000; Su, Menuz and Carlson, 2009; Ali *et al.*, 2015; Depetris-Chauvin, Galagovsky and Grosjean, 2015). The olfactory sensilla are diverse and specialized for various chemical class of odorants, as basiconic house neurons responding to food related odours, ceoloconic senilla house neirons responding to acids and amines,and trichoid sensilla mostly houses neurons responding to pheromone (Shields and Hildebrand, 2000; van der Goes van Naters and Carlson, 2007; Ali *et al.*, 2015). The dendrite of the OSNs express olfactory receptors which detect the odorant molecules and initiate the olfactory signal processing (Leal, 2013; Missbach et al., 2014; Menuz, & Carlson, 2009). Activated olfactory receptors initiate signal transduction of the chemical information into a cascade of an electrical signal which can be interpreted by the insect's brain for appropriate behaviour (Jacquin-Joly and Merlin, 2004; De Bruyne and Baker, 2008; Pelosi *et al.*, 2014). Chemosensation is well conserved process in all living organisms, and olfaction is the most important sensory modality for organisms such as insect where it modulates their life cycle and life histories. In disease carrying insects, sense of smell mediates vector-host interaction and therefore underlies the epidemiology of several vector-borne diseases around the world (Carey and Carlson, 2011; Masiga *et al.*, 2015). Hence, understanding olfactory pathways in insects can contribute to develop or to improve

olfactory-based tools for vector control and diseases management. Since the first insect odorant receptors identification (de Bruyne, Clyne and Carlson, 1999; Vosshall *et al.*, 1999), enormous progress has been done to deorphanize olfactory receptors and to elucidate molecular basis of olfaction (de Bruyne, Foster and Carlson, 2001; E. a Hallem, Ho and Carlson, 2004; Ghaninia *et al.*, 2008; Wang *et al.*, 2010). However, deorphanization of olfactory receptors of non-model insect is still a challenge, which is the main objective of my thesis.

Tsetse flies (Diptera: Glossinidae) are the most important cyclical vectors of the various trypanosomes which are a protozoan blood parasites of the genus Trypanosoma (Deirdre P Walshe *et al.*, 2009). The trypanosomes are the causative agent of the debilitating disease called Animal African Trypanosomiasis (AAT) or nagana in livestock and Human African Trypanosomiasis (HAT) or sleeping sickness in humans (Leak, 1999; M. J. Lehane., 2005; Solano, Kaba, Ravel, Naomi A Dyer, *et al.*, 2010). In Africa, ten million km^2 areas are infested by the tsetse (Simarro *et al.*, 2010). According to World Health Organisation (WHO), 55.1 million people at continental level are at risk to be infected by the Human African Trypanosomiasis. About 10.4 million people are living in areas where HAT is still considered a public health problem (Kuzoe, F A., Schofield, 2004). However, in recent years there is a reduction in new cases of reported HAT annually (Burri, 2020; Franco *et al.*, 2020). For the Animal African Trypanosomiasis (AAT) or nagana, about 50 million cattle and 10 million of small ruminants are permanently at risk of becoming infected by the AAT (Cecchi *et al.*, 2014). Animal trypanosomiasis remains a serious problem, according to the Programme Against African Trypanosomiasis (PAAT), 3 million cattle die every year (Schofield and Kabayo, 2008; Morrison *et al.*, 2017). The overall annual losses in livestock and crop production due to nagana are estimated as high as USD 4.5 billion (Schofield and Kabayo, 2008). Therefore, tsetse and Trypanosomiasis (T&T) constitute a major constraint to livestock production and the main factor preventing the establishment of sustainable agricultural systems in sub-Saharan Africa (Krafsur, 2009; Shaw, 2009). The lack of vaccines and high costs of disease treatment associated with the development of resistance by the parasites, make disease control, via a vector management more reliable option. Currently, vector control is achieved through sequential aerial spraying (SAT), ground spraying, insecticide-treated targets or insecticide-treated animals as live baits, the use of traps and the sterile insect technique (SIT) (Takken and Weiss, 1974a; Calkins and Parker, 2005; Bouyer *et al.*, 2010). The other option for trypanosomes control is through the manipulation of the chemosensory

basis of vector behaviour toward their hosts. In this line, the International Centre of Insect Physiology and Ecology has invented an innovative repellent collar technology for the control of savanna tsetse flies and trypanosomiasis. This method reduced the drug use to 50% and the trypanosomiasis prevalence by 75% (Saini *et al.*, 2017). The new spatial repellent is a blend of chemicals cues identified from tsetse flies unpreferred hosts, waterbuck (*Kobus defasa*) (Gikonyo *et al.*, 2002, 2003). The molecules of the blend include δ-octalactone, guaiacol, geranyl acetone, and pentanoic acid (Gikonyo et al. 2003; Bett, Saini, and Hassanali 2015; Wachira et al. 2016). However, within each multi-component class of compounds (carboxylic acids, ketones, and phenols), significant variations in intrinsic individual repellency to *G. pallidipes* were found (Bett, Saini and Hassanali, 2015). Moreover, others study shown that subtle structural changes alter their activity from repellence to attraction (Benson M. Wachira *et al.*, 2016). Furthermore, the continuous use of only one type of tsetse repellents might also lead to repellent resistance flies as has been demonstrated in other insects for well-known repellent DEET (Reeder *et al.*, 2001; Klun *et al.*, 2004; Nina M. Stanczyk *et al.*, 2010). A significant challenge in finding new or to improve the tsetse attractant and repellents is that the target olfactory sensory neurons through which the attractants (Vale, 1984; Vale, Hall and Gough, 1988) and repellents (Saini *et al.*, 2017) operate are unknown. We also do not have effective attractants or repellents for many of the tsetse species, for example, riverine tsetse species, which are both medically and veterinary important vectors. However, recent progress in tsetse genomes will open opportunity to make functional characterization of *Glossina sp* olfactory receptors (George F.O. Obiero *et al.*, 2014; Watanabe J. et al., 2014; Macharia *et al.*, 2016; Attardo *et al.*, 2019). Hence, the main focus of this PhD project was to do functional characterization of tsetse olfactory receptors expressed on the olfactory sensory neurons (OSN) of *G. f. fuscipes* using ecologically relevant odorants, the tsetse repellent and attractant to predict the odorants receptors (ORs) responsible for the detection of the repellent and attractant, odorants that vary in their valence.

1.2. Literature review

1.2.1. Tsetse fly morphology, physiology, taxonomy and economic importance

1.2.1.1 Tsetse fly life cycle, morphology and physiology

Tsetse flies (genus Glossina) are bloodsucking insects that belong to Dipteran order. They are morphologically easily distinguishable by their forward-projecting piercing proboscis on the

head that is capable of puncturing skin (Vreysen *et al.*, 2013) (Figure 2.1D). Tsetse flies are robust insects, ranging from 6 to 16 mm in length (Pollock, 1982) but the males are generally smaller than females with body mass that varies according to species. Their colour ranges from brown to dark brown with a dark marking in the thorax. The body is coated with a rigid exoskeleton, the cuticle, a chitinous membrane secreted by the epidermis (Pollock, 1982). Tsetse fly wings are completely folded one on top of other at the resting position. Unlike the others dipteran species, tsetse fly female is viviparous where the eggs, the first instar and second instar larvae are developed in the uterus (Figure 1.1). This embryonic development takes a period of 10 days (Leak, 1999). The third instar larva is deposited on moist soil or sand in shaded places where it will immediately turn into a pupa. A teneral fly will emerge from the pupae 22–60 days later, depending on ecological conditions of the habitat. Adult females mate only once in their life and produce a larva every 10 days (Launois *et al.*, 2004) (Figure 1.1). Tsetse flies can live up to 100 days in the optimum field conditions.

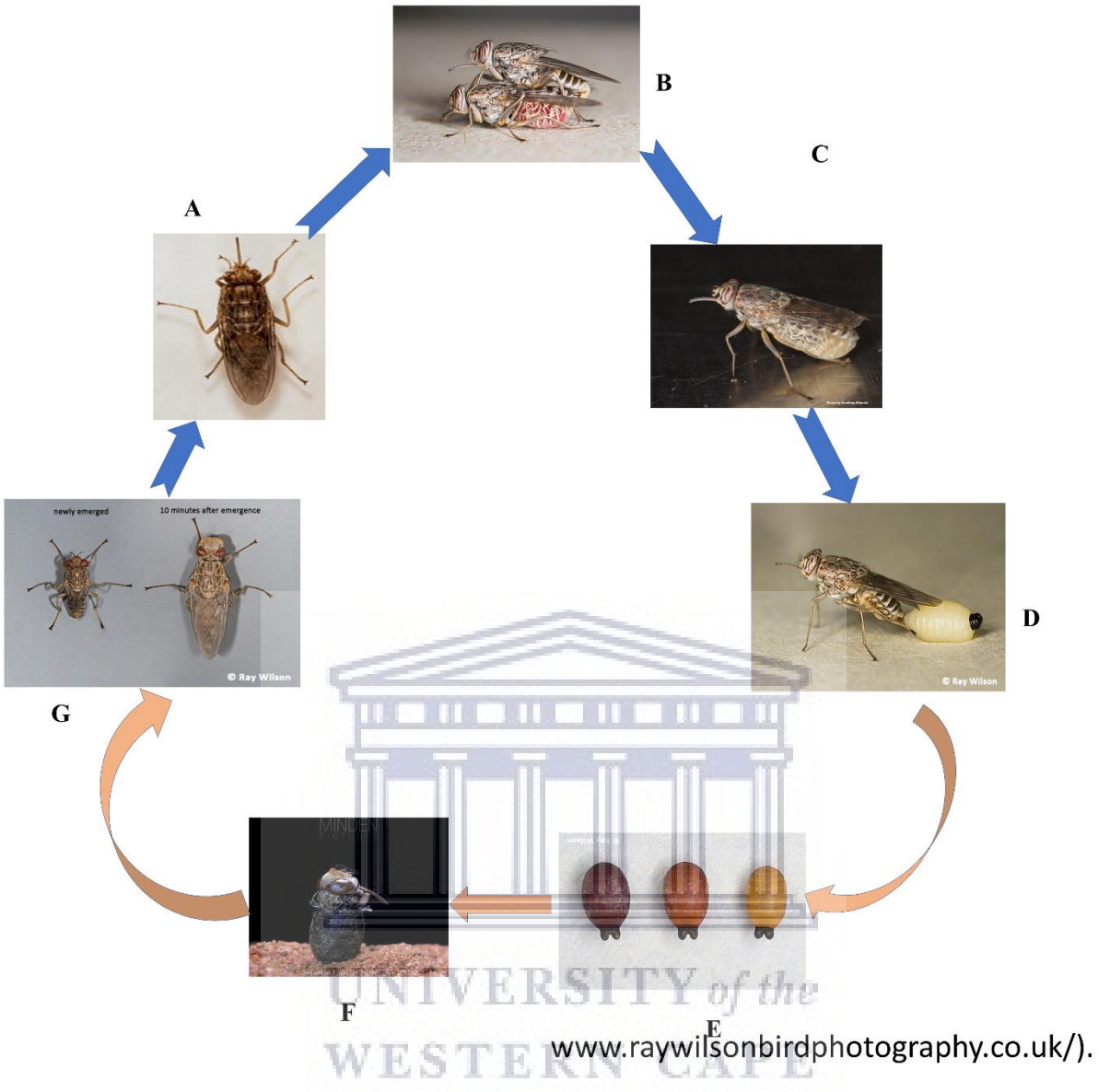

Figure 1.1 Tsetse life cycles, A-G summarise the life cycle of tsetse from adult (A) to a teneral fly (G).

1.2.1.2. Tsetse fly taxonomy and distribution

Tsetse flies are dipterans which belong to the super family of the Hippoboscoidea family *Glossinidae* and genus *Glossina* Wiedemann 1830 with 33 species and subspecies (Gooding and Kufsur, 2005). There are 31 species strictly distributed in sub-Saharan Africa (Figure 1.2). These 31 species are subdivided into three extant subgenera *Austenina* Townsend, *Nemorhina* Robineau-Desvoidy, and *Glossina* Wiedemann that correspond respectively to the *Fusca, Palpalis, and Morsitans* groups. Furthermore, tsetse flies are classified into three groups according to their morphology and their ecological habitat. The *Morsitans* flies are

found in savannah woodlands; the *Palpalis* flies are more often found in riverine vegetation and the *Fusca* flies usually found in the forested habitats(Rogers DJ, 2004; Cecchi *et al.*, 2014) (Figure 1.2).

Figure 1.2 Tsetse distribution map in Sub-Saharan Africa (Adapted from Mugenyi 2015)

1.2.1.3. Medical and veterinary importance of tsetse fly

Tsetse flies are the biological vector of a deadly protozoan parasite known as trypanosomes. The trypanosomes are responsible for parasitic disease called African Trypanosomiasis (AT) affecting humans and animals. Human African Trypanosomiasis (HAT) or sleeping sickness threatens millions of people in more than 30 countries in sub-Saharan Africa (Simarro, Jannin and Cattand, 2008). People at risk of contracting sleeping sickness live in rural areas where adequate health services are limited and difficult to access for early diagnosis and treatment of the potential cases. Control effort has considerably reduced human trypanosomiasis (Bouteille and Buguet, 2014; Burri, 2020). In 2018, only 997 new cases were reported and still estimated at over 50 million people at the risk of contracting the disease. At least 50 million of cattle are at the risk of contracting nagana in Africa. Due to the high morbidity of trypanosomiasis, approximately 35 million doses of trypanocidedrugs are used annually to treat cattle (Geerts *et al.*, 2001; Ngumbi and Silayo, 2017; Bengaly *et al.*, 2018; Raftery *et al.*, 2018). Hence, the annual losses in terms of cattle production alone exceed USD 1 billion (Schofield and

Kabayo, 2008; Shaw *et al.*, 2014; Cecchi *et al.*, 2015). Other than the disease affecting livestock and people, tsetse flies and the disease they transmit represent a major constraint for agricultural production and cause of food insecurity in the vast and fertile zone of sub-Saharan Africa (Holmes 2013;Hordofa and Haile 2017; Egeru et al. 2020). Taken together, direct and indirect economic losses due to tsetse and African trypanosomiasis are estimated at more than USD 4 billion per year in terms of agricultural Gross Domestic Product (GDP) (Schofield and Kabayo, 2008; Shaw, 2009; Holmes, 2013; Hordofa and Haile, 2017).

1.2.1.4. Tsetse and African Trypanosomiasis control: Past, present and the future directives

In the past, some of the extreme control methods such as bush clearing (tsetse habitat destruction) or elimination of wild animals (tsetse reservoir hosts) were conducted (Rogers, Hendrickx and Slingenbergh, 1994). Theses interventions were followed by extensive insecticide ground spraying (Meyer *et al.*, 2016). But those methods have been abandoned because of their ecological and environmental impacts. To date, no vaccine is available against African Trypanosomiasis, conventional parasite control measures are based on the use of trypanocides such as isometamidium chloride, diminazene aceturate and homidium (bromide and chloride) (Roderick *et al.*, 2000; Raftery *et al.*, 2018). Unfortunately, resistance to the trypanocide drugs used in cattle has been reported in some countries of sub-Saharan Africa (Geerts *et al.*, 2001), the resistant could happen from miss use of the drug such as underdose, symptom based drug administration due to lack of veterinary services in rural Sub-Saharan African countries. Thus, to manage African Trypanosomiasis vectors control plays an important role.

Pour-on (application of insecticides on cattle) or selective spraying on legs and belly where flies land to bite are another effective means of vector control saving funds and minimizing the impact of pesticide on the environment. Furthermore, residual chemicals in the animals and their products is a concern (Rowlands *et al.*, 2001). Currently, vector control is achieved through sequential aerial spraying (SAT), ground spraying, insecticide-treated targets, the use of traps (Meyer *et al.*, 2016) and the sterile insect technique (SIT) (Takken V, Oladunmade MA, Dengwat L, Feldmann HU, Onah JA, Tenabe SO, 1986; Calkins and Parker, 2005; Klassen, 2005). The other and reliable option for trypanosomes control is through the manipulation of the chemosensory basis of vector behaviour toward their hosts, such as attractant and repellent. The future directive is to identify, optimize attractants and repellents

in a push-pull system to effectively control different species of tsetse fly by investigating the various tsetse flies chemical ecology.

1.2.2. Sensory ecology of tsetse fly

In the insect, olfaction underlies behaviours that are critical for their biological needs such as mating, host recognition and selection for feeding. It is known that some species of tsetse flies feed on wide range of animals, but some species have characteristic feeding behaviours regardless of the host abundance (Weitz, 1963; Bett, Saini and Hassanali, 2015). Some authors have explained that the host choice is governed by the olfactory response to the host volatile odours (Vale, Hall and Gough, 1988; Harraca, Syed and Guerin, 2009). Furthermore, they also use the sense of smell to find their mate (Benton, 2006; Pellegrino and Nakagawa, 2009; Sachse and Krieger, 2011) as well as larvipositional sites (Saini *et al.*, 1996; Renda *et al.*, 2016). The application of odorants for behavioural control for tsetse flies has not been fully realized yet due to lack of information on the olfactory system of tsetse flies, such as the functional characterization of their odorant receptors, (sensilla types on both antenna, and maxillary palp, proboscis etc) and also due to species complexity. However, recently a number of papers published on tsetse genome (George F O Obiero *et al.*, 2014; Watanabe J. et al., 2014; Macharia *et al.*, 2016; Attardo *et al.*, 2019). In tsetse flies, morphological characterization of antennal sensilla of species from *G. m. morsitans* done by (Chahda *et al.*, 2019a) has shown several types of sensilla housed in the antennae: trichoid,a, basiconic and ceoloconic are found on the flagellum. They have also demonstrated some variability in the number of olfactory sensilla among the sexes. Indeed, we can also hypothesize that there is a variability in the number of olfactory sensilla among the species. Furthermore, diverse types of olfactory receptors and other proteins such as odorant binding proteins (OBPs) have been characterised from different tsetse species (George F.O. Obiero *et al.*, 2014; Attardo *et al.*, 2019; Liu *et al.*, 2020). However, their expression at the olfactory organs such as sensillum and their function is elusive/not known. In my chapter 2, I described the sensilla morphology, in chapter 4 the function of OBPs and in Chapter 3 the function of Odorant receptors of *G. f. fuscipes*.

1.2.3. Basic concepts in insect olfaction

In nature, insects rely on their chemical senses to drive their several key behaviours which include food source detection, potential mate location, and identification of a suitable egg-laying site and avoidance of natural enemies (Stensmyr and Hansson, 2011). The olfactory

system is well adapted to perceive, identify, evaluate and transmit complex chemical information from semiochemicals found in the environment into the brain to make important behavioural decision. However, the exact mechanisms by which odours induce instinctive behaviors are largely unknown.

Insects detect semiochemicals by olfactory receptors, which are interface between the environment and the nervous system, olfactory sensory neurons found in sensory hairs called sensilla. The sensilla house and protects neurons and several chemosensory proteins such us olfactory receptors (ORs), odorant binding proteins (OBPs), odorant degrading enzymes (ODEs), at the periphery, chemosensory proteins detect and send the signal, to brain to make behavioural decision. For behavioural decision beside olfaction other multiple sensory modalities also contribute for host location (Keesey *et al.*, 2019).

1.2.3.1. The ultra-structure of in insect olfactory sensilla

The olfactory organ of an insect is primarily located on their head. It formed by a pair of the antennae and maxillary palp. Those appendages house an innervated hair structures called sensilla which are considered as the sensory organ in most arthropod. Although, antennae and maxillary palp are considered as main olfactory organ in insect, sensory hairs can also be found on insect legs, wings and ovipositor (Van Der Goes Van Naters and Carlson, 2006; Seenivasagan *et al.*, 2009; Xia *et al.*, 2015). Olfactory organs can also bear gustatory, mechano and thermoreception sensilla (de Brito Sanchez, 2011; Raad *et al.*, 2016). The number and the distribution of the sensilla on the olfactory organ vary according to the insect species and their ecological niches (Rogers and Simpson, 1997). For example, *D. melanogaster* has around 400 sensilla housing approximately 1200 olfactory sensory neurons (OSNs) (Shanbhag, Müller and Steinbrecht, 1999, 2000; Steinbrecht, 2017) while *Manduca sexta* has around 100,000 sensilla with 250,000 OSNs (Sanes and Hildebrand, 1976). In blood-feeding insect like stable fly, over 20,000 of sensilla were counted on the antenna (Tangtrakulwanich *et al.*, 2011). A sensillum, according to its type can house many sensory neurons which largely vary among insects. The classification of sensilla is based on the external appearance under microscope as well as the wall structure as it appears in the transmission electron microscope (Shanbhag, Müller and Steinbrecht, 1999). Therefore, sensilla can be subdivided into single-walled (SW), double-walled (DW).

- Single-walled sensilla

Single-walled sensilla found in insects are: sensilla trichoid, sensilla chaetica, sensilla basiconic, sensilla placodea, sensilla ampulleaca and sensilla auricillica. Sensilla trichoid are found on the antenna, maxillary palp, legs and ovipositor of most insects. They are usually thin with a rather sharply pointed tip and measure 30-600 µm. *Sensilla trichoid* arise directly from the cuticle and don't possess a flexible socket (Steinbrecht, 1996). They are considered as olfactory sensilla if the cuticular wall is pierced by pores. Sensilla with flexible socket and without pores are considered as pure mechanoreceptors. Sensilla basiconic are often short (30-600 µm) and rounded or blunt tip. Basiconic sensilla don't have flexible socket and the cuticular wall bears numerous pores. They are considered to be exclusively olfactory sensilla. They can be divided into subtypes according to their shape and size. In drosophila there are 10 basiconic sensilla on the antenna alone, classified based on their size and shape, as for example small, large and thin basiconic sensilla (Shanbhag, Müller and Steinbrecht, 1999).

Sensilla chaetica are thick-walled aporous sensilla type. They arise from a flexible socket and are innervated by a single receptor cell. Sensilla chaetica is considered to be taste sensilla (TP) (De, Linardi and Chiarini-Garcia, 2002).

- Double-walled sensilla

They are the shortest sensilla found in insect (6-12 µm) and the olfactory sensilla with double walled are called *sensilla coeloconic*. *Sensilla coeloconic* also called grooved peg are a pyramid shape types sensilla with the wall consisting of cuticular finger-like meeting each other at the tip. Their structure appears as a double wall with pores. Additionally, they can arise from the antennal surface or stand on a small socket in some insect (Shanbhag, Müller and Steinbrecht, 1999; Seenivasagan *et al.*, 2009). Sensilla coeloconic are olfactory sensilla and they can house up to 5 sensory neurons.

1.2.4. The molecular components of olfaction in insect

1.2.4.1. Odorant binding proteins (OBPs) and chemosensory proteins

Olfactory sensillum house various number of olfactory sensory neurons (OSNs) depending to the sensilla types and the insect species. The OSNs project their dendrites into the sensillum shaft and the cell bodies of the OSNs are surrounded by three support cells (trichogen, tormogen, thecogen) (Shanbhag, Müller and Steinbrecht, 2000). The support cells serve several functions including formation of the sensillum cuticle early in development and the secretion of perireceptor proteins (CSPs, OBPs, ODEs) and signalling ions late in

development of the insect (Shanbhag, Müller and Steinbrecht, 2000; Shanbhag, Smith and Steinbrecht, 2005). The axons of OSNs converge onto the first olfactory information processing units called glomeruli in the antennal lobe (AL) of the brain. Odorant-binding proteins (OBPs) refer to a large unrelated families of polypeptides expressed in the support cells and abundantly secreted into the sensillum lymph in insects (Pelosi, 1996; Pelosi *et al.*, 2006, 2014; Fan *et al.*, 2011; Leal, 2013; Larter *et al.*, 2016). OBPs of insects contain around 130–140 residues; they are made of six α-helical barrels. They are characterised by conserved six-cysteine signature (C1-X20-35-C2-X3-C3 -X20-30-C4 -X8-12-C5-X8-C6) paired into three interlocked disulfide bridges (Graham and Davies, 2002). OBPs have been first identified in giant silk moth *Antheraea polyphemus* where it was referred pheromone binding proteins (PBP) (Vogt and Riddiford, 1981). With development and the improvement of biochemical and molecular biological studies, additional proteins belonging to OBP family were identified in several insects (Graham and Davies, 2002; Hekmat-Scafe *et al.*, 2002; Pelosi, Calvello and Ban, 2005; Xu, Cornel and Leal, 2010; Swarup, Williams and Anholt, 2011; Macharia *et al.*, 2016). Odorant binding proteins are mainly grouped into pheromone binding proteins (PBPs) and General Odorant binding proteins (GOBPs). However, specific binding proteins known as antennal binding proteins X (ABPX) (Krieger *et al.*, 1996) and antennal specific protein (ASP) (Briand *et al.*, 2002) are also ranged as odorant binding proteins. The role of OBPs in olfactory physiology is still elusive (Steinbrecht, 1996; Swarup, Williams and Anholt, 2011; Larter *et al.*, 2016; Sun, Xiao and Carlson, 2018). Traditionally, it was presumed that OBPs binds, solubilise and transport odorants to the olfactory receptors through the sensillum lymph. This hypothesis is sustained by the fact that most odorant molecules are hydrophobic and therefore should be solubilised in the aqueous sensillar lymph in order to reach the dendritic membrane. In vitro experiments have demonstrated that OBPs can bind to several odorant molecules (Wojtasek and Leal, 1999; Leal *et al.*, 2005; Oliveira *et al.*, 2018). Additionally, it has been clearly established in *Drosophila melanogaster* that LUSH binds to the pheromone cVA (Xu *et al.*, 2005; Stowers and Logan, 2008) and RNAi knockdown of OBPs in mosquitos and Drosophila reduced the sensitivity of the sensory and behavioural responses (Biessmann *et al.*, 2010; Pelletier *et al.*, 2010; J. *et al.*, 2012; Swarup *et al.*, 2014; Liu *et al.*, 2020) demonstrating their role in odour reception and perception. However, a recent study has demonstrated a robust olfactory and behavioural response of *Drosophila melanogaster* in the absence of OBPs (Xia, Sun, and Carlson 2019) suggesting that OBPs may have other additional functions, as response regulators. Others function such as protecting odorant molecules from degrading enzymes and filtering them are also proposed

(Kaissling, 2013). To sum up, OBPs exact functions are still unclear and there is great gap to fill in terms of knowledge in OBPs role in insect olfaction.

Chemosensory Proteins (CSPs) similar to OBPS but are smaller (around 100–120 residues) and present a conserved pattern of four cysteine instead of six in the OBPs. CSPs are also made of α-helical segments but folded differently from that of OBPs (Lartigue *et al.*, 2002; Tomaselli *et al.*, 2006; Liu *et al.*, 2012),their exact role in olfaction is elusive to date.

1.2.4.2. Odorant Degrading Enzymes (ODEs)

Odorant degrading enzymes (ODEs) are enzymatic proteins (esterase) found in the sensillum lymph (Leal, 2013). They are presumed to be principally responsible for the rapid degradation of the odorant molecules in the sensillum lymph (Durand *et al.*, 2011; Chertemps *et al.*, 2012; Younus *et al.*, 2014; Fraichard *et al.*, 2020). By this rapid inactivation of odorant molecules in the sensillum lymph, ODEs are vital in the maintenance of sensitivity of the olfactory system. To date they haven't been extensively studied and their specificity towards odorants molecules and their mode of action is still elusive in the literature. However, *Drosophila melanogaster* is esterase 6 (EST6) which act as an ODE was successfully shown to degrade sex and aggregation pheromone cis-vaccenyl acetate (cVA) (Chertemps *et al.*, 2012).

1.2.4.3. Olfactory receptor proteins (ORs)

Odorant receptors are membrane proteins located on the dendritic membrane of the OSNs. They have been first identified in rats by Buck and Alex in 1991 using Polymerase Chain Reaction (PCR) and described as class A rhodopsin-like family of G protein-coupled receptors (GPCRs) (Buck and Axel, 1991). The encoded proteins were named olfactory receptors (ORs) because they are expressed in olfactory sensory neurons, and later named as odorant receptors (ORs) because they are presumably recognise odorant molecules (Kato and Touhara, 2009). However, some ORs can be found in non-olfactory tissues and can also detect molecules other than odorant molecules. Although, ORs share the same name in vertebrate and in invertebrate, it is clearly established that insect ORs are very distinct from mammalian GPCRs. Insect ORs were long thought to be similar to vertebrates ORs although they differ topologically(Benton, 2006). Indeed, insect OR proteins, the N-terminus is cytoplasmic, whereas the C-terminus is extracellular while the orientation within the plasma membrane is inverted in mammals (Kaupp 2010; Kato and Touhara 2009). Additionally, insect ORs are heterodimeric constructs composed of an odour-specific receptor protein and a

ubiquitous co-receptor protein (Orco) (Mattias C Larsson *et al.*, 2004; Sargsyan, Getahun, Llanos, Shannon B Olsson, *et al.*, 2011; Bohbot and Dickens, 2012; Missbach *et al.*, 2014). Genome wide analysis has revealed large number of different odorant receptors in vertebrate as well as in invertebrate. Humans have approximately 1000 functional olfactory receptors like genes while mice got around 1400 genes (Yoshihito Niimura, 2012). The number varies considerably between organisms. For example, in insects, *Drosophila melanogaster* has 60 ORs while up to 350 ORs are found in ants. In *Glossina sp*, the number of ORs genes varies from 42 to 45 genes according to the species. However, among this large number of olfactory receptors genes, several have been characterised as pseudogenes in vertebrate as well as in invertebrate. For example in human olfactory repertoire, 600 genes considered as pseudogenes (Gilad *et al.,* 2003). Although, pseudogenes are presumed to be non-functional DNA, recent studies have questioned their none functionality, by demonstrating pseudogenes encodes a functional receptor (Prieto-Godino *et al.*, 2016). Since the identification of the first OR in *Drosophila melanogaster* (Clyne *et al.*, 1999; Vosshall *et al.*, 1999) enormous progress has been done in their functional study and their diversity across insect. The protein sequence of the ubiquitous co-receptor protein (Orco) is highly conserved among insect (Soffan *et al.*, 2018) but ORs are generally divergent and hardly share more than 20% sequence similarity between different insect at the order level (Robertson, Warr and Carlson, 2003). To date, there are consistent data demonstrating the role of ORs-Orco in olfactory signalling model insect insects such as *Drosophila melanogaster* and mosquitos. However, enormous effort needs to be done in functional understanding of ORs in many non-model insects.

1.2.4.4. Gustatory receptors

The Gustatory receptors (GRs) also called taste receptors encoding seven-transmembrane chemoreceptors expressed mostly in gustatory sensilla receptor neurons. They were first characterised in *Drosophila melanogaster* (Clyne, Warr and Carlson, 2000). Similar to ORs, they share a common amino residue motif in the seventh transmembrane plus C terminal domains (Leslie B. Vosshall and Stocker 2007). They can function as monomer (Touhara, 2009; Sato, Tanaka and Touhara, 2011; Sato, 2012) or as an obligate heteromers of two or more receptors. GRs are also found on several organs such as wing and legs (Seada *et al.*, 2018). Recent study by (Dweck and Carlson, 2020) demonstrated a complex taste odour identification and how co-expressed up to four GRs interact to shape the response (Dweck and Carlson, 2020). However, Unlike ORs, GRs functions have yet to be extensively studied.

1.2.4.5. Ionotropic receptors (IRs)

Ionotropic receptors (IRs) are receptors homologous to ionotropic glutamate receptors, they are localized in coeloconic sensilla and often found in arista and in the sacculus (Sato, 2012). IRs are highly conserved three trans-membrane proteins expressed with co-receptors Ir8a and Ir25a (Benton *et al.*, 2009; Rytz, Croset and Benton, 2013; Abuin *et al.*, 2019) and responding to compounds such as acids and amines (Silbering et al., 2011; Prieto-Godino et al. 2017).

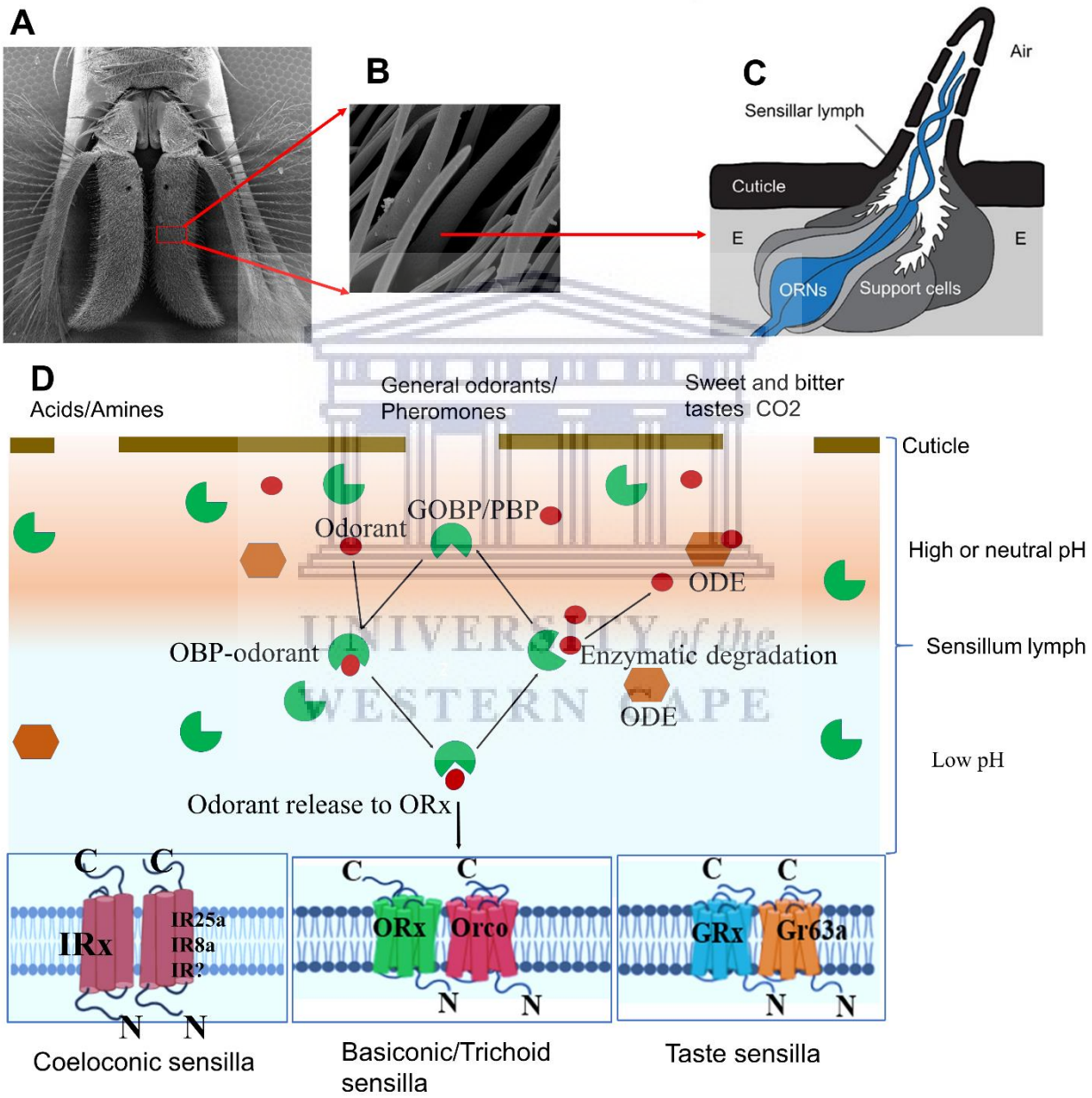

Figure 1.3 Brief Physiology of the olfaction in insect. A. antenna of tsetse fly (original photo), B. Olfactory sensilla of tsetse fly (original photo), C. Anatomy of olfactory sensillum adapted from (Shanbhag, Müller and Steinbrecht, 1999; Larter *et al.*, 2016), D. Olfactory

processing in insect and different chemosensory proteins and their putative roles, adapted from (Suh, Bohbot and Zwiebel, 2014).

1.2.5 Chemosensory signal transduction

Chemosensory transduction is the process by which chemical stimuli are detected and converted into internal signals that elicit changes in cellular membrane properties. Chemosensory transduction takes place within sensory neurons and involves multistep mechanism by which a cascade of biochemical events that convert extracellular signals into cellular response (Stengl, 1992; Pelosi *et al.*, 2006). The transduction starts by the adsorption of an odour molecule by sensilla on the antenna followed the penetration of the molecule through pores in to the sensillum lymph (Pellegrino and Nakagawa, 2009; Rospars *et al.*, 2010; Suh, Bohbot and Zwiebel, 2014; Stengl and Stengl, 2019). The molecules are the then transported to sensory nerve endings called dendrites where odorant receptors are located. It has been long time proposed that OBPs bind to the hydrophobic odorant and transports them through the sensillum lymph to the odorant receptors. But recent findings have shown OBPs are multifunctional ,they contribute to sensitivity as well as OBPS might get involved in the response regulation (Xu *et al.*, 2005; Stowers and Logan, 2008; Sun, Xiao and Carlson, 2018; Xiao, Sun and Carlson, 2019). The next olfactory protein involved in the signal conversion is odorant receptor. The interaction between odorant molecule and olfactory receptor is transduced into sequence of action potentials which is transmitted to the antennal lobe (AL) via sensory neurons axon (Figure 1.4). The signal transduction pathway, involves activation, termination, adaptation (Nakagawa and Vosshall, 2009; Ronderos and Smith, 2009; Fleischer and Krieger, 2018), however there is no consensus about the mechanism of each step of the process. In insect olfactory neurons, two major signal transduction pathways involved: Ionotropic and metabotropic pathway. In ionotropic signal transduction, the receptor functions both as receptor and ion channel, which is fast but less sensitive (Sato *et al.*, 2008; Wicher *et al.*, 2008, 2009). Metabotropic signal involves secondary messengers (Wicher *et al.*, 2008). However, insect ORs is sensitive can detect one molecule, thus the integration of inotropic and metabotropic signalling make it fast at the same time sensitive (Wicher *et al.*, 2008; Getahun *et al.*, 2016).

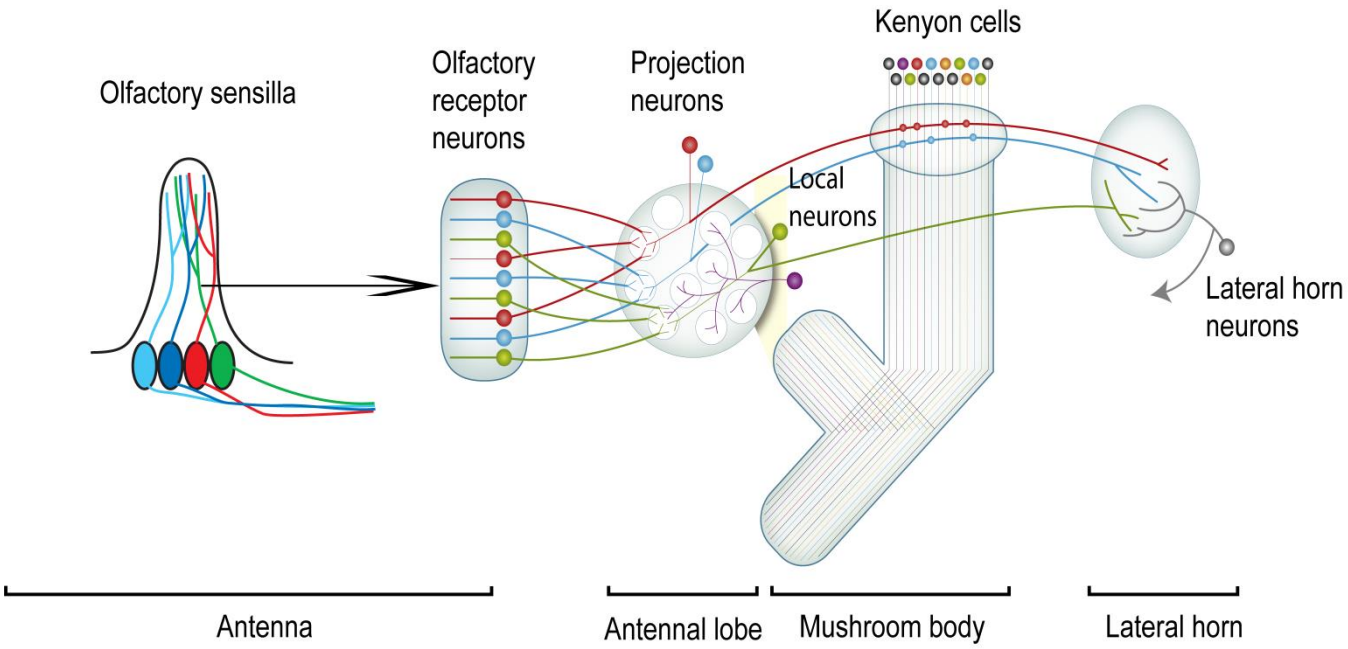

Figure 1.4. Schematic representation olfactory information processing in insect (*Drosophila melanogaster*) adapted from (Masse, Turner and Jefferis, 2009)

1.2.6. The olfactory code in insect

1.2.6.1. Odorant mixtures perception

The chemical environment is composed of thousands of different compounds emitted from various organisms in the environment of various chemical classes, which are detected by olfactory system in multiple ways. Both the chemical and receptors varies in their selectivity, some odours may activate a selective receptor and some receptors might be specific, that make chemical communication complex. Odour information gathered by multiple receptors are processed through multiple receptor-glomerular connection in the antennal lobe, which is the first order olfactory center, then it will be further processed, in the higher brain centers to make a decision by the target insect (Galizia and Rössler 2010, Seki et al., 2017). The perception of tsetse repellent (four components) result in antifeedant and repellence is not clear. The WRB blend elicited a robust antifeedant response in tsetse flies feeding when it is presented as a blend, furthermore, elicited a distinct neuronal response from its individual constituents at OSNs , showing the integration of olfactory information beginning at the periphery, as also shown in Drosophila (Su *et al.*, 2011; Getahun *et al.*, 2012), moths (Kramer, 1992), and in beetles (Nikonov and Leal, 2002) . In odours that induce innate attraction or aversion are processed via a combinatorial code comprising multiple receptor and glomeruli. For instance, a highly attractant mixture induces a specific activation pattern

among a combination of glomeruli (Stökl *et al.*, 2010). Similarly, for successful foraging a mixture of odorants needed for *Manduca sexta* (Lei *et al.*, 2009) that involves coordinated response of multiple glomeruli.

Furthermore, several ecologically relevant odours dedicate pathway leading to an innate behavior, such as the male-produced pheromone 11-cis-vaccenyl acetate (cVA), the mating enhancing pheromone methyl laurate, CO_2, acids, ammonia and amines, parasitoid odours, and the microbial odorant geosmin (Jones *et al.*, 2007; Laughlin *et al.*, 2008; Hany K.M. Dweck *et al.*, 2015).

1.2.6. Cracking the olfactory code: current tools

Due to the importance of the insect olfactory system in insect-host interaction, it is clear that understanding the sense of smell in insect can lead to better olfactory-based strategies for vector and pest control. Since the discovery of the insect odorant receptor in 1999, enormous efforts have been made to decipher their function and to elucidate mechanisms of insect olfaction. Thus, several techniques have been developed over the past decade and are still being improved over time.

1.2.6.1. Expression in Drosophila OSNs

This technique takes advantage of the recent advances in the understanding of insect olfactory system made in *Drosophila melanogaster*. It is based on a mutant and empty antennal neuron which lack it endogenous receptors (Or22a) (Dobritsa *et al.*, 2003). The odorant receptor of interest is then introduced specifically into the empty neuron using GAL4/UAS system (E. a Hallem, Ho and Carlson, 2004). This system allows for functional analysis of any OR using single-sensillum electrophysiological recording. The "empty neuron" system is robust in the functional characterization of insect ORs where various properties of the receptor and the receptor neuron are maintained. Hence, the empty neuron system has since been used to screen several members of the ORs family in *D. melanogaster* and *An. gambiae*, as well as a handful of other olfactory receptors from various insects (E. a Hallem, Ho and Carlson, 2004; Hallem and Carlson, 2006; Qiu *et al.*, 2006; Wang *et al.*, 2010; Chahda *et al.*, 2019a). The system currently simplified using CISPR/Cas9 (Chahda *et al.*, 2019a).

1.2.7.2. Activity Imaging in Vivo from Neurons

This technique is a functional imaging analysis monitoring the neuronal activity from neurons and/or from olfactory processing centers such as glomeruli or the other higher brain centers.

The technique uses fluorescent calcium indicators which allow visualizing the spatial and temporal aspects of odours representations in whole neuronal population (Grienberger and Konnerth, 2012; Knaden *et al.*, 2012; Balkenius, Johansson and Balkenius, 2015). In model organism like *Drosophila melanogaster*, different calcium sensitive proteins can be genetically express using GAL4-UAS system. It has been suggested that this methodology is very suitable for ligand identification for ORs present in OSNs that are difficult to access with an electrode. It can also be used in non-genetic model organism using calcium sensitive dyes staining techniques. Functional imaging has been successfully used to profile neuronal activity patterns in the antennal lobe of different moth species (Knaden *et al.*, 2012) in bees (Paoli *et al.*, 2018) and in cockroach (Paoli *et al.*, 2020).

1.2.7.3. Expression in Cell Systems

For functional characterisation of ORs, *in vitro* systems involving the expression of ORs in cell culture platforms have also been used. These classic ion channel expression system culture can be human embryonic kidney cells, (Sargsyan, Getahun, Llanos, Shannon B. Olsson, *et al.*, 2011; Corcoran *et al.*, 2014; Miazzi *et al.*, 2019), *Xenopus laevis* oocyte (Misawa *et al.*, 2010; Luetje *et al.*, 2013) and *Spodoptera frugiperda* Sf9 cells (Guo, Zhao and Jiang, 2018). Basically, the cDNA sequence of the ORs of the interest is sub-cloned into a cloning vector and microinjected into the expression the systems. This method allows to directly measure receptor and ion conductance two-electrode voltage clamp electrophysiology (TEVC), whole cell patch clamp electrophysiology or on excised membranes. To date, several receptors have been characterised using oocytes and human embryonic kidney (HEK). However, these in vitro expression systems present some limitations. Indeed, these cell systems require the solubilisation of odours for aqueous delivery and miss some important factors of in vivo OSNs since they are non-neuronal cells. They also lack components of the sensillum lymph.

1.2.7.4. Deorphanization of receptors based on expression alterations of mRNA levels (DREAM)

In non-model insect's functional characterization of olfactory receptors is a challenge. However, recently, it has been demonstrated that odorant exposure induced alterations in mRNA levels; therefore, it is possible to identify ligand–receptor interactions in vivo, by analysing receptor expression after odorant exposure (Weid *et al.*, 2015; Koerte *et al.*, 2018; Diallo *et al.*, 2020). The technique known as **D**eorphanization of **R**eceptors based on **E**xpression **A**lterations of **m**RNA levels (DREAM) does not involve any other genetic tools. The technique is particularly useful for the ORs functional characterisation in non-model insect and it less time consuming for technique for high throughput deorphanization of odorants receptors. DREAM can be complemented and benefit from other deorphanization techniques such as empty neuron expression. This technique has been used in this thesis and discussed in chapter 3.

1.2.7.5 In Silico Screening

Computational screening is widely used to identify potential drug target in neuropharmacology. These virtual screening involves molecular docking and machine learning to identify receptor-ligand properties in a high-throughput manner. Indeed, molecular interactions such as ligand-protein play important roles in many essential biological processes, such as signal transduction, transport, cell regulation, gene expression control. When an odorant receptor interacts with an odorant, it leads to the formation of stable protein-ligand complex which generate a very low binding energy. It is possible to computationally predict the ligand-protein binding mode(Cavasotto and Phatak, 2009; Jalily Hasani and Barakat, 2017). This computational prediction is known as molecular docking. It is done in two steps: the prediction of the tertiary structure (3D) of proteins and the docking itself.

- **Tertiary structure 3D prediction by Homology modelling**

The 3D structure can be experimentally solved using as X-ray crystallography or NMR. Unfortunately, it is currently expensive and time-consuming to obtain complex structures of all the receptors. Also, membrane proteins like odorant receptors are particularly challenging due to the difficulties in their purification and their crystallization (Ash *et al.*, 2004; Butterwick *et al.*, 2018). Another alternative is to predict the structure of the proteins of interest from its amino acid sequence with experimentally resolved structures of related proteins using homology modelling also called comparative modelling (Akansha Saxena;

Rajender S. Sangwan and Asnjay Mishra, 2013; Jalily Hasani and Barakat, 2017). Homology modelling computationally is done in 5 steps and can be summarised as follow (i) identification of template; (ii) single or multiple sequence alignments; (iii) model building for the target based on the 3D structure of the template; (iv) model refinement, analysis of alignments, gap deletions and additions, and (v) model validation (Figure 2.4).

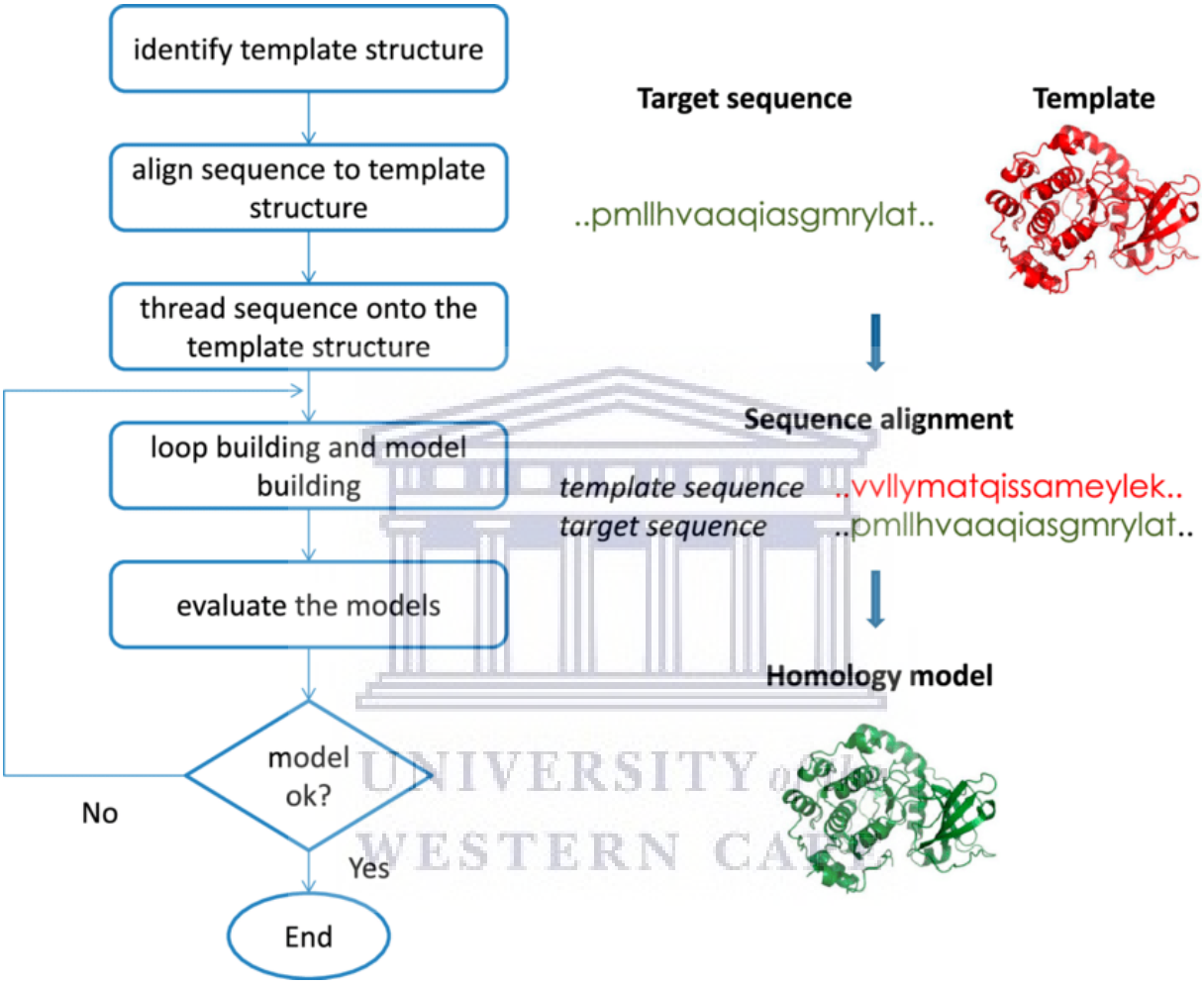

Figure 1.5 Summary of homology modelling steps using proteins sequences (Sliwoski *et al.*, 2014).

- **Molecular docking**

Molecular docking is a bioinformatics simulation which predicts the stable conformation in the interaction of two or more molecules. The simulation predicts optimized docked conformer based upon total energy of the system formed by the interaction of the molecules (R. D. Taylor;P.J.Jewsbury & J. W. Essex, 2002; Guedes, de Magalhães and Dardenne, 2014). In this technique, the main objective is to generate the optimized conformation of the ligand-receptor complex with less binding free energy. The binding free energy can be attained using

scoring algorithms and functions. Due to the breakthrough in structural biology and the increasing power of computer, different algorithms and scoring functions have been developed and improved (Sousa, Fernandes and Ramos, 2006; Tripathi and Misra, 2017; Naqvi *et al.*, 2019). Molecular docking involves two main steps which are the prediction of ligand conformation and orientation (the pose) by search algorithms and assessment of binding affinity by scoring functions.

a. Search algorithms used in docking simulation

In this first step, the efficient binding modes or poses are generated from different ligand conformations and orientations. The choice of the algorithms is related to the molecules and the receptor flexibility; hence therefore two methods are often used: methods for "rigid-body" docking and methods for "flexible docking". The methods used for "rigid-body" calculate the intermolecular interactions of molecular complexes (Ligand-receptor) considered molecules as rigid bodies. Therefore, they consider geometry match between molecules and are based on Shape Matching (SM) and Fast Fourier Transform (FFT). Shape Matching (SM) algorithm search for shape complementarities between two molecules and then measure the surface complementarity. For example, two molecules (ligand and receptor) will have similar shape if they have equal size or if the inner part of the ligand surface matches with the outer part of the receptor surface (Axenopoulos *et al.*, 2011). Hence molecules with similar shape are expected to result in similar fields. Several programs such as DOCK (Ewing *et al.*, 2001; Allen, Dokholyan and Bowers, 2016), MSDOCK (Sauton *et al.*, 2008), EUDOC (Pang *et al.*, 2001) utilize this approach as docking algorithms. The Fast Fourier Transform (FFT) method involves atomic coordinates for the digital representation of molecule by three-dimensional discrete functions that distinguish between surface and the interior of the molecule. Algorithm also calculates correlation function that assesses the degree of molecular surface overlap and penetration considering 6 degrees of freedom using Fourier transformation (Katchalski-Katzir *et al.*, 1992). Program like FTDOCK (Moont, Gabb and Sternberg, 1999) ZDOCK (Chen, Li and Weng, 2003; Vreven *et al.*, 2017) are using this algorithm.

Flexible docking takes into account the translational and conformational degrees of freedom of the molecules making molecular docking with flexible ligand and flexible receptor more accurate approximation of in vivo complexes formation, especially when conformational changes upon formation are present. Basically, there are three types of flexible ligand docking approaches (Lorber and Shoichet, 2008). The first approach, the systematic methods ligand is

split into several fragments that are separately docked in the receptor site and covalently linked to reassemble the ligand; it is one of the most used methods by the docking software. The second approach is the random or stochastic methods which include algorithm like Monte Carlo, simulated annealing, evolutionary algorithms, Particle Swarm Optimization. They are standard minimization methods that search for global energy minimum of the conformers, generate random changes in the ligand conformation and thermodynamically explore different states and selects energetically favourable states to create more reliable protein-ligand complexes. The most popular dock programs that used this approach are MCDOCK, AutoDock, GOLD and ICM-pro. The last approach in the flexible body docking is the deterministic methods. It is a recent method which implements Newton equation simulation and therefore quantifies the properties of a system at a precision and on a time scale. Programs like CDOCKER, CHARMM use this algorithm for molecular docking.

b. Scoring functions used in molecular docking

The scoring follows the poses and the conformations of receptor-ligand generated by the search algorithms. The scoring function algorithms calculate the energy that estimates the binding affinity between two biomolecules generated by the binding constant (Kd) and the Gibbs free energy (ΔGL). There are four main scoring functions: force-field based (first principle based approach), empirical, knowledge-based and consensus scoring (Gohlke, Hendlich and Klebe, 2000; Brooijmans and Kuntz, 2003; Huang and Zou, 2010; Xuan-Yu Meng, Hong-Xing Zhang, Mihaly Mezei, 2011). Scoring functions algorithm determines the binding energy by summing the contributions of bonded and non-bonded terms using *ab initio* method to calculate the energy associated with each term of the function using the equations of classical mechanics in a general master function. The empirical scoring functions reproduce experimental affinity data based the correlation of the free energy of binding to a set of non-related variables (Pason and Sotriffer, 2016). Empirical scoring functions are generally simpler than force filed based functions, therefore binding score calculations of former are faster. As these functions are dependent on experimental data set used for parameterization it is not necessary that the binding affinity will be predicted correctly for structurally different ligands from those exploited in the training set. The knowledge-based are statistical methods, where the parameters of the potential functions are extracted from the structural information of experimentally determined atomic structures. This approach implicitly incorporates physical interactions (electrostatic, van der Walls, cation-π interactions), where high frequency of occurrence/non-occurrence of specific atom-atom

interactions is assumed to represent favourable/unfavourable contact (Brooijmans and Kuntz, 2003; Huang and Zou, 2010). The last scoring function, consensus scoring, combines results from various scoring functions to improve the performance of scoring in molecular docking. It uses the advantages and simultaneously attenuates the shortcomings of each method which make it an improved scoring function in molecular docking. It has been shown that this scoring function outperformed the others previously cited (Feher, 2006).

- **Molecular dynamic simulations**

Molecular dynamics (MD) simulations predict how every atom in a protein or other molecular system will move over time based on a general model of the physics governing interatomic interactions (Karplus and McCammon, 2002; Hollingsworth and Dror, 2018). It helps to validate the molecular docking and to understand the molecular insight of different interacts between the receptor and the ligand.

1.2.7.6. Functional characterisation of odorant receptors using Chemoinformatics

Given to the recent advances in machine learning, another computational screening pipeline known as chemical informatics has emerged. With this technique, the computer first analyses three-dimensional structures of the known ligands for a known odorant receptor to identify shared structural features of actives. Then, the computer next screens large chemical structure databases to identify ligands that are close in structure to known actives (Cao *et al.*, 2015; Gasteiger, 2016; Lo *et al.*, 2018). This computational prediction has been validated at >70% by electrophysiology. Although, the technique requires odorant receptors that have been already deorphanized, it can be very helpful in the screening of large database for the identification of new attractant or repellent for insect.

1.3. Rational of the thesis

Animal and human trypanosomiasis is a complex disease caused by multiple species and strains of trypanosomes and transmitted by multiple tsetse species. Tsetse flies such as *G.f.fuscipes* are the vectors of both human and animal trypanosomiasis, which cause sleeping sickness in humans and nagana in domestic animals. The lack of vaccine, drug resistant development and the continous impact of trypanosomiasis, spured the search for alternative control technicques such as repellents, and attractants. Insect spatial repellents can reduce the transmission of insect vector borne diseases such as trypanosomiasis by blocking contact between blood-seeking insects and their hosts. In this regard, International Centre of Insect

Physiology and Ecology (ICIPE) has successfully developed spatial novel repellent from unpreferred animals. However, the target olfactory recptors and odorant binding proteins and the olfactory sensory neurons through which the repellents reception and transduction are unknown. In order to improve the tsetse repellents and develop new ones, it is important to investigate the mechanism through which the repellents are perceived at the receptor level. Hence the main focus of my PhD Thesis will be to morphologically charaterize the olfactory sensilla, functionally charaterize the *G.f.fuscipes* odorant receptors and odorant binding proteins using behaviorally well charaterized odorants, tsetse repellent and comparing it with known attractant to find the receptors and olfactory sensory neurons responsible for the detection of the repellent components, and to dissect the contribution of each blend components to odour valence and specific behavior.

1.3.1. Objectives of the thesis

1.3.1.1. Main objective

This study aims to morphologically describe the olfactory sensilla, functionally characterise the olfactory sensory neurons, olfactory receptors and odorant binding proteins of *G. f. fuscipes* that will enable us to find the neurons, receptors and binding proteins responsible for the detection of the waterbuck repellent components and attractant.

1.3.1.2. Specifics objectives

i. Morphological characterization of *G. f. fuscipes* olfactory sensilla using scanning electron microscopy.
ii. Functional characterization of *G. f. fuscipes* olfactory sensory neurons housed in the different olfactory sensilla and identification of the putative odorant receptors responsible for the detection of Waterbuck repellent compounds and attractant.
iii. Identification of candidate odorant binding proteins involved in the coding of Waterbuck repellent compounds in *G. f. fuscipes.*

Chapter Two

Morphological characterization of antennal sensilla in *Glossina fuscipes fuscipes*

Abstract

Tsetse fly (Diptera: Glossinidae) are the primary vector of trypanosomes, the parasites responsible for African Trypanosomiasis which causes sleeping sickness in humans and nagana in domestic animals. The transmission of the infectious pathogens depends on the proper location of host by the vector. Tsetse flies locate their host mostly using the sense of smell, olfactory sensilla are the structural and functional unit of the fly "nose" the antenna, they are the one that are exposed to the environment, that enable the insect to communicate with the environment using receptors expressed on the dendrite of olfactory sensory neurons, which are housed in sensillum shaft.Thus the type, diversity of olfactory sensilla, number affects the behavioural response of tsetse flies to their hosts. The types of olfactory sensilla, diversity, distribution of *G. f. fuscipes*, riverine tsetse fly, the main vector of human and animal trypanosomiasis is not described morphologically. Using scanning microscopy, we characterised the sensillar types on the dorsal side of the *G. f. fuscipes*, it demonstrates that *G. f. fuscipes* is equipped with diverse and rich olfactory sensillar types, we identified three types of olfactory sensillar from ceoloconic, basiconic and trichoid sensilla, the latter two are also with diverse types, that various in length, width and number of pores per a given area. The basiconic sensilla were distributed in all the surface of the third antennal segment, with higher density at the base and middle, but their number decrease at the tip. However, trichoid sensilla are almost uniformly distributed on antennal surface. While the coeloconic sensilla are restrictively distributed at the apical and middle section of the flagellum. Furthermore, we demonstrated that there is slight numerical difference between male and female, there are more basiconic and trichoid sensillar in male than female. Similarly, the number of coeloconic sensilla is the same between male and female. There is a sensory pit on the antenna; housing basiconic sensilla that are different in morphology from the basiconic sensilla found on the surface of the antennal, they are club-like in shape, wider and shorter. Also, they are only present in male and completely absent in female. The difference in number, size, pore density,

distribution and types of olfactory sensilla might induce both physiological and behavioral difference between male and female *G. f. fuscipes*.

Key words: Antennae, Olfactory sensilla, Glossina sp, flagellum, diversity and abundance.

2.1. Introduction

Tsetse fly (Diptera: Glossinidae) are the primary vector for trypanosomes, the parasites responsible for African Trypanosomiasis that causes sleeping sickness in Humans and nagana in domestic animals (Solano, Kaba, Ravel, Naomi A Dyer, *et al.*, 2010). The transmission of the infectious pathogens is much dependent on the contact between vector and host. For all tsetse olfactory stimuli play an important role in host location. However, it is known that sensitivity to the olfactory stimuli varies between species, sexes and even according to the habitat for some species. For example, riverine species of palpalis group are less sensitive to the odour baits developed for the savannah species (Esterhuizen *et al.*, 2011; Rayaisse *et al.*, 2011). Also, the savannah species *Glossina pallidipes* Austen and *Glossina m. morsitans* Westwood is differently attracted to odorants compound isolated from buffaloes and oxen urine (Hassanali *et al.*, 1986; Cork *et al.*, 1988). These species respond differently to, for example, men (Vale, 1974), acetophenone (Vale, 1980b), natural urines and synthetic phenols (Vale, 1974, 1980). Furthermore, the attractants used for the savannah tsetse are less or not attractive to palpalis species and sometimes even repel them (Hall *et al.*, 1984; Rayaisse *et al.*, 2011). Savanah species are also known to have some avoidance behaviour to some vertebrate such as waterbuck, zebra etc. Semiochemicals have been identified from tsetse unprefered host to develop repellent for the savannah species (Gikonyo *et al.*, 2002; Bett, Saini and Hassanali, 2015; Saini *et al.*, 2017). However, these semiochemicals, repel less efficiently in tsetse from riverine species (Mbewe *et al.*, 2019), but strong antifeedant effect to both *G. pallidipes* and *G. f. fuscipes* (Diallo *et al.*, 2020), even if *G. pallidipes* was more sensitive than *G. f. fuscipes* (Diallo *et al.*, 2020). The fact that tsetse fly species are showing specific behaviour to different odour stimuli indicates that they can clearly discriminate and perceive the same odour present in their environment differently. In insects, odorants are recognized by olfactory sensory neurons (OSNs) located in the dendrites of olfactory sensory cells within the sensilla present on the surface of the chemosensory appendages such the antennae and the maxillary palp (Galizia and Rössler, 2010). The observed olfactory behaviour difference between savannah and riverine tsetse species might be attributed by the type, number, size,

distribution of olfactory sensilla, and then the olfactory sensory neurons housed. For example, Drosophila with bigger antenna performed better in odour navigation (Keesey *et al.*, 2019). However, the morphology of riverine tsetse fly, *G. f. fuscipes* sensilla are not well characterised. Chadha et al., 2019 has demonstrated *G. m. morsitans*, a savannah species is equipped with the diverse olfactory sensilla. Evidently, species of tsetse fly code differently the stimuli from their environment. In insects, odorants are recognized by olfactory sensory neurons (OSNs) located in the dendrites of olfactory sensory cells within the sensilla present on the surface of the chemosensory appendages such the antennae and the maxillary palp (Galizia and Rössler, 2010). The type, the number and the distribution of the sensilla vary according to the insects and species (Suh, Bohbot and Zwiebel, 2014). For example, *Drosophila melanogaster* has 410 olfactory sensilla present on the antenna and about 60 olfactory sensilla on the maxillary palp while *Manduca sexta* got about 2200 sensilla (Shields and Hildebrand, 1999, 2001).

In tsetse fly (Glossinidae), a clear description of the olfactory sensilla is not yet known. Furthermore, the olfactory code in this vector remains unclear. The main objective of this study was to morphologically characterise the olfactory sensilla of *G. f. fuscipes*. In this study, we report the diversity and the abundance of the olfactory sensilla on the antennal surface and sensory pit of *G. f. fuscipes.*

2.2. Material and Methods

Scanning Electron Microscope

Teneral flies (0 day) were anesthetized in CO2, placed in 5 ml 25% EtOH, and incubated for 12–24 h at room temperature. In an EtOH row, the flies were further dehydrated in 50%, 75%, and two times 100% EtOH for 12–24 h each at room temperature.

Samples were dried at their critical point and mounted samples with T. V. tube coat (Ted Pella) onto the SEM stubs. After mounting, samples were sputter-coated with a 25-nm-thick platinum coat. Images of the sensillum types on the third antennal segment and the maxillary palp were acquired using a LEO 1450 VP scanning electron microscope with 10 kV and 24 mm working distance (Carl Zeiss). The antenna length, diameter, and sensilla number for each segment and the length, diameter and density of were evaluated using Image J/Figi. To obtain accurate data, the sensilla in different images were marked using the GIMP2.

The numerical data were analysed, and differences between the sexes and species were separated using a Mann–Whitney U -test (Siegel and Castellan, 1988) as the data were not distributed normally. The statistical analyses were performed using R 3.3.2 version

2.3. Result and discussions

2.3.1. Results

General morphology of the antennal

Our scanning electron microscopy in *G. f. fuscipes*, demonstrated that the antennae are a pair of an organ placed at the front of the head and located between two large compound eyes. The antenna is divided into three basic parts: scape, pedicel and flagellum. The scape is the basal segment that articulates with the head capsule. The second segment is the pedicel, and it is directly attached to the scape. The flagellum is the longest segment (env. 700 µm) where the arista is attached (Figure 2.1) and also densely covered with various morphological classes of sensilla including olfactory and mechano sensilla. Flagellum also contains sensory pit (1 per flagellum) and sacculus (2 per flagellum). The arista is a long highly branched structure like an eyelash. The arista has twenty-seven (27) row branched hairs on its upper side (Figure 2.1).

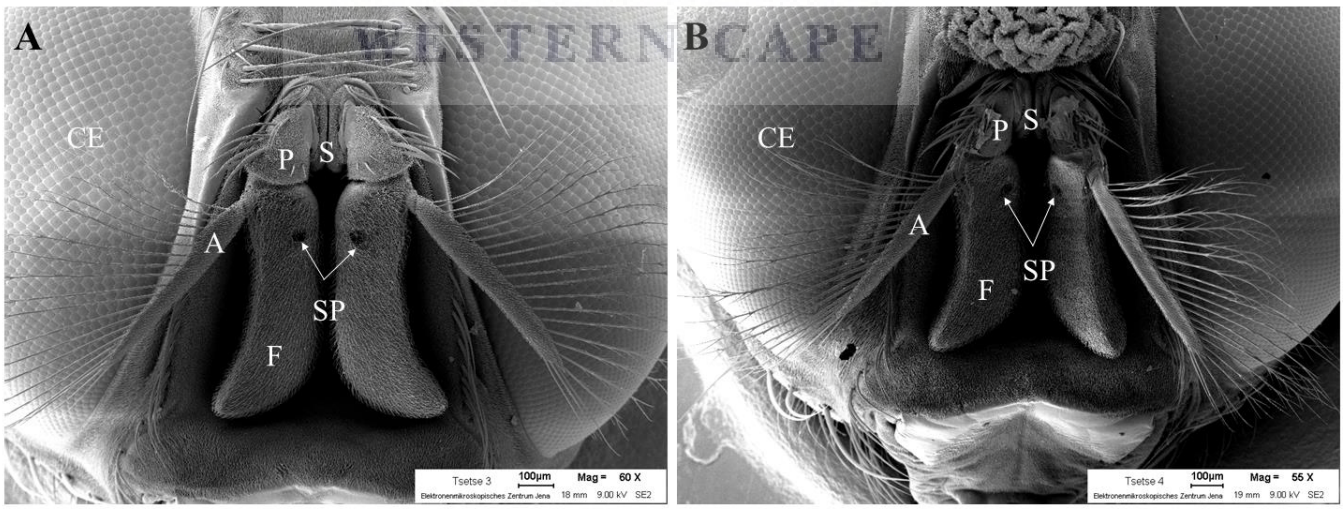

Figure 2.1: Antennal morphology of *G. f. fuscipes.* **A**: Arista, **CE**: Compound eye, **F**: Flagellum, **P**: Pedicel, **SP**: Sensory pit, **S**: Scape

Antennal sensilla types and abundance

Three different types of olfactory sensilla were identified on the antenna of male and female of *G. f. fuscipes*. These sensilla include two types of sensilla trichoid (long and short trichoid sensilla), three types of sensilla basiconic (long- thick basiconic sensilla, short, thick basiconic sensilla and long- thin basiconic sensilla), sensory pit basiconic sensilla and one type of coeloconic sensilla. All the sensilla have been differentiated and classified according to their external appearance (Shanbhag, Müller and Steinbrecht, 1999; Stocker, 2001; Chahda *et al.*, 2019a).

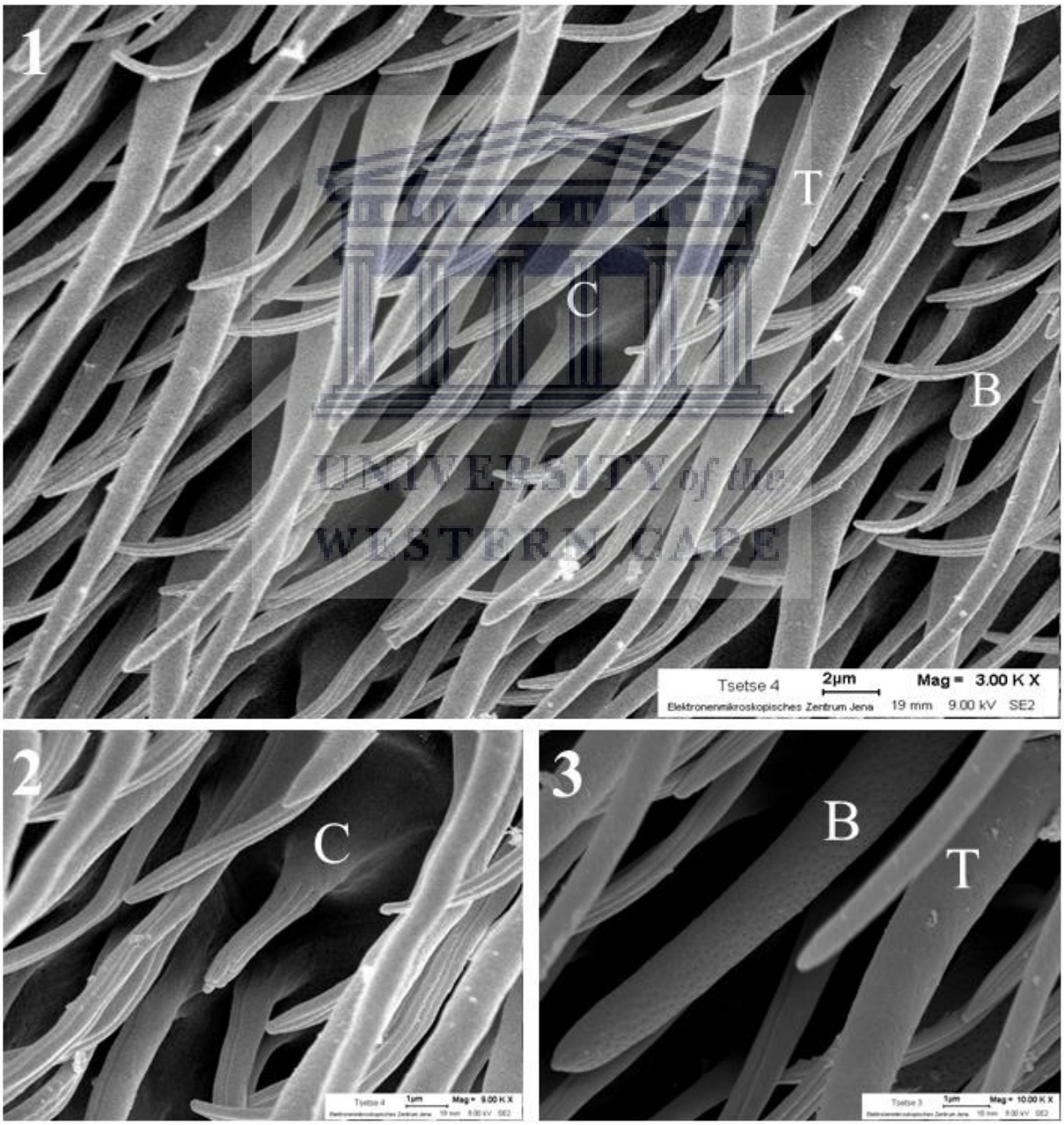

Figure 2.2. Electromicrograph of *Glossina f. fuscipes* flagellum showing different types of antennal sensilla. **1-** Tree main types of sensilla found on the flagellum, **T**=trichoid sensilla, **B**= Basiconic sensilla, **C**= coeloconic sensilla. **2-** Aporous coeloconic sensilla highlighting cuticular finger-like meeting each other at the tip. **3-** Multipous basiconic and trichoid sensilla showing more pores in basiconic sensilla than trichoid sensilla.

Table 2.1. Summary of length mean, basal diameter and the tip shape of different sensilla in *G. f. fuscipes* male and female, **M**=male, **F**=female, **LT**=long trichoid, **ST**=short trichoid, **TLB**= thick large basiconic, **TB**= thin basiconic, **TSB**= tick short basiconic and **C**=coeloconic.

Sex	sensilla	Length mean±se	Basal diameter	Tip
M	LT	32.1 ± 0.23	2.5 ± 0.03	Sharp
	ST	19.0 ± 0.47	2.0 ± 0.05	Sharp
	TLB	12.4 ± 0.12	1.9 ± 0.03	Blunt
	TB	16.2 ± 0.80	2.0 ± 0.05	Blunt
	TSB	12.4 ± 0.12	1.9 ± 0.03	Blunt
	C	4.7 ± 0.17	1.8 ± 0.06	Blunt
F	LT	31.2 ± 0.21	2.5 ± 0.03	Sharp
	ST	19.9 ± 0.56	2.2 ± 0.05	Sharp
	TLB	14.5 ± 0.49	2.0 ± 0.03	Blunt
	TB	13.2 ± 0.34	1.7 ± 0.04	Blunt
	TSB	8.1 ± 0.17	1.6 ± 0.03	Blunt
	C	4.5 ± 0.17	1.8 ± 0.10	Blunt

Sensilla Trichoid (ST)

Sensilla trichoid are single walled (SW) type sensilla and they represent the longest cuticular apparatus of all the types of sensilla. They are long and thin with a rather sharply pointed tip. They arise directly from the cuticle and don't have a flexible socket. According to their length, we identified two types of trichoid sensilla on the antenna of *Glossina f. fuscipes*: Long trichoid sensilla and short trichoid sensilla. Long trichoid (LT) sensilla are gradually curved distally with a pointed tip. LT sensilla are robust of 32.1 ± 3.50 µm long with basal diameter of 1.5-4 µm (Table 2.1). They are mainly distributed in the distal and the medial part of flagellum. In terms of abundance, sexual dimorphism was observed; 892 LT sensilla were counted on the female flagellum while 678 were found in the male. Their walls are smooth, with pores and no grooves on the surface (Figure 2.2). Short trichoid sensilla (ST) are different from the long trichoid sensilla in their length, they measure 19 ± 1.7 µm in length and in 2 ± 0.35 µm diameter. In *G. f. fuscipes* antenna, 171 ST were counted in female and 115 in male on the dorsal side of the flagellum.

Sensilla Basiconic (Sb)

Basiconic Sensilla are single walled olfactory sensilla type ((Figure 2.2, Table 2.1). They are multi-porous sensilla throughout their walls. The number of pores various from 36 to 40 per µm^2 but this number is considerably reduced at the tip (20/µm$^{2)}$. The basiconic sensilla observed on the flagellum of *G. f fuscipes* they are straight and have blunt tip. They are U shaped with two arms arising directly from the cuticle with a non-flexible socket. Based on their morphology and length we classified the *G. f. fuscipes* basiconic sensilla into three types. Thick Long basiconic sensilla (TLB) characterised as a long (14.5 ± 2.8 µm), straight or slightly curved with a blunt tip with basal diameter of 1.5-4 µm (Figure 2.2, Table 2.1). These sensilla are distributed on flagellum slightly more abundant in male (n=304) than female (n=244). Furthermore, female *G. f. fuscipes* LB (14.5 ± 2.8 µm) is slightly longer than that of male *G. f. fuscipes* LB (12.4 ± 1.69 µm) (Table 1).

Thin basiconic (TB) sensilla are slightly similar to large basiconic (LB) sensilla. This type of sensillum is thin as compared to the large basiconic sensilla. However, their abundance and distribution on the antenna are similar to LB sensilla. We counted 313 thin basiconic sensilla on the flagellum of G. *f. fuscipes* male and 115 for *G. f. fuscipes* female, showing the same as LB, it is more in female than male.

The last type of basiconic sensilla are think and short sensilla (TSB). These types of sensilla are 5-10 µm long and 1.6 ± 0.25 µm wide at the base. They are also U shaped and blunt tipped (Figure 2.2). Like the other type of basiconic sensilla, they are more abundant in female than male. We identified 130 Tick and short basiconic sensilla in female and only 29 in male.

Coeloconic sensilla

The coeloconic sensilla found on the antenna of *G. f. fuscipes* are grooved with a pyramid shape (Figure 2). This type sensillum are characterised with several grooves consisting of cuticular finger-like meeting each other at the tip. Their structure appears as a double wall with no pores. Coeloconic sensilla were the shortest (4.5 ± 0.17µm) sensilla on the antenna of *G. f. fuscipes*. Female and male had on average 31 and 27 coeloconic sensilla, respectively. The coeloconic sensilla on the surface of the *G. f. fuscipes* antenna appear more uniform in size and shape. They are the lowest in their abundance as compared to basiconic and trichoid sensilla.

Club shaped Pit basiconic sensilla

The sensilla found in the sensory pit are classified as basiconic type but is very distinct in morphology from those basiconic sensilla found on the surface of the flagellum. They are shorter 3.5-4 µm in length and 1.6-1.7 µm, even though we are not able to measure due to their location, but they are wider, and has wide and round tip, they have multiple pores (Figure 2.3). We also demonstrate there is sexual dimorphism in these sensilla type, female pits contain different sensilla from that of male (Figure 2.3). The sensory pit also houses coeloconic sensilla, which are grooved.

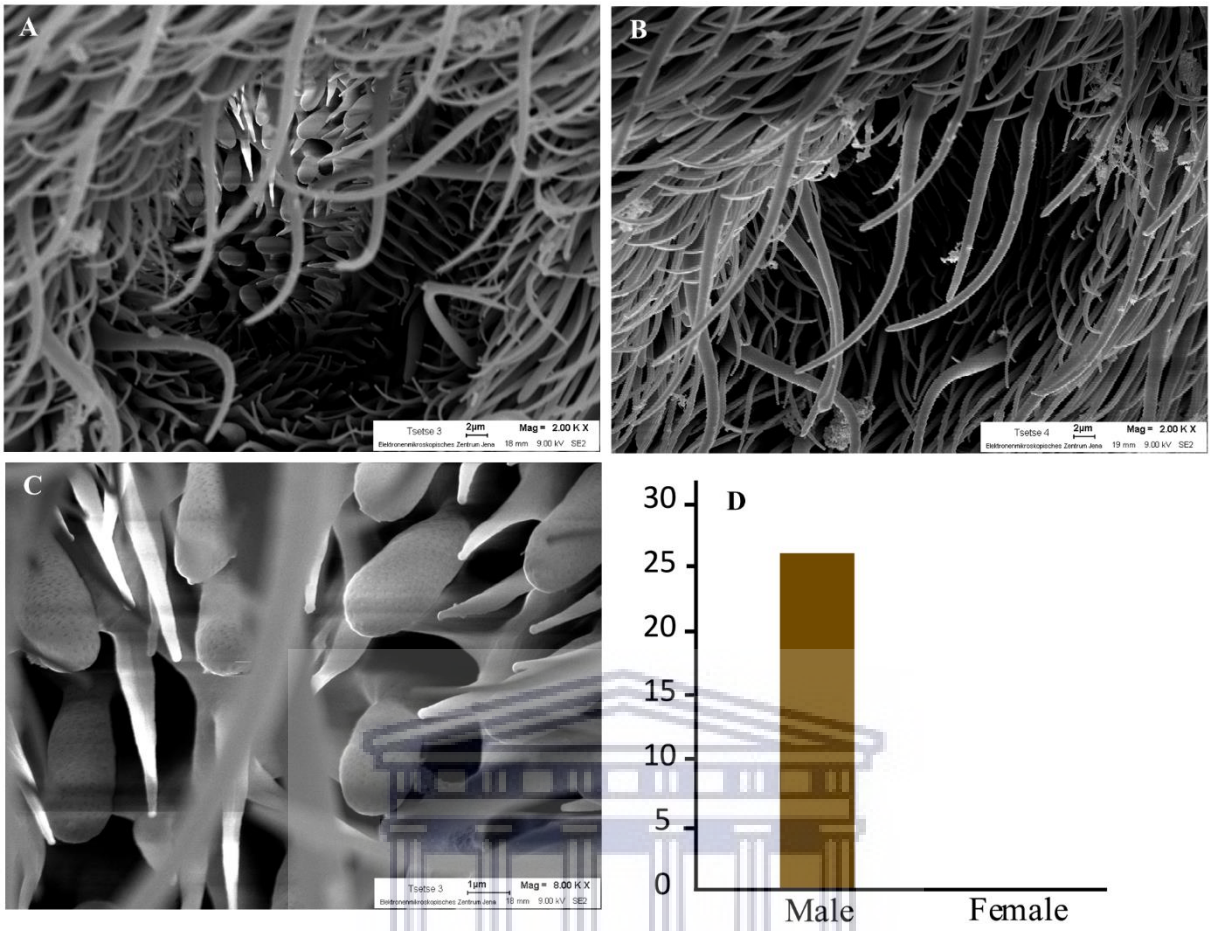

Figure 2.3: Pit structure in *Glossina fuscipes fuscipes* male and female. A=male, B=female, C= electromicrograph showing porous sensilla. D= number of sensilla counted in the pit.

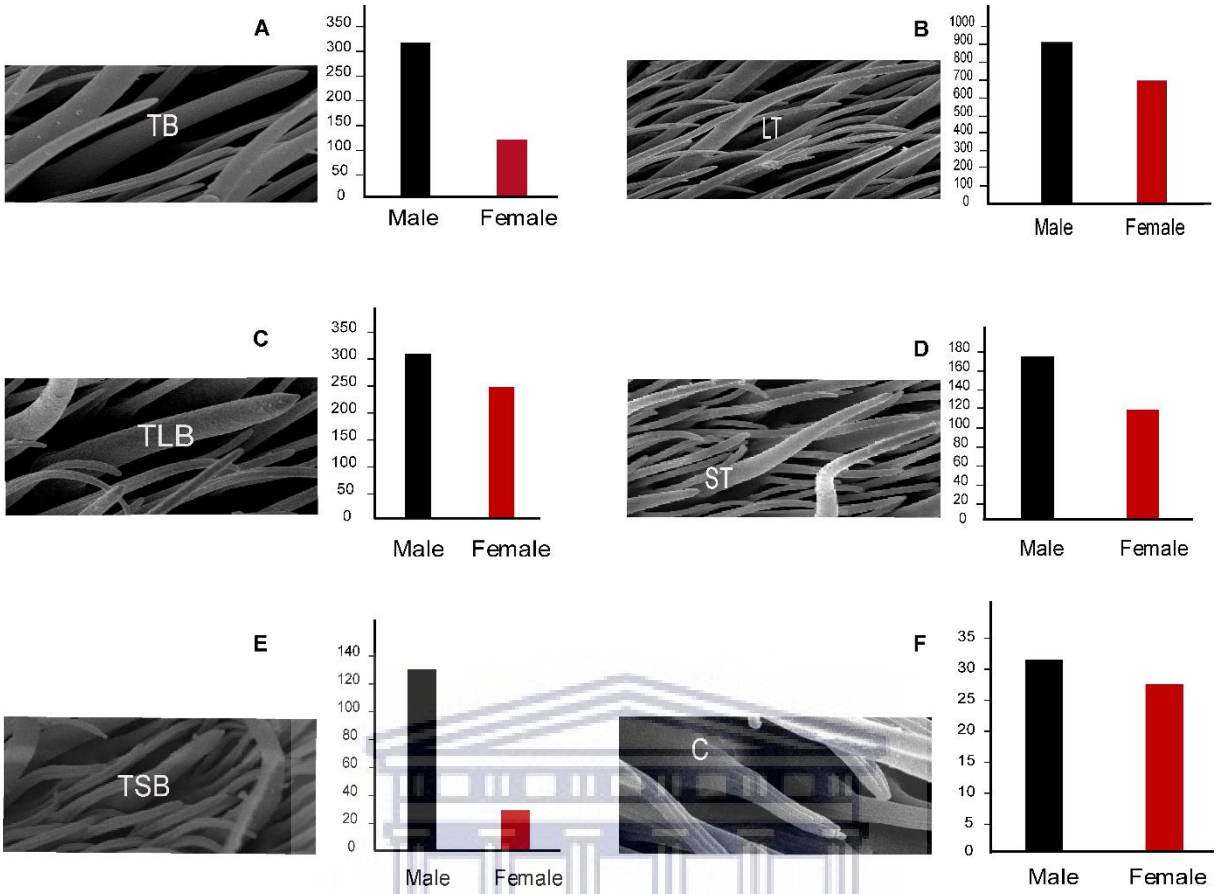

Figure 2.4. Graph showing the abundance of different type of sensilla in male and female. A – F representing respectively, Thin Basiconic (TB), Long Trichoid (LT), Thick Large Basiconic (TLB), Short Trichoid (ST), Thick and Short Basiconic (TSB), Coeloconic (C).

2.3.2. Discussion

Herein, we investigated the sensilla diversity and abundance on the antennal surface of *G. f. fuscipes.* Firstly, we found that the general morphology of the antennae of *G. f. fuscipes* is similar to the basic structure described in *G. palpalis and G. tachinoides* by Isaac et al., 2015 and to *G. m. morsitans*, described by (Chahda *et al.*, 2019b). No apparent sexual dimorphism was observed in the antennal morphology of male and female *G. f. fuscipes*, as well as in size, both have sensory pit, and sacculus (Figure 2.3). The antenna structure constituted three segments are the scape, pedicel and unsegmented flagella is also the same with that of stable fly (Tangtrakulwanich *et al.*, 2011), with that of the house fly (Sukontason *et al.*, 2004) and with that of fruit fly (Shanbhag, Müller and Steinbrecht, 1999).

Even though tsetse and drosophila various in evolutionary history and also mode of feeding, reproduction they do share similar morphology sensilla.

Similarly, to Drosophila melanogaster, three olfactory sensilla, the trichoid, basiconic and coeloconic types, were found on the antenna of the *G. f. fuscipes.* The basiconic sensilla of *G. f. fuscipes*, vary in their morphology some have got rounded tips, others have pointed tips, they also vary in their pore density and length. Interestingly the pores were arranged differently as compared to that of Drosophila melanogaster, which are place in a line continuously, while that of *G. f. fuscipes* not in line. However, unlike Drosophila, the basiconic sensilla on the surface of the *G. f. fuscipes* antenna appear more diverse in size, and morphology. This makes it different from *G. m. morsitans* that has more uniform basiconic sensilla (Chahda *et al.*, 2019a). Basiconic sensilla houses neurons responding to food and other ecological relevant signals, such as danger, ovipositional sites etc. (Marcus C. Stensmyr *et al.*, 2003; Vosshall and Stocker, 2007; Touhara, 2009; Diallo *et al.*, 2020). For all of them, except coeloconic, sexual dimorphisms were observed in their abundance on the flagellum. Sexual dimorphism in the antennal sensilla diversity and abundance has also been demonstrated in other insects (Di Giulio *et al.*, 2012; Zhang *et al.*, 2015; Polidori *et al.*, 2016; Galvani *et al.*, 2017; Wang *et al.*, 2018; Nowińska and Brożek, 2019). Similar results have been found in *Glossina palpalis* and *Glossina tachinoides*, that belong to the same tsetse group as *G. f. fuscipes* (Isaac *et al.*, 2015). Indeed, electroantennogram responses of females were higher than males in *G. f. fuscipes* than female (Voskamp, Van Der Goes Van Naters and Den Otter, 1999; Ouedraogo and den Otter, 2018). Also, recently, (Mbewe *et al.*, 2019) reported that *G. f. fuscipes* female are more sensitive to 4-methylguaiacol and certain repellent compounds in waterbuck odour than male. This result elicits a clear evidence of the importance of olfactory sensilla abundance in tsetse behaviour. The types of antenna sensilla observed on the antenna of *G. f. fuscipes* were similar to the others observed on the antenna of the other dipterans such as *Stomoxys calcitrans* and fruit fly with minor differences. For the trichoid sensilla, we observed only two subtypes in both male and female. However, 3 subtypes were found in *Stomoxys calcitrans* (Tangtrakulwanich *et al.*, 2011) and in *Drosophila melanogaster* (Shanbhag, Müller and Steinbrecht, 2000) using a silver staining method, detected wall pores on trichoid sensilla.

We demonstrated the antenna of *G. f. fuscipes* have sensory pits, that are equipped with distinct olfactory sensilla, which agrees with (Chahda *et al.*, 2019a). The placement of olfactory sensilla in specialized deep sensory pit structure, such as tsetse pits is not clear, especially when they supposed to be exposed to the environment to get in contact with the odours, Chadha et al., 2019 has demonstrated these pits contains olfactory receptors responding to food odorant, showing their olfactory function. Sensory pits are also shown in

other insects such as blow fly *Lucilia cuprina* and the flesh fly *Parasarcophaga dux* (Sukontason *et al.*, 2004; Zhang *et al.*, 2014, 2016). However, we found sexual dimorphism in the sensory pits; the female does not have those club shaped basiconic sensilla in their pit (Figure 2.3). The sexual dimorphism and the function of those sensilla housed in the sensory pits need to be investigated.

Sensory pits are found in antennae of other calyptrate flies besides tsetse, including the blow fly *Lucilia cuprina* and the flesh fly *Parasarcophaga dux* (Sukontason *et al.*, 2004; Hassan *et al.*, 2013). Pits are also found in none fly insect antenna, for instance on the third segment of the labial palp of *Manduca sexta*. Interestingly, the pit is lined with sensilla that respond to carbon dioxide and other important sources such as food and oviposition sites (Guerenstein, Christensen and Hildebrand, 2004; Chahda *et al.*, 2019b). However, sensory pits are absent in Drosophila flagellum.

Grooved coeloconic sensilla, which measured about ~4.5 µm in length, were also found on the antenna of *G. f. fuscipes*, with lower abundance and also restricted distribution, mostly at the base as compared to the other two olfactory sensilla. Structurally similar coeloconic sensillar type found in *G. m. morsitans* and *Drosophila* (Shanbhag, Müller and Steinbrecht, 2000; Chahda *et al.*, 2019a). We characterised only those found on the surface of the antenna, as opposed to the pit and sacculus, by Chadha et al., 2019. Coeloconic sensilla in Drosophila houses neurons that respond to amines and organic acids (Yao, Ignell and Carlson, 2005; Prieto-Godino *et al.*, 2017) it will be interesting to investigated, if coeloconic sensilla houses neurons responding to acids and amines and to demonstrate that interaction is conserved between these two evolutionary distinct flies. Even though our count might not be exhaustive, male got higher number of coeloconic than females, this is also interesting to find out the effect on the physiological response to acids and amines.

G. f. fuscipes have more diverse trichoid sensilla as compared to *G. m. morsitans*, which various in their morphology, as well as length, most of them are wide at the base and tapering at the tip, but they also got curly shape, while other are straight. Most insect showed sexual dimorphisms in the number of trichoid sensilla, being more in male than female for example (Drosophila, moth, mosquitos), because trichoid sensilla detect most of the time pheromone, which is most of the time produced by the female, thus requires high sensitivity. Similarly, we documented sexual dimorphism in male and female, as male have more trichoid sensilla than the female.

Chapter Three

Functional characterisation of characterization and prediction of the odorant receptors responsible for the detection of the tsetse repellent

This chapter has been published in Frontier Cellular Neuroscience as:

Cellular and Molecular Targets of Waterbuck Repellent Blend Odors in Antennae of *Glossina fuscipes fuscipes* Newstead, 1910

doi: 10.3389/fncel.2020.00137

3.1. Introduction

Blood feeding insects such as tsetse flies have a differential feeding preference to some animals over others regardless of their abundance (Weitz, 1963).

Such behaviour is a response to odours and can lead to the identification of attractants and repellents for vector control. The spatial repellent for some tsetse fly species is a blend of semiochemicals identified from a non-host, waterbuck (*Kobus defasa*) (Gikonyo *et al.*, 2002, 2003; Mwangi, Gikonyo and Ndiege, 2008). The repellent formulation is a blend that consists of δ-octalactone, guaiacol, geranyl acetone, and pentanoic acid (Bett, Saini and Hassanali, 2015; Saini *et al.*, 2017). Until recently the main tools for tsetse control were, odour baited traps and targets (Kuzoe, F A., Schofield, 2004). Additionally, recent use of the tsetse repellent compounds (WRC), has reduced the transmission of animal trypanosomiasis in cattle by reducing contact between blood-seeking *Glossina pallidipes* and cattle hosts (Saini *et al.*, 2017) and showed to reduce trap catches of *G. f. fuscipes* (Mbewe *et al.*, 2019). The cellular and molecular mechanisms of the spatial repellent odours is not well understood, yet such knowledge will enable us to improve the efficacy of existing repellent blend or to identify novel repellents for control of various tsetse fly species of both medical and veterinary importance. Olfactory research on *Glossina* spp. is fragmented (Chahda *et al.*, 2019b; Soni, Sebastian Chahda and Carlson, 2019). However, recent research on tsetse genomes has opened new opportunities to make functional characterisation of *Glossina* odorant receptors possible (ORs) (Aksoy et al., 2014; Attardo et al., 2019; Macharia et al., 2016; Obiero et al., 2014; Watanabe et al., 2014).

In *Drosophila*, since the first insect odorant receptors (ORs) were identified (de Bruyne, Clyne and Carlson, 1999; Vosshall *et al.*, 1999), enormous progress has been made to functionally characterise almost the entire ORs repertoires and elucidate the molecular basis of olfaction in this insect species (E. a Hallem, Ho and Carlson, 2004). The limited genetic tools available for non-model insects have limited functional characterisation studies of odorant receptors in economically important insects such as tsetse flies. A recently developed technique (Weid *et al.*, 2015) that compares change in mRNA due to odour stimulation could be a useful tool to identify potential ORs genes in non-model insects. In this technique, when live insects were exposed to a given odorant, the expression levels of mRNA transcript of ORs activated by the odour were altered; some were up-regulated, with others down-regulated (Weid *et al.*, 2015; Koerte *et al.*, 2018).

The main aim of this research was to describe the cellular and molecular basis of tsetse repellent odours coding using *Glossina f. fuscipes*, an important vector of both human and African Animal trypanosomiasis, based on activity-dependent changes in OR mRNA transcripts. Here, we show that exposure of *G. f. fuscipes* to the tsetse repellent blend changes mRNA transcript of several odorant receptors and the blend components elicit a strong antifeedant behaviour and physiological response in *G. f. fuscipes*.

3.2. Materials and methods

3.2.1. Biological material

The tsetse flies used in this study were obtained from the colony maintained at the insectary of the International Centre of Insect Physiology and Ecology (*icipe*). The flies were maintained at 24±1ºC, 75–80% relative humidity and were fed three times per week by membrane feeding with defibrinated bovine blood collected from local slaughterhouse.

3.2.2. Antifeeding bioassay

This experiment was performed using non-teneral flies (9-10 days old). Before the anti-feeding bioassay, the flies were starved for three days. In vitro feeding was done using a silicone membrane feeding system following standard mass rearing procedures (Feldmann, 1994; FAO/IAEA, 2006). To prepare the treated membrane, 100 µl of the diluted chemical at 10^{-3} v/v or the solvent was applied on 2116 cm^2 of a silicone membrane. The 4-component WRC comprised of δ -octalactone, geranylacetone, guaiacol and pentanoic acid roughly in a

ratio of 3:1:2:3 respectively as found naturally in the waterbuck odour (Gikonyo *et al.*, 2002). The chemicals were first loaded into the feeding membrane and spread to the whole surface of the membrane using cotton wool. The feeding started five minutes later after the application of the chemical on the silicon membrane when the solvent had evaporated. For each treatment, flies of the same age were separated into two groups of 20 flies each in a 1:1 sex ratio. The first group was fed on the treated silicone membrane, while the second group (control group) was fed on membrane treated with solvent only. The feeding of the two groups was simultaneously done and for each group, flies were individually fed and weighed before and after feeding. The feeding efficiency was calculated by the difference in weight of the individual fly before and after feeding. Using the feeding efficiency, the feeding index (FI) was calculated as (T-C)/(T+C) with T representing the amount of blood taken by the fly when a membrane is treated with given compound and C represents the amount of blood taken by the fly on an untreated membrane (solvent only). As previously done by (Dweck *et al.*, 2013; Ebrahim *et al.*, 2015) deviation of the feeding index from zero was tested with a Student's t-test ($P < 0.05$). The distribution of the data was checked using Shapiro test. The Student's t-test followed by Cohen's D test were performed on independent samples corresponding to different treatment. Multiple testing was not performed in any sample hence no p-value adjustment was required. The statistics were generated using R software (Team, 2018), (version 3.5.1), www.R-project.org.

Single sensillum recording

Both male and female *G. f. fuscipes*, 5–7 days old and starved for two days, were used. The single sensillum recording was done as described previously (de Bruyne, Clyne and Carlson, 1999; E. a Hallem, Ho and Carlson, 2004; Getahun *et al.*, 2012; Chahda *et al.*, 2019a; Soni, Sebastian Chahda and Carlson, 2019). Only 1 recording was mode from a single fly to avoid response adaptation from multiple stimulations. Briefly, the flies were mounted in a cut pipette tip (blue) with the head protruding and a small amount of wax placed at the back of the tip to prevent retraction of the fly. The pipette was then fixed onto a microscope slide with wax, and the antennae fixed on a coverslip with a sharpened glass electrode. A sharpened tungsten electrode was placed in the eye for grounding; a second recording electrode was brought in to contact with the base of the sensillum using a PM 10 Piezo manipulator. The electrodes were sharpened using saturated potassium nitrite (KNO_2) solution. The sensilla were observed with an Olympus BX-51WI microscope (Olympus Corporation, Tokyo, Japan) at 1000x magnification. Odorants were diluted in dichloromethane (DCM) at 10^{-3} v/v except for pentanoic acid, which was diluted in distilled water at 10^{-3} v/v from which 10µl of the

diluted odorant were pipetted on 1 cm diameter filter paper disk placed in glass Pasteur pipettes. Flies were stimulated by placing the tip of a cartridge into a glass tube which delivered a stream of humidified air (0.5 L/min) to the fly's antenna (Getahun *et al.*, 2012). The odours were delivered by puffing using Syntech stimulus delivery system. The odour stimulus was administered as 0.5 s pulse of charcoal-filtered air (5.9 ml/s) by placing the tip of the glass Pasteur pipette through a hole in a tube carrying a purified air stream. The signal was amplified (Syntech UN-06, http://www.syntech.nl), digitally converted (SyntechIDAC-4).The responses (spikes/s) were analysed by counting the number of spikes, 1 second during the 0.5 second stimulation minus 1 second before stimulation offline using the Autospike software (Olsson and Hansson, 2013). For spike count, neurons were sorted based on their amplitude. Responses of individual OSNs were calculated as the increase (or decrease) in impulse rate (spikes per second) relative to the pre-stimulus rate. Each sensillum was tested with all odorants. We used AutoSpike v3.9 signal acquisition software, Syntech Ockenfels, Germany).

3.2.3. Chemicals

The synthetic chemicals: Geranylacetone, δ-octalactone, guaiacol, pentanoic acid and1-octen-3-ol were purchased from Sigma-Aldrich at highest available purity. Geranylacetone, δ-octalactone, guaiacol, and 1-octen-3-ol were diluted in absolute ethanol (99.8%) (Conde, 2014; Karlsson and Friedman, 2017) and pentanoic acid in distilled water.

3.2.4. Odorants exposure and RNA extraction

The flies were exposed to different odorants for 5 h (Weid *et al.*, 2015; Koerte *et al.*, 2018) in a Plexiglas cubic cage (13.5 x 13.5 x 20 cm). Males and females were exposed in a separate cage under mass-rearing conditions and 25 flies were placed per cage for the odorant exposure. After exposure, flies were chilled at -80º C for 5 min and their antennae were removed on ice. The main reason why we targeted antenna is because the WRC is a spatial repellent. Antennae were removed from 150 flies (male-female ratio 1:1) representing three biological replicates. Dissected antennae of male and female were mixed and collected in 2.0-mL microcentrifuge tubes. The microcentrifuges were stored in liquid nitrogen during the antennae dissection to preserve the integrity of RNA transcripts. After dissection, samples were homogenised with a bead mill using Tissue Lyser LT, Qiagen for 10 min at 50 Hz. The samples were centrifuged at 13000 g for 5 min, and the 350 µl of the homogenate was used

for the total RNA isolation. Total RNA was isolated using TRIzol Reagent (Invitrogen, Thermo Scientific), following the manufacturer's instructions. RNA concentration and purity were evaluated using spectrophotometer (GeneQuant Pro RNA/DNA calculator, Amersham Biosciences, Cambridge, U.K.) measuring absorbance at A260 and A280 nm. Before converting to cDNA, RNA was temporarily stored at -80°C in nuclease-free water.

3.2.5. Quantitative real-time RT-PCR assay and data analysis

The total RNA was reverse transcribed from 500 ng in a 20 µl reaction mixture using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. The cDNA was amplified in 12.5 µl of 1× SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's instruction. The primers (Supplementary data 1) sets were designed with Primer3 software and optimised with gradient PCR using Kyratec Thermal cycler). qPCR experiment was performed with QuantStudio 3 using the comparative ΔΔCT method as previously described (Bustin and Nolan, 2004). A previous study (Koerte *et al.*, 2018) has used ORCO and CAM as reference genes and found that their mRNA transcripts levels could be altered by the exposure to chemicals. It was then suggested that the choice of ORCO as reference gene might be one of the factors that could affect the efficiency of the DREAM technique. Briefly, DREAM refers to **D**eorphanization of **r**eceptors based on **e**xpression **a**lterations of **m**RNA levels) which allow to identify the chemosensory receptors interacting with an odorant in a high-throughput manner instead of a deorphanization of single ligand-receptor pairs at a time. The method is based on the comparison the mRNA transcript levels of ORs between treated (exposed insects) and control (unexposed) insects using RT-qPCR (Koerte et al., 2018; Weid et al., 2015). Hence, in this study, we used b-actin as reference gene for our ΔΔCT calculation.

3.2.6. Ortholog comparison and in silico prediction of ligand-receptor interactions

Orthologs of odorant receptors were identified using Vectorbase (https://www.vectorbase.org) and Flybase (https://flybase.org).The receptor response profiles in *D. melanogaster* were identified in the Database of Odorant Responses (DoOR) (Münch and Galizia, 2016).

Homology modelling of the studied proteins was performed using fold recognition algorithm present in Phyre2 server (Kelley *et al.*, 2015). The "Intensive mode" which combine the ab-initio techniques was used to perform complete modelling of the entire proteins. The Olfactory co-receptor (Orco) structure (PDB ID: 6C70) from *Apocrypta bakeri* (Butterwick *et al.*, 2018) was used as a template for structure predictions. The template structure was

obtained at its high resolution (3.5 Å) from Protein Data Bank (Berman *et al.*, 2000). The quality of our predicted models was evaluated using SAVES v5.0 (https:// servicesn.mbi.ucla.edu/SAVES/) tools (Supplementary data 2).

Predicted 3-D models were optimised and molecular docking was performed using ICM-Pro software (Ruben Abagyan, 1994) version 3.8.7, MolSoft LCC San Diego, CA (http://www.molsoft.com/). The binding pockets were identified using ICM Pocket Finder before the molecular docking. The binding pocket was chosen within the extracellular part between and helix 2 and 3 (Lua *et al.*, 2016; Batra *et al.*, 2019). Membrane topologies were analysed using psipred-MEMSATSVM (http://bioinf.cs.ucl.ac.uk/psipred/).

3.2.7. Molecular dynamics (MD) simulations

For each WRC component, the best scoring complex was selected and subjected to molecular dynamics (MD) simulations using GROningen MAchine for Chemical Simulations (GROMACS) 5.1.2 (Pronk *et al.*, 2013). However, for δ- octalactone, we included one more complex with a different scoring given that we used its analogues as reference in Drosophila receptors. Primarily, the GROningen MOlecular Simulation (GROMOS)96 53a6 force field (Oostenbrink *et al.*, 2004) was used to generate the topologies of the protein structures in the docking based generated different complexes. Moreover, the topologies of the studied ligand compounds were generated using the PRODRG server (Schüttelkopf and Van Aalten, 2004). But the PRODRG does not contain server the functionality of generating the partial charges of the studied ligands, therefore, using the (Density functional Theory (DFT) method implemented in GAUSSIAN which utilised the B3LYP 6-31G (d,p) basis set and the CHELPG program (Frisch, M. J., Trucks, G. W., Schlegel, H. B., Scuseria, G. E., Robb, M. A., Cheeseman, J. R., Fox, 2009) was used for correction. After successful topology generation of the docked complexes, they were solvated using SPC/E water model (Zielkiewicz, 2005a) and then neutralised by adding the suitable number of sodium (NA) and chlorine (CL). Consequently, the systems were subjected to energy minimisation step using combined steepest descent as well as conjugate gradient algorithms, with a convergence criterion of 0.005 kcal/mol. Prior to the equilibration step the position restraints were applied to the structure of the ligands in the minimised system ligands before the equilibration phase (Idrees *et al.*, 2018; Shahbaaz *et al.*, 2018; Shahbaaz, Nkaule and Christoffels, 2019).

The equilibration step was carried out into the combined stages of NVT (constant volume) and NPT (constant pressure) ensemble conditions, each at 100 ps time scale. The temperature of 300 K was maintained for the system using Berendsen weak coupling method, and pressure

of 1 bar was maintained utilising Parrinello-Rahman barostat in the equilibration stage. The LINear Constraint Solver (LINCS) algorithm was used for the generation of final conformational production stage for 100 ns timescale, and trajectories were generated, which were analysed in order to understand the behaviour of each complex in the explicit water environment. The changes in the H-bonds, as well as the Root Mean Square Deviations (RMSD), and Radius of Gyration (Rg) of the complex systems were analysed (Idrees *et al.*, 2018; Shahbaaz, Nkaule and Christoffels, 2019). Furthermore, the Molecular mechanics Poisson–Boltzmann surface area (MM-PBSA) protocols implemented in g_mmpbsa package (Kumari *et al.*, 2014a) was used for the calculation of free energy of binding protein and the ligand molecules.

3.3. Results

3.3.1. Repellent odorants reduce tsetse fly blood feeding

Since WRB has a strong spatial repellent effect on *G. pallidipes* and reduced feeding (Bett, Saini and Hassanali, 2015; Saini *et al.*, 2017), we tested if it also influenced the blood feeding behaviour of this tsetse fly species and the related species *G. f. fuscipes* (Fig. 1A). We found that the feeding behaviour was significantly inhibited in *G. pallidipes* (t-test, P = 2.2e-16, d = 15.73, n = 20) relative to the control. Likewise, the feeding behaviour of *G. f. fuscipes* was also inhibited (t-test, P = 5.08e-13, d = 3.83, n = 20) (Fig, 1B). The feeding index of the flies fed on the treated membrane was -0.93 and - 0.74 in *G. pallidipes* and *G. f. fuscipes*, respectively. We then tested the contribution of each component of the WRB in this anti-feeding effect in subtractive assays. Removal of guaiacol from the blend (WRB-GU) did not affect the feeding inhibition in *G. f. fuscipes* flies (FI = -0.65, t-test, P = 3.356e-08, d = 1.98). However, removal of pentanoic acid (WRB-PA) or δ-octalactone (WRB-DO) significantly reduced the anti-feeding effect in this tsetse fly species compared to the antifeedant activity elicited by the full blend. The feeding index of flies fed on membrane treated with WRB minus pentanoic acid was -0.38 (t- test, P = 0.002755, d = 0.99) and -0.39 (t- test, P =0 0.001772, d = 1.03) for WRB minus δ-octalactone (Fig.1C). On the other hand, removal of geranylacetone from the WRB (WRB-GA) significantly reduced the antifeedant effect of the blend (FI = -0.26, t-test, P= 0.05536, d= 0.45) (Fig.1C).

Next, we assessed whether the blood feeding inhibition could be due to the presence of novel odours on the membrane. To do this, we tested the known tsetse fly attractant, 1-octen-3-ol,

and preferred hosts (Buffalo/ox) volatiles nonanal, decanal and octanal (Gikonyo *et al.*, 2002) as a positive control in identical assays. We found that decanal and octanal had no effect on the feeding efficiency, i.e. no inhibition or enhancement. However, nonanal and 1-octen-3-ol enhanced the feeding efficiency in *G. f. fuscipes* compared to the control (FI = 0.33; t-test, *P* = 0.004312, d = 1.36). (Fig. 1D). These results confirm that the feeding inhibition in Fig (1B-C) was not due to the presence of novel odours on the membrane, but due to the presence of specific odours, in our case the WRB blend.

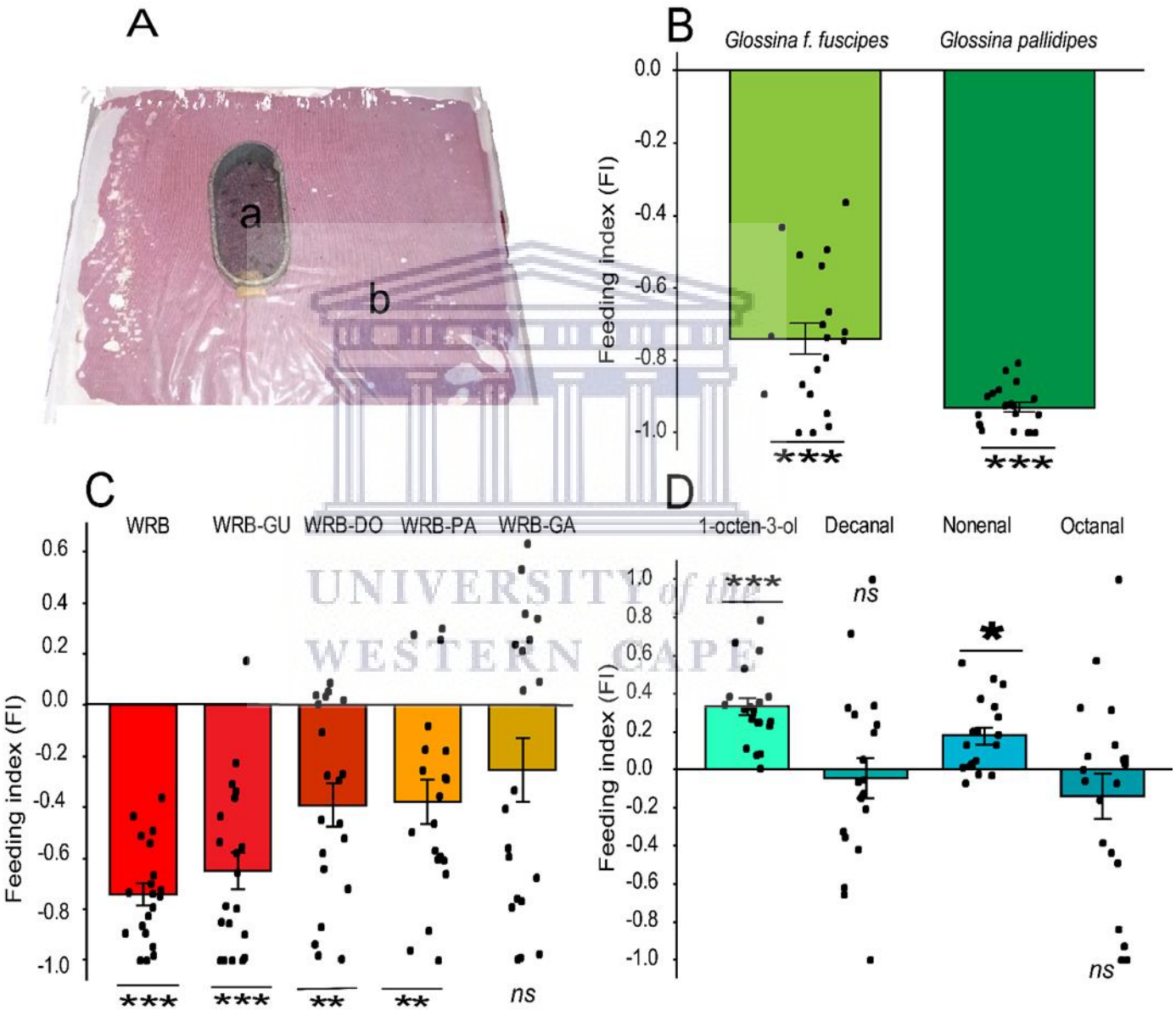

Figure 1: Anti-feeding effect of Waterbuck Repellent Blend (WRB).

(A). Anti-feeding bioassay setup; (a) represents the feeding cage, single fly/cage (original photo). (b) Indicates the feeding tray containing sterile blood covered by a silicone membrane.

(B) Feeding index (FI) of *G. f. fuscipes* and *G. pallidipes* fed on membrane treated with WRB. WRB = Waterbuck Repellent Blend. Deviation of the feeding index from zero was tested with a Student's t-test ($P < 0.05$). (C) Feeding index (FI) of *G. f. fuscipes* fed on membrane treated with WRB and showing the contribution of each compound to the anti-feeding effect. WRB-GU = WRB minus Guaiacol; WRB-DO = WRB minus δ-octalactone; WRB-PA = WRB minus pentanoic acid; WRB-GA = WRB minus geranylacetone (D) Feeding index (FI) of *G. f. fuscipes* fed on membrane treated with positive controls (an attractant). Deviation of the feeding index from zero was tested with a Student's t-test ($P < 0.05$). The number of stars indicate the level of significance, *** shows $P < 0.0001$, ** indicates $P < 0.001$, and * $P < 0.05$, d represents the effect of size (Cohen's D) and ns means non-significant. Error bars represent standard error, n=20 for each test. The graphs and the statistics were generated using R software (Team, 2018), (version 3.5.1), www.R-project.org.

3.3.2. Exposure to tsetse repellents odorants induced change in receptors of mRNA transcripts level

We used activity dependent change of mRNA transcript level (Weid *et al.*, 2015; Koerte *et al.*, 2018) in 27 odorant receptors in *G. f. fuscipes* to identify potential receptors of WRB and 1-octen-3-ol. These 27 odorant receptors were selected because of their Polymerase Chain Reaction (PCR) efficiency during optimisation of primers. We used the WRB components δ -octalactone, geranylacetone, guaiacol and pentanoic acid, mixed at the following ratio of 3:1:2:3, respectively according to their abundance in waterbuck odour (Gikonyo *et al.*, 2002, 2003; Mwangi, Gikonyo and Ndiege, 2008; Bett, Saini and Hassanali, 2015) and the attractant 1-octen-3-ol (Vale and Hall, 1985), which has a different odour valence in this experiment. We found that the mRNA transcripts of odorant receptors were differentially affected by the various odorant exposures after 5 h. The exposure of flies to δ-octalactone induced down-regulation of six ORs mRNA; however, 9 ORs mRNA transcripts were up-regulated (Fig. 2A). The exposure to geranylacetone altered the mRNA transcripts levels of 20 ORs in total, whereby nine were up-regulated with 11 down-regulated (Fig. 2B). Exposure to guaiacol affected the transcript levels of 22 ORs, in which 15 of them were up-regulated, while 7 ORs mRNA transcripts were down-regulated (Fig. 2C). However, the number of ORs that were up- and down-regulated were not significantly different ($P > 0.05$). Exposure to pentanoic acid affected 14 ORs mRNA transcripts; four were up-regulated, whereas 10 were down-regulated (Fig. 2D). In contrast, the attractant chemical 1-octen-3-ol significantly up-regulated mRNA transcripts of 21 ORs, but down-regulated mRNA of only one odorant

receptor transcripts (*GffOr94b*) (Fig. 2E), (χ^2, 18.8, df=1, P <0.0001). The gene expression patterns are well represented in the heatmap (Fig. 3). The positive control, gene i.e., the co-receptor Orco mRNA expression was not affected by the odour exposure in all exposed flies (Fig. 2A-E).

To correlate feeding inhibition and ORs mRNA gene expression alteration, we performed a principal component analysis (PCA). The PCA was based on the log fold change in ORs mRNA transcripts expression. The PCA analysis separated the three components (geranylacetone, δ-octalactone and pentanoic acid) that significantly deterred fly feeding from the blend component (guaiacol) that had no effect on feeding. Interestingly, the receptors activated profiles of attractant, feeding stimulant, 1-octen-3-ol was clearly discriminated by principal component analysis (PCA) from the other odorants that inhibited blood feeding (Fig.2F). However, the two compounds 1-octen-3-ol and guaiacol which elicited no detectable feeding inhibition induced over expression of ORs mRNA transcripts in several ORs as compared to those down-regulated, but that of guaiacol is not significantly different.

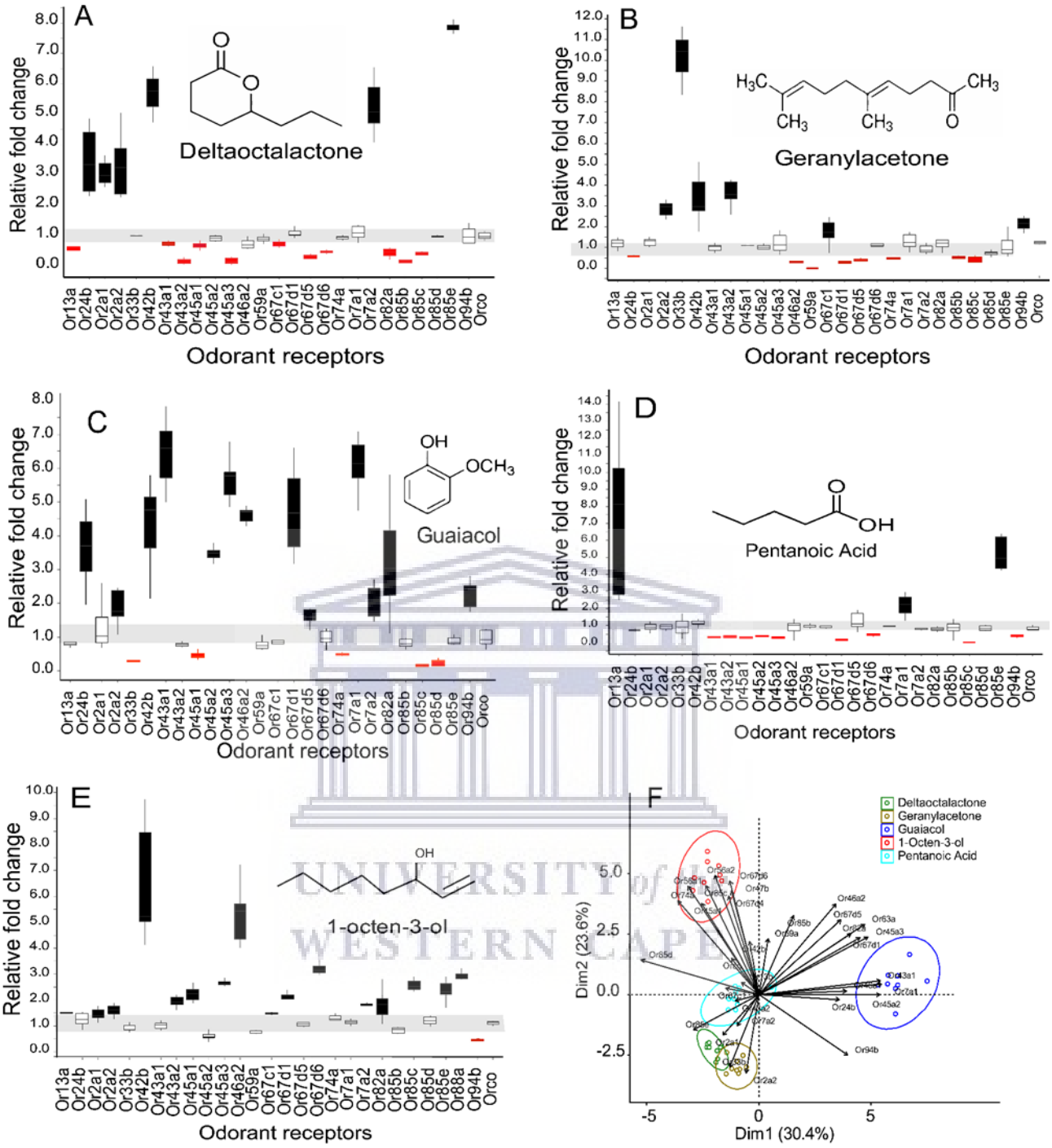

Figure 2: Expression pattern of *G. f. fuscipes* odorant receptors after exposure to WRB components and 1-octen-3-ol. The horizontal grey zone corresponds to ORs mRNA transcript values that were not affected. (**A**) Expression pattern of ORs to δ-octalactone. (**B**) Expression pattern of ORs to geranylacetone. (**C**) Expression pattern of ORs to guaiacol. (**D**) Expression patterns of ORs to pentanoic acid. (**E**) Expression pattern of ORs to 1-octen-3-ol. (**F**) PCA plot showing the clustering pattern of the five tested ligands based on the fold change of the mRNA of ORs (**A-E**).

The PCA explained 53.6% of the total variation. Odorant receptors that are not falling in one of the clusters circles (Fig. 2F) shows that the mRNA transcripts level was not affected by the odorant exposure. The graphs and the statistics were generated using R software21, (version 3.5.1), www.R-project.org. The principal components analysis (PCA) was performed using two R packages called "FactoMineR" and "Factoextra (Kassambara, no date) .

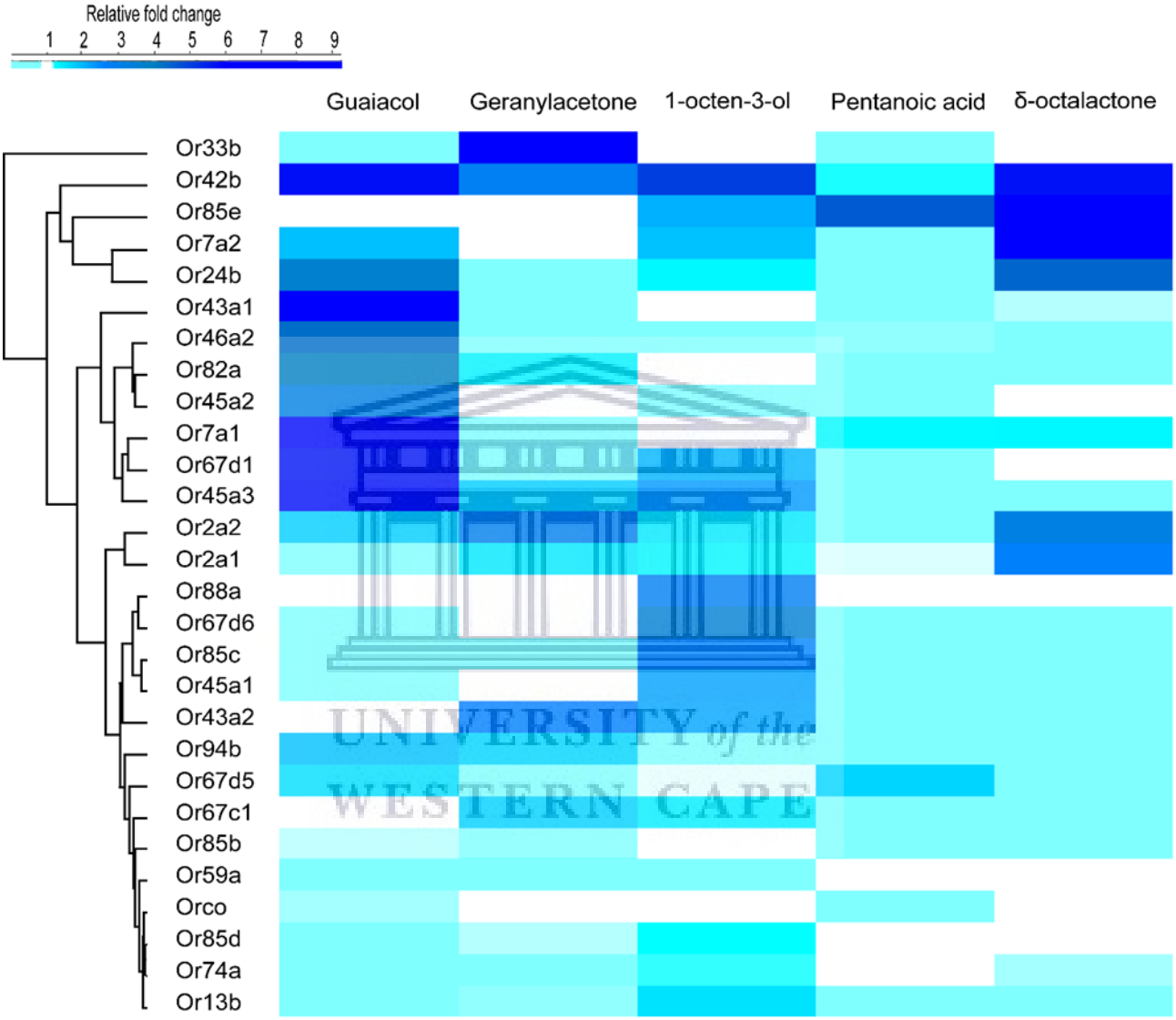

Figure 3: Heat map showing the differential expression of *G. f. fuscipes* odorant receptors across the five odorants, generated using R software (Team, 2018) (version 3.5.1) www.R-project.org, edited using adobe illustrator CS5.1.

3.3.3. Comparison of the response profile of G. f. fuscipes receptors to their orthologous receptors in Drosophila melanogaster

We aimed to compare the ligand-receptor pairing in our study to their deorphanized orthologs in *Drosophila melanogaster that* has functionally well characterised ORs for comparison with *G. f. fuscipes* odorant receptor orthologs genes. For *D. melanogaster*, there is an online platform (DoOR: Database of Odorant Responses) that provides an extensive database for known ligand-odorant receptor pairs.

In our activity-dependent change in mRNA expression, exposure to pentanoic acid affected many ORs mRNA transcripts, in which most were down-regulated and a few up-regulated. Similarly, in *D. melanogaster* pentanoic acid activates several ORs (DoOR). Comparing our data to pentanoic acid (Kreher, Kwon and Carlson, 2005; Hallem and Carlson, 2006; Silbering *et al.*, 2008; Galizia and Rössler, 2010), we found substantial similarity between 9 ORs whose mRNA expression were altered: (GffOr7a1, GffOr7a2, GffOr13a, GffOr43a1, GffOr43a2, GffOr43a3, GffOr45a1, GffOr45a2 and GffOr63a in *G. f. fuscipes* (Fig. 2D, Fig.3) and their orthologs in *D. melanogaster* (DoOR). In the DoOR database, only DmOr19b has been reported as receptors of geranylacetone in *D. melanogaster*. Its Orthologs in *G. f. fuscipes*, which is GffOr2a2, also elicited a change in mRNA expression level to geranylacetone exposure. Additionally, other receptors of *G. f. fuscipes* were affected by the geranylacetone exposure (Fig. 2B, Fig. 3). The orthologs of the remaining *G. f. fuscipes* odorants receptors that responded to geranylacetone in our study were not reported as receptors of geranylacetone in the DoOR database. Guaiacol has four receptors in *D. melanogaster* (DmOr7a, DmOr19b, DmOr22a and DmOr71a), according to previous studies (Marcus C Stensmyr *et al.*, 2003; Hany K.M. Dweck *et al.*, 2015) GffOr7a1 and GffOr7a2 orthologs of DmOr7a, GffOr42b ortholog of DmOr22a and GffOr2a1 ortholog of DmOr19b were all up-regulated after exposure to guaiacol in our study. The following ORs of *D. melanogaster*, DmOr47b, DmOr33b, DmOr35a and DmOr85b are orthologs of GffOr47b, GffOr33b, GffOr74a and GffOr85c, respectively do not respond to guaiacol but elicited a response to the related the compound, 4-ethylguaiacol.

The ORs for δ-octalactone are not reported yet in DoOR; however, the receptors for some of its analogues have been documented in DoOR database. Comparing the G. *f. fuscipes* ORs affected by δ-octalactone exposure to the response profile of some of the orthologs in *D. melanogaster*, we found similarities between *D. melanogaster* Or35a, Or19a, Or22a with the following GffOr85b, GffOr45a3, GffOr24b, and GffOr7a2, as potential receptor of δ-octalactone in *G. f. fuscipes*. The attractant compound, 1-octen-3-ol affected several ORs

mRNA transcripts in most of them by up-regulation (Fig. 2E, Fig. 3). Similarly, in *D. melanogaster,* 1-octen-3-ol activated many ORs. The change in mRNA transcripts of GffOr13a, GffOr42b, and GffOr88a match with the following orthologs ORs in *D. melanogaster,* DmOr43a2, DmOr43a1, and DmOr59a, which are 1-octen-3-ol receptors. Showing GffOr13a, GffOr42b, and GffOr88a are potential 1-octen-3-ol receptors in *G. f. fuscipes*.

3.3.4. *In silico* prediction of ligand-odorants receptors interaction.

We further compared the response profile of *G. f. fuscipes* and their orthologs ORs in *D. melanogaster* using molecular docking to predict the potential odorant receptors and ligand interactions. In *G. f. fuscipes,* all the receptors whose ORs expression were up-regulated or down-regulated to after exposure to the WRB components, were docked within extracellular loop-2 and 3. Before ligand-odorant interaction studies, we checked the topology of the odorant receptors using psipred-MEMSATSVM (http://bioinf.cs.ucl.ac.uk/psipred/).

As *D. melanogaster* odorant receptors are well deorphanized; we first identified the best receptors for our ligand in DoOR database. We compared the binding affinity score of *G. f. fuscipes* to *D. melanogaster* receptors binding scores. The binding affinity scores were considered as reference. Since no receptors have been reported for δ-octalactone in the DoOR database, we chose its analogue compounds γ-octalactone and hexa-octalactone receptors, DmOr35a, DmOr19b and DmOr22a in *D. melanogaster*. The binding affinities of these receptors with δ-octalactone were -22.14Kcal/mol, -16.59Kcal/mol and -12.59Kcal/mol, respectively. In *G. f. fuscipes,* five receptors showed similar binding affinity (Table 1). DmOr19b is known as the receptor of geranyl acetone, the only reported receptor. Its binding affinity with its ligand is -14.15 Kcal/mol. In *G. f. fuscipes*, GffOr2a2, GffOr59a and GffOr33b interacted with geranylacetone (Table 1). We found almost equal binding affinity (env. -15.5Kcal/mol), when we docked guaiacol to 3 of its receptors in *D. melanogaster*. In *G. f. fuscipes,* guaiacol showed high binding affinity of -25.84Kcal/mol, -16.43Kcal/mol, and -15.65Kcal/mol with GffOr46a2, GffOr67d1 and GffOr45a3, respectively. *In D. melanogaster*, we selected DmOr7a, DmOr22a and DmOr71a as reference receptors for pentanoic acid. The docking of pentanoic acid to the selected receptors showed binding energy between -15.75Kcal/mol and -25.84Kcal/mol. In *G. f. fuscipes*, we found that four receptors have similar or higher binding efficiency (Table 1). 1-Octen-3-ol is known to be detected by

several receptors. Based on the binding score of three receptors of *D. melanogaster*, we identified GffOr13a, GffOr42b and GffOr88a as potential receptors of 1-octen-3-ol in *G. f. fuscipes.*

Table 1: Docking scores of the potential receptors for WRB components and 1-octen-3-ol identified in *G. f. fuscipes* through ligand receptors interactions

Ligands	***Drosophila melanogaster***		***Glossina f. fuscipes***	
	Docking score (Kcal/mol)	ORs	Docking score (Kcal/mol)	ORs
δ-octalactone	-22.14	Or35a	-21.51	Or85b
	-16.59	Or19a	-17.24	Or45a3
	-12.59	Or22a	-16.19	Or24b
			-14.33	Or7a2
Geranylacetone	-14.15	Or19b	-16.38	Or2a2
			-15.21	Or59a
			-14.11	Or33b
Guaiacol	-15.33	Or7a	-25.84	Or46a2
	-15.52	Or22a	-16.43	Or67d1
	-15.52	Or71a	-15.65	Or45a3
Pentanoic acid	-16.53	Or7a	-25.84	Or45a2
	-16.20	Or45a	-21.21	Or67d6
	-15.75	Or67a	-21.51	Or43a1
			-15.82	Or67d1
1-octen-3-ol	-17.51	Or43a2	-19.94	Or13a
	-11.31	Or43a1	-18.77	Or42b
	-10.63	Or59a	-17.62	Or88a

3.3.5. Molecular Dynamic (MD) simulations

- ***Hydrogen bonding pattern of docked ORx-ligand complexes***

The 100 ns MD simulations were performed for the validation of the docking based generated parameters and the patterns of the hydrogen bonding between the protein and ligand were studied during the course of MD simulations (Fig. 4). MD simulations highlighted the changes observed in the structure of the studied protein with highest structural stability was observed in the GffOr85b_δ-octalactone complex. The Hydrogen bonding is involved in a diversity of the cellular functionalities as it regulates the molecular interactions in the metabolic processes. Therefore, the understanding of the molecular functions such as ligand binding effects requires the analyses of the hydrogen bond perturbations. The

GffOr2a2_Geranylacetone showed the presence of three H-bonds, while in GffOr24b_δ-octalactone one H-bonds were observed. In GffOr45a2_Pentanoic_acid complex, the hydrogen bonds were observed till 20 ns time period and up to 70 ns lesser number of H-bonds, but afterwards, a constant three H-bonds were observed (Fig. 4). Moreover, the GffOr46a2_Guaiacol, GffOr85b_δ-octalactone and GffOr13a_1_octen_3_ol showed similar H-bonds pattern with the number raised to one. These observations indicated that the binding of GffOr2a2_Geranylacetone is more favourable compared to the other studied systems.

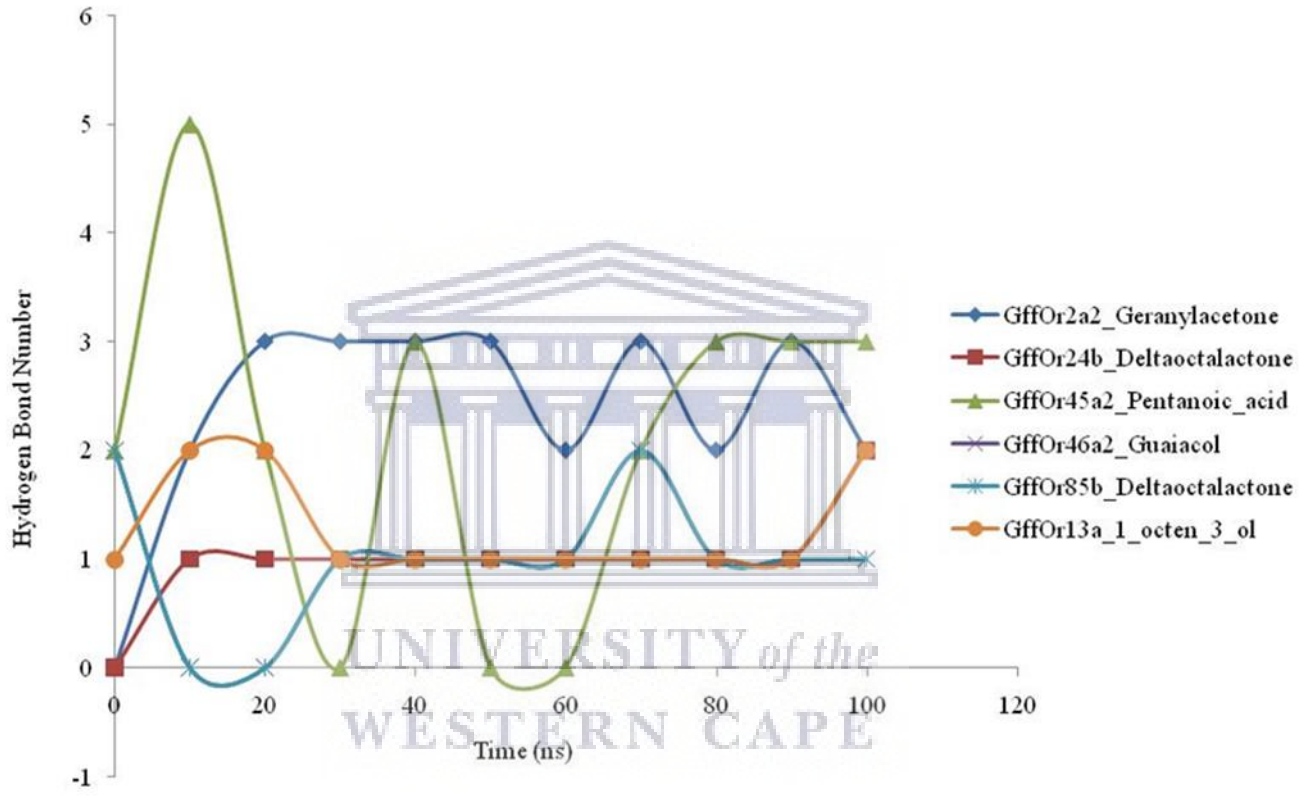

Figure 4: The time lapse curve highlighting the changes observed in the number of hydrogen bonding between the receptors and the studied ligands during the course of 100 ns MD simulations.

- ***Evaluation of complex compactness and stability***

The radius of gyration (Rg) was computed using "gmx gyrate" module of the GROMACS which illustrated the stability of the protein by calculating the compactness of the system, which is the reflection of the stable nature of the protein (Fig. 6A). The variations in the Rg values were observed around 2.5 nm for GffOr2a2_Geranylacetone system, while in GffOr24b_δ-octalactone the Rg values fluctuated between 2.6 nm - 2.7 nm which was higher than the rest of the system, indicative of less compactness in the respective system (Fig. 5A).

In GffOr45a2_Pentanoic_acid, the Rg values fluctuated around 2.4 nm up to 40 ns but afterwards rose up to 2.6 nm and then gradually decreased and became stable between 2.4 nm - 2.5 nm after 70 ns time period (Fig. 5A). Similarly, for GffOr46a2_Guaiacol, the Rg values varied around 2.4 nm, but in GffOr85b_δ-octalactone, the highest compactness was observed, which was indicative of the obtained Rg values present between 2.2 nm - 2.3 nm. Moreover, the GffOr13a_1_octen_3_ol system showed the lowest compactness among the studied complexes, indicating lesser degree of protein folding (Fig. 5A).

Furthermore, the conformational stability of the studied docked systems was further assessed using Root Mean Square Deviation (RMSD) values (Fig. 5B). It was observed that GffOr45a2_Pewntanoic_acid interaction was the least stable in its nature during 100 ns MD simulations with RMSD values continuously fluctuating, which elevated sharply after 40 ns time period but stabilised after 70 ns (Fig.5B). The GffOr2a2_Geranylacetone, GffOr24b_δ-octalactone and GffOr13a_1_octen_3_ol showed a relatively similar pattern, indicating similar stability was present in the systems, but GffOr46a2_Guaiacol was slightly more stable than the respective systems (Fig. 5B). The GffOr85b_δ-octalactone achieved the highest stability as observed from the measured RMSD values (Fig. 5B).

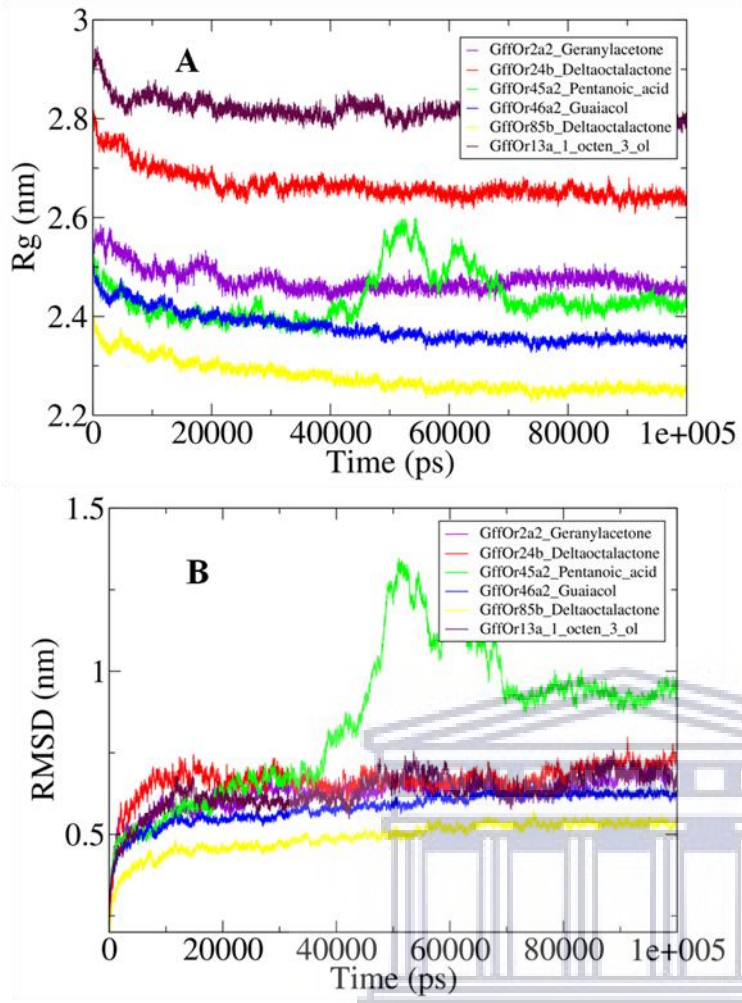

Figure 5: The complexes stability assessment curves with (A) highlighting the changes observed in the pattern of Radius of Gyration (Rg) (B) illustrating the fluctuations in the patterns of Root Mean Square Deviation (RMSD) values.

- ***Time evolution of system energies***

The Molecular mechanics Poisson–Boltzmann surface area (MM/PBSA) based algorithm was used to calculate the interaction energies, as an indication of the binding strength between the proteins and the ligands (Fig. 6). The MM/PBSA calculate the free energies of the interactions by combining three energetic terms, namely, potential energy in the vacuum, solvation energies in the implicit solvation model and configurational entropy associated with complex formation (Kumari *et al.*, 2014a). In GffOr2a2_Geranylacetone complex, the total free energy of interactions was observed between -100 kJ/mol - -150 kJ/mol, while for GffOr24b_δ-octalactone the energy was observed around -200 kJ/mol. In the GffOr45a2_Pentanoic_acid system, the total energy was observed between -1000 kJ/mol and -500 kJ/mol up to 20 ns but afterwards, the interaction became unfavourable, indicating changes in the energy values (Fig.

6). Similarly, in GffOr46a2_Guaiacol, the total free energy of binding observed was around -150 kJ/mol. The lowest interaction energies of around -300 kJ/mol were observed in GffOr85b_δ-octalactone system, indicating the relatively favourable nature of binding between the respective protein and ligand. In addition, the total energy of GffOr13a_1_octen_3_ol observed was between -50 kJ/mol and -100 kJ/mol, with a lesser contribution of the electrostatic energy.

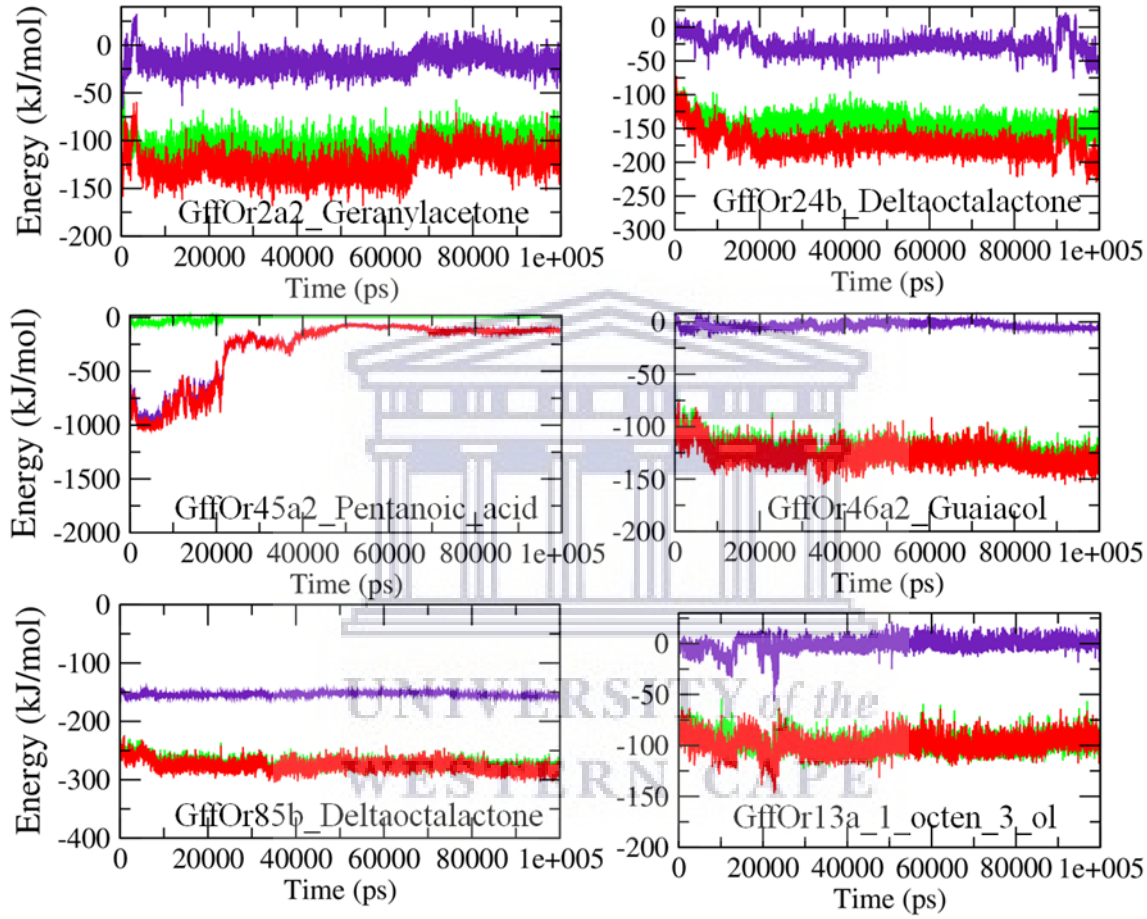

Figure 6: The MM/PBSA based energy curves highlighting the changes observed in the pattern of the interaction energies observed between the studied receptors and ligands during the course of 100 ns MD simulations. (Green - Vander Waals energy, Indigo - Electrostatic energy, Red - Total energy).

3.3.6. Docking-based odorant receptor-ligand interaction sites studies

To explore the binding interface of the selected top five scorings docked complex, 2D interaction diagram were built using ICM-Pro software (Version 3.8-7, MolSoft LCC San Diego, CA, www.molsoft.com). Several predominantly hydrophobic interactions were

observed between the tested ligand and putative receptor binding site residues. Our predicted ligand-binding pockets consisted mostly of hydrophobic residues, with a few hydrogen bonds, supporting the MD data, except the Geranylacetone-GffOr2a2 which did not show any hydrogen bond formation, with unknown reason. We found that geranylacetone could possibly interact with GffOr2a2 at up to 9 possible interactions sites, including at G139, S143, I 304 and F305 (Fig. 7A). δ-Octalactone-Or24b and δ-octalactone-GffOr85b showed respectively 6 and 8 interactions sites. The residues F210, M277, F295, I299 and P345 could be potential interaction sites. Likewise, the complex δ-octalactone-Or24b, I113, C101, W114 and G112 are predicted potential interaction sites. Also, 2 H-bonding were observed in δ-octalactone-GffOr85b, but 1H-bonding in δ-octalactone-Or24b (Fig. 7B-C). The less stable complex, pentanoic acid-GffOr45a2 showed possible interactions with F110, S103 and L107. Additionally, 2 H-bonding were observed at I105 and S102 for this complex (Fig. 7D). The complex Guaiacol-Or46a2 showed 5 possible interactions residues, K69, S174, V177, C291 and M387 and 1 H-bonding at Q390 (Fig. 7E). 1-Octen-3-ol-GffOr13a revealed 9 (L94, M154, L158, I294, L298, S295, S326, S327 and Y323) interaction sites. We also noted 2 H-bonding at residues M91 and Q330 for 1-octen-3-ol-GffOr13a complex (Fig. 7E).

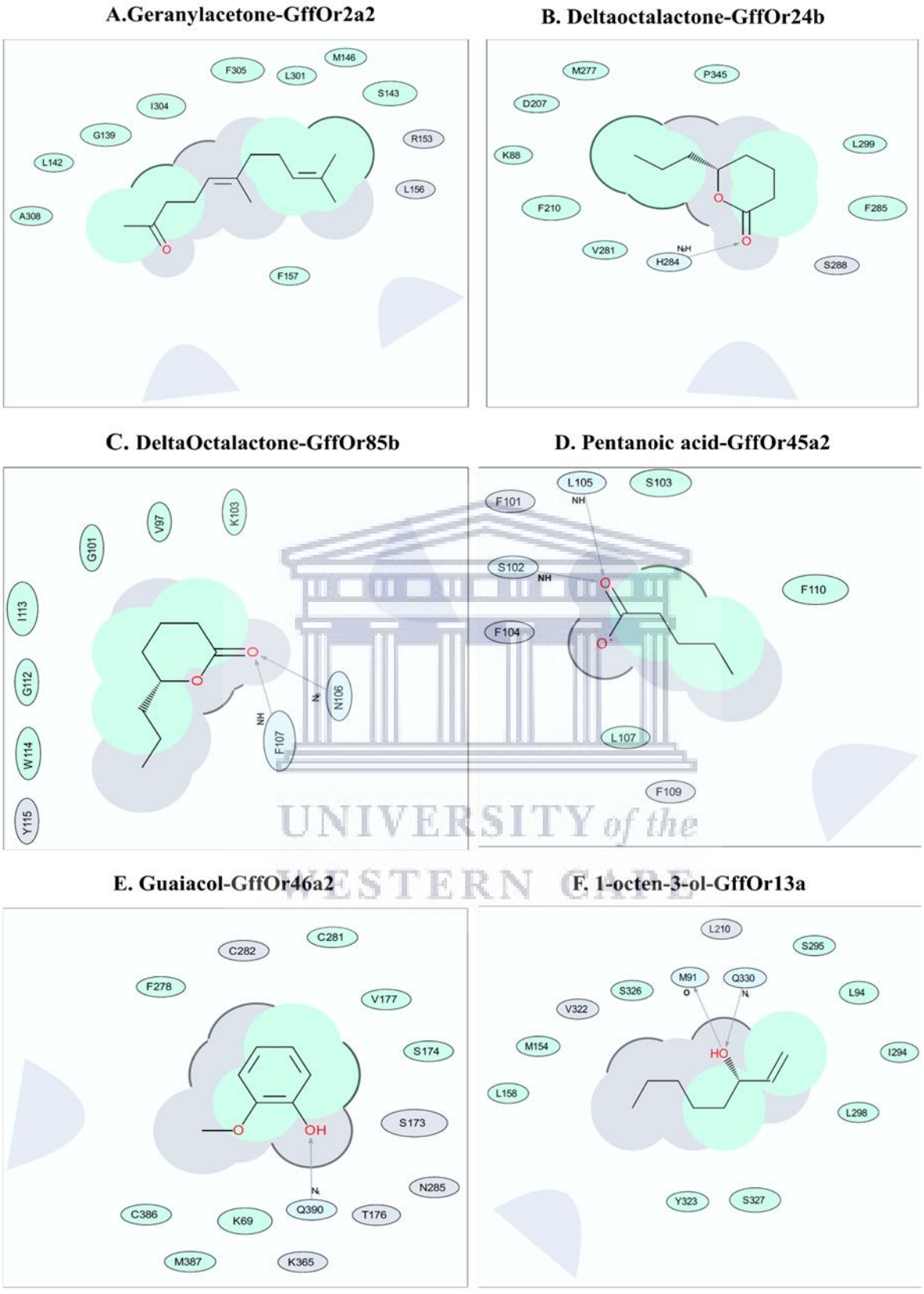

Figure 7. Bioinformatics analysis for the interaction of the different ligands to their putative odorant receptors. Amino acid residues in interaction within 5Å of ligands as depicted by

ligand interaction diagram for pairs (A) Geranyl acetone-GffOr2a2, (B) δ-octalactone_GffOr24b (C) δ-octalactone-GffOr85b, (D) pentanoic acid-GffOr45a2, (E) Guaiacol-GffOr46a2 and (F) 1-octen-3-ol-GffOr13a. Generated using ICM-Pro software (Ruben Abagyan, 1994) (**Version 3.8-7**, MolSoft LCC San Diego, CA, www.molsoft.com). Green shading represents the hydrophobic region.

- White dashed arrows represent hydrogen bonds.
- Grey parabolas represent accessible surface for large areas.
- The broken thick line around ligand shape indicates an accessible surface.
- Size of residue ellipse represents the strength of the contact.

3.3.7. Olfactory sensory neuronal response

Having identified the change in transcript levels of ORs mRNA, followed by docking and MD analysis, and identified putative OR, next, we analysed the physiological response of the WRB, the four components and 1-octen-3-ol using single sensillum recording techniques. Dilution levels at 10^{-3} v/v were used to validate the presence of receptor proteins in the olfactory sensilla of *G. f. fuscipes*. The electrophysiological recording was done only from large basiconic sensilla (Fig. 8A), we found these sensilla types distributed well all over the antennal region and basiconic sensilla house odorant receptors responding to host odours (Fig. 8A). From the targeted sensilla (n =14), most of the sensilla housed one to two OSNs per sensillum based on their spike amplitude. The targeted sensillum consistently showed spontaneous action potential. The Odour-OSN interaction resulted into different response dynamics and spikes magnitude. For example, some odour (1-octen-3-ol) resulted in a prolonged response and activated all tested sensilla, while others resulted into a phasic response, i.e geranyl acetone (Fig. 8E). Delta-octalactone, gave a response of 172 spikes/sec, while 1-octen-3-ol 102 spikes/sec in some sensillum. Similalry, WRB blend elected a response of 188 spikes/sec in one sensillum. However, geranyl acetone did not elicit a strong response; the maximum from all tested sensilla was 53 spikes/sec. Pentanoic acid similarly elicited a maximum response of 74 spikes/sec. Guaiacol, which is a WRB component, resulted up to 159 spikes/sec. The olfactory sensory neurons housed in the targeted sensillum varied in their response spectrum, some are selective even at the tested concentration (SB14, SB7, and SB10), while others responded to most of the tested odours (SB1, SB2, SB11) (Fig. 8I). Furthermore, the WRB blend, elicited a distinct response from its constituents response (Fig 8F and I).

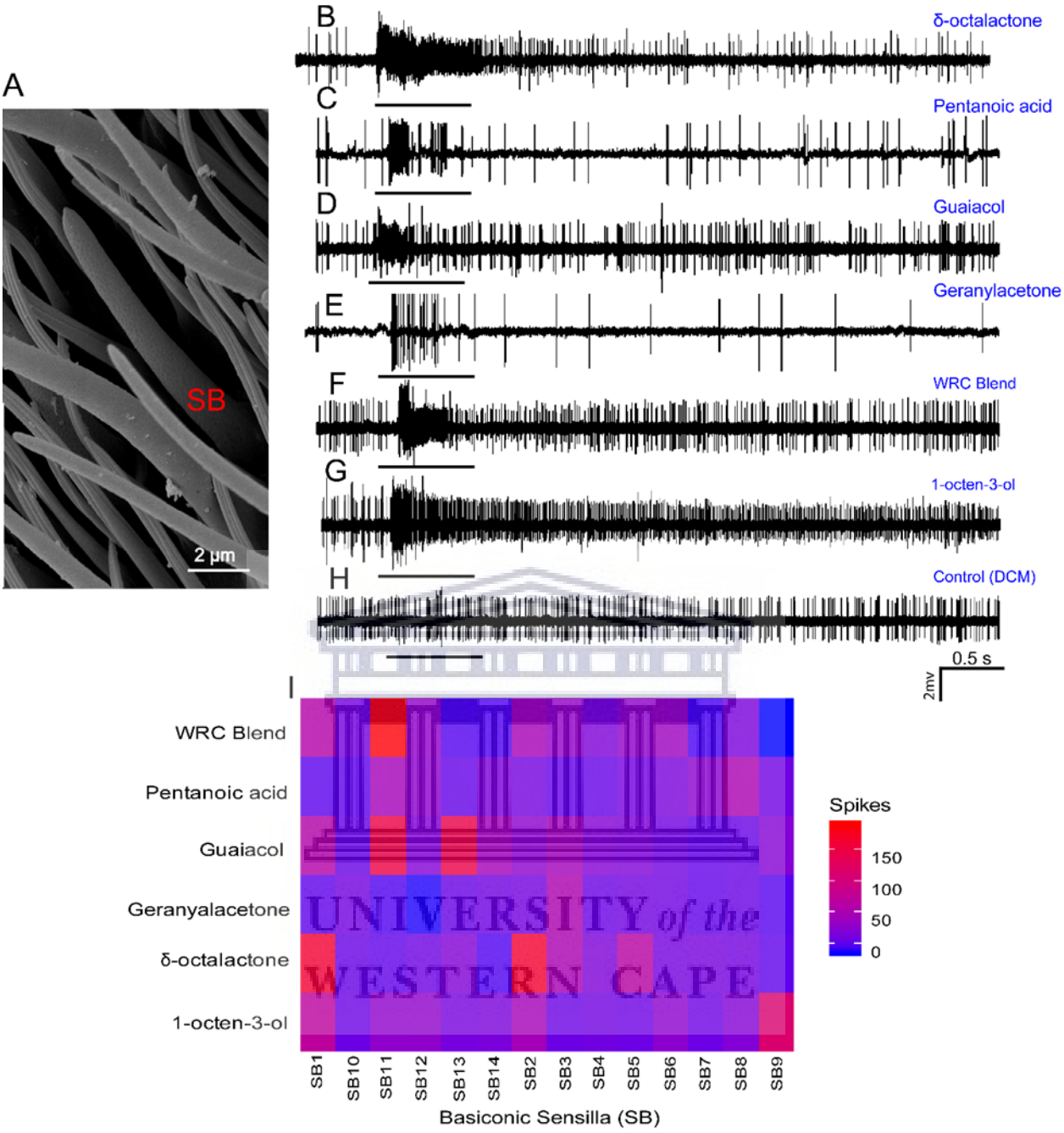

Figure 8: Cellular response patterns of basiconic sensilla of *G. f. fuscipes* to different chemicals. ***A.*** *Scanning electron micrograph of the basiconic Sensilla (SB) of G. f. fuscipes.* ***B–H.*** Representative Single sensillum Recording (SSR) traces, showing responses to the indicated odorant and the control (DCM). **I.** Heatmap of olfactory sensory neurons responses patterns of 14 basiconic sensilla of *G. f. fuscipes* elicited by different odours used in DREAM techniques, generated using R software (Team, 2018) (version 3.5.1), www.R-project.org, edited using adobe illustrator CS5.1.

3.4. Discussion

In this study, we analysed how tsetse repellent components are coded in the olfactory sensilla of the tsetse fly *G. f. fuscipes*. The tsetse repellents showed strong antifeedant activity to *G. f. fuscipes*. Of the WRB components, pentanoic acid, δ-octalactone and geranylacetone, contributed strongly to the antifeeding effect, with geranylacetone appearing to serve as the key odour contributing greatly to the feeding inhibition effect of the WRB. Nonetheless, our results show that these three components are essential and enough to elicit the strongest antifeedant activity of the WRB. Since removal of guaiacol did not affect the feeding of *G. f. fuscipes* on treated blood, our results suggest that guaiacol plays no role in the antifeedant effect of the WRB.

In field trap capture studies showed that removal of δ-octalactone from the blend reduced the repellency of the WRB on *G. pallidipes* (Bett, Saini and Hassanali, 2015). Likewise, when tested singly, pentanoic acid and geranyl acetone were found to reduce trap catches of *G. pallidipes* (Bett, Saini and Hassanali, 2015). A recent study on *G. f. fuscipes* under field conditions showed that WRB reduced trap catches by 33% and the different components had a different contribution to spatial repellency of the WRB (Mbewe *et al.*, 2019). Our results show the additional effect of WRB as an antifeedant, besides its spatial repellency. Similar results have been found in mosquitoes using DEET, whereby DEET affected the feeding behaviour of mosquitoes, in addition to its spatial repellency (Wei *et al.*, 2017). Similarly, geosmin inhibited the feeding of *D. melanogaster*, functioning as an antifeedant, operating via the olfactory system, besides its strong repellency (Stensmyr *et al.*, 2012). Furthermore, in mosquitoes, olfactory sensilla are associated with other organs such as the stylet, suggesting that olfaction plays a role in odour perception at close range (Won Jung *et al.*, 2015). Because, tsetse flies and other insects express gustatory receptors (GRs) over their entire bodies (Macharia et al., 2016; Obiero et al., 2014;Vosshall and Stocker, 2007), so taste might also play a role in blood feeding as well as in its inhibition, at short range in feeding that needs further investigation.

We characterised the change in transcript expression change of the entire Odorant receptors in the antenna (ORs) of *G. f. fuscipes* to determine which ORs are involved in the detection of WRB. We found that in *G. f. fuscipes*, following *in vivo* exposure to WRB volatile chemicals in an open cage, olfactory receptor genes that interacted with the given odour responded in two ways. Some of the ORs mRNA transcripts were down-regulated, while others

overexpressed their mRNA transcripts as reported previously (Weid *et al.*, 2015; Ibarra-Soria *et al.*, 2017; Dewan *et al.*, 2018; Koerte *et al.*, 2018). To account for this significant reduction in mRNA transcripts, we hypothesise that it has an adaptive function in response to olfactory over stimulation. It would represent a form of neural plasticity that would desensitise neurons, similar to other molecular adaptations that take place at the olfactory transduction or processing levels (Leinders-Zufall *et al.*, 2000; Kato and Touhara, 2009; Getahun *et al.*, 2012; Weid *et al.*, 2015). Since a previous dose-response experiment in *D. melanogaster* after odour exposure did not show a change in sensitivity (Koerte *et al.*, 2018), we cannot rule out this hypothesis without carrying an ecological setting studies that challenge the OSNs sensitivity.

Additionally, mRNA transcripts of some ORs significantly increased by up to 10-fold change. Similarly**,** (Ibarra-Soria *et al.*, 2017; Koerte *et al.*, 2018) has shown that some ORs mRNA transcript increased due to odour exposure. The differential (up and down) regulation in transcript levels of the various ORs is not clear, but it shows their involvement in the detection of the odour to which the insects were exposed. Similarly, (Ibarra-Soria *et al.*, 2017) showed stimulation with odours resulted in modulation of many olfactory receptors genes, whereby some up-regulated and in other their mRNA transcript level down-regulated. The opposite change in ORs mRNA transcript to the same compound shows, there is an individualised response in the olfactory sensory neurons of *G. f. fuscipes*, which might provide the olfactory system freedom of odour coding and neuronal diversity. The current hypothesis about up and down-regulation of ORs mRNA transcript is not clear. According to previous studies (Weid *et al.*, 2015; Koerte *et al.*, 2018), up-regulation is because of OSNs inhibition. Interpreting ORs transcript up-regulation is because OSN inhibition is difficult. We showed almost all of the targeted OSN response to be excitatory (Fig. 8) which could be due to targeting a subset of sensilla, as demonstrated in the present study. Furthermore, because in the present study, we stimulated the entire receptor repertoires (the whole sensilla), we could potentially have generated a mixed response. Our alternative hypothesis is the OSNs plasticity to handle the high influx of odours encountered. For example, in the moth pheromone system, sensillum housing OSNs which respond to the major pheromone component have a high pore density accompanied by high ORs expression to handle the maximal ranges of molecular flux imparted by major pheromone component in every plume strand (Baker, Domingue and Myrick, 2012). Furthermore, prior exposure to a given odour that creates a rich olfactory experience shapes the OSNs tuned to the exposed odour to be more sensitive and enhances its

discriminative power, showing exposure-dependent adaptation at the level of the receptor neuron (Iyengar *et al.*, 2010).

Deorphanization of odorant receptors of non-model insect is a challenge, but the DREAM method developed by Weid et al., 2015 allows for its use in non-model insects, like *G. f. fuscipes*. Furthermore, according to Koerte et al., 2018, there is a good correlation between the change in mRNA and receptor-ligand interaction in the model Drosophila is about 69%. However, Koerte et al., 2018 also noted the limitations of DREAM in predicting potential receptor false positive and false negative results. For example, the change in mRNA is influenced by both concentration and exposure time (Weid et al., 2015), thus the use of high concentration might result in false positive, as ORs are less specific at higher concentration (Hallem et al., 2004). Additionally, with exposure time, since the change in mRNA is reversible (Weid *et al.*, 2015), if ORs are exposed for a long time the required change in mRNA might not be capturedIn the present study, we combined the DREAM technique with molecular docking and compared the corresponding orthologs of *D. melanogaster* to allow us to predict putative ORs. Combining the three methods significantly reduced the number of putative receptors for each odour into a few possible receptors when compared to using the DREAM technique alone (Table 1). Additionally, our molecular docking results showed a strong affinity between ligands and identified putative receptors. The Molecular dynamics results of the top scoring docked OR-ligand complex showed a stable complex and strong binding affinity, which demonstrates the reliability of our docking scores. Hence, the identified putative receptors selected in Table 1 could be some of the receptors of the ligands used in this study. Similarly, the orthologous receptors from *D. melanogaster* also responded to the given odours or their analogues of δ-octalactone (DoOR) showing that DREAM combined with molecular docking, followed by orthologs comparison and physiological studies can predict potential receptors for a given odour in a non-model insect such as *G. f. fuscipes*. Interestingly, Orco gene expression was not affected by all the tested odorants, showing it can be used as a reference gene for these odorant receptors. This shows it is not directly involved in these ligand interactions, as previously showed (Nichols *et al.*, 2011), but important for the ORx-Orco functionality and behavioural response (Mattias C. Larsson *et al.*, 2004; Getahun *et al.*, 2016; Fandino *et al.*, 2019).

With regard to the correlation of the antifeedant effect with up- and down-regulation of mRNA, odours that inhibited blood feeding also affected the mRNA transcript in a mixed

response both by up and down-regulation of mRNA transcript. On the other hand, the WRB components that did not inhibit feeding modified the ORs mRNA transcript by up-regulating after homology modelling followed by orthologs comparison. Our results show that odours with strong antifeedant effects and feeding stimulants are coded differently at the molecular level. In the future, it is important to address how the activation of these receptors elicit repellent and antifeedant behaviour. Various studies in *D. melanogaster* showed different mechanisms of repellency, that is, the activation of the dedicated olfactory circuit (Stensmyr *et al.*, 2012). Furthermore, other researchers showed that a given odour valence changed due to its concentration, and correlated with the recruitment of additional glomeruli (Semmelhack and Wang, 2009; Strutz *et al.*, 2014) that changed the odour valence from attractant to repellent.

Tsetse flies exhibit a reduced number of ORs as compared to other Dipteran flies (Attardo *et al.*, 2019). We have analysed the WRB, its constituents, and 1-octen-3-ol coding in the large basiconic sensilla expressed on the antenna of *G. f. fuscipes*. Different response dynamics were elicited by the different tested odours on the same sensillum. The different response of the given OSN to different odours suggests that physico-chemical properties of the constituent odorants may influence their interaction with receptors. Similar to previous findings (Soni, Sebastian Chahda and Carlson, 2019), tsetse attractant, 1-octen-3-ol activated most of the tested sensilla, and also resulted in prolonged responses in some sensilla. The WRB blend elicited a distinct response from its individual constituents, showing the integration of olfactory information beginning at the periphery, as also shown in Drosophila (Su *et al.*, 2011; Getahun *et al.*, 2012) moths (Kramer, 1992) and in beetles (Nikonov and Leal, 2002). Our results are consistent with other conclusions (Soni, Sebastian Chahda and Carlson, 2019) about the absence of strong response from targeted sensilla.

Our results show that some of the OSNs of *G. f. fuscipes* are less specific, whereby one odorant receptor can respond to multiple ligands, and a single ligand can activate multiple odorant receptors (Fig. 2 and 8). Similarly, OSNs of *G. morsitans. morsitans* have been found to be broadly tuned to diverse chemical classes (Soni, Sebastian Chahda and Carlson, 2019). Likewise, in other insects, it has been demonstrated that non-pheromone volatiles can activate multiple odorant receptors and non-pheromone receptors can also detect more than one chemical including insect repellents and attractants (Firestein C7587, 2001; Hallem and Carlson, 2006; Bohbot and Dickens, 2012). Recently, in *G. morsitans morsitans*, GmmOr9

was shown to respond to chemically diverse odours, acetone, 2-butanone and 2-propanol, and 1-octen-3-ol, the later activated all targeted sensilla (Chahda *et al.*, 2019b; Soni, Sebastian Chahda and Carlson, 2019). Similar results were recently found in *G. f. fuscipes* and *G. pallidipes* (Ouedraogo and den Otter, 2018). The odorant concentration [10^{-3}] dilution v/v at which we exposed our flies could have induced responses in the majority of the receptors and might be higher than the ecological concentration they encounter in their natural environment. However, their generalist response has to be challenged by using other physiological set-ups such as GC-single sensillum recording (Stensmyr *et al.*, 2012; Dweck *et al.*, 2013) or at low concentration stimulation (De Bruyne and Baker, 2008; Getahun *et al.*, 2016). However, as previously reported (Soni, Sebastian Chahda and Carlson, 2019), the spike number response seems less even when tested with high concentrations as compared to Drosophila, for unknown reasons. In the future it is necessary to characterise their responses using different approaches, such as to express these putative receptors in an empty neuron system (Chahda *et al.*, 2019b), such system will enable us to validate the identification of these potential/putative receptors using DREAM combined with molecular docking. Also, it is important to show the reduction or up-regulation in mRNA, into a corresponding decrease in ORs expression on the dendrite of OSNs in *G. f. fuscipes* sensillum shaft, and the opposite in these ORs with their mRNA significantly increased. The continuous use of only one type of tsetse repellents might also lead to repellent resistance flies as has been demonstrated in other insects for the well-known repellent DEET (Reeder *et al.*, 2001; Nina M Stanczyk *et al.*, 2010). Thus, the identification of the cellular and molecular target of this strong spatial repellent and antifeedant, WRB could lead to the discovery of alternative repellents, by targeting the same receptors.

3.5. Conclusions

In conclusion, our study has demonstrated that WRB has a strong antifeedant effect beside its spatial repellency. Furthermore, the DREAM technique, combined with molecular docking, molecular dynamics, Orthologs comparison and electrophysiology has enabled us to predict the putative ORs involved in coding of this behaviourally well characterised odorant in the non-model tsetse fly *G. f. fuscipes.* Our molecular and physiological analysis of ORs mRNA alteration patterns evoked by repellents and attractants odorants suggests that they vary at the molecular and cellular level by the identity of the activated odorant receptors.

Chapter four

Structural insights into functional analysis of *Glossina fuscipes fuscipes* (Newstead, 1910) odorants binding proteins

Abstract

Olfaction is orchestrated at different stage and involves various proteins at each step. For example, Odorant binding proteins (OBPs) are soluble proteins found in sensillum lymph that might encounter odorants before reaching the odorant receptors. In tsetse flies, the function of OBPs in olfaction is less understood. Herein, we investigated the role of OBPs in Glossina fuscipes fuscipes olfaction, main vector of sleeping sickness using multidisciplinary approaches. Our tissue expression study demonstrated that GffLush was conserved in legs and antenna in both sexes, whereas GffObp44 and GffObp69 were expressed in the legs but absent in the antenna. GffObp99 absent in female antenna but expressed in male antenna. Short odorants exposure induced a fast alteration in the transcription of OBP genes. Furthermore, we successfully silenced specific OBP expressed in antenna via dsRNAi feeding to decipher its function. We found that silencing OBPs that interact with 1-octen-3-ol significantly abolished flies' attraction to 1-octen-3-ol a known attractant for tsetse fly. However, OBPs that demonstrated weak interaction with 1-octen-3-ol did not affect the behavioural response even though it was successfully silenced. Thus, OBPs selective interaction with ligands, their expression in the antenna and significant impact on behaviour when silenced demonstrated their direct involvement in olfaction.

Keywords: Glossina sp, Odorant binding proteins, gene expression, structural properties, molecular docking, dsRNAi.

4.1. Introduction

The terrestrial life style of insects has necessitated the adjustment of the olfactory system such as the evolution of odorant receptors and OBPs in flying insects (Missbach *et al.*, 2014, 2015; Getahun *et al.*, 2016). The OBPs which have evolved independently from OR and earlier function is not well understood especially in medically important non model insects, such as tsetse flies biological vector of human and animal trypanosomiasis. The *Glossina sp* genome expresses several chemosensory proteins including odorant-binding proteins (OBPs) (Liu *et al.*, 2012; George F.O. Obiero *et al.*, 2014; Macharia *et al.*, 2016; Attardo *et al.*, 2019). Human African trypanosomiasis (HAT) is caused by two closely related parasites that are transmitted by tsetse flies (Leak, 1999; Solano, Kaba, Ravel, Naomi A. Dyer, *et al.*, 2010). In this regards, *Glossina fuscipes fuscipes* is the most important vector of HAT (Rogers DJ, 2004; Gooding and Krafsur, 2005; Krafsur, Marquez and Ouma, 2008; Krafsur, 2009). More precisely, *G. f. fuscipes* is involved in the transmission of 90 % of HAT (Aksoy *et al.*, 2013; Tirados *et al.*, 2015). This tsetse species is also known to have an opportunistic blood-feeding behaviour on livestock (Clausen *et al.*, 1998) and therefore contributes to the transmission of African Animal Trypanosomiasis (AAT). To date, no vaccine is available for HAT and AAT; and the vector control tools, offer a highly valued approach to the disease control. Currently, vector control is achieved through sequential aerial spraying (SAT), ground spraying, insecticide-treated targets or insecticide-treated animals as live baits, the use of traps and the sterile insect technique (SIT) (Takken and Weiss, 1974b; Rogers, Hendrickx and Slingenbergh, 1994; Klassen, 2005). Particularly, traps and targets have been widely used in tsetse control campaigns in many countries across Africa despite the fact that type of target and their efficacy largely vary according to the specie and the geographical location [20–24]. For example, targets usually with 1.0 × 1.0 m in size have been found to be effective for *G. pallidipes* Austen and *G. morsitans morsitans* Westwood [20,21] while small target with 0.25 × 0.25 m in size catch more *G fuscipes fuscipes* [22,24,25]. It has been suggested that an addition of an appropriate attractants odours such as CO2, acetone, 1-octen-3-ol [2,20,6–28] and phenols [29–31] could improve the efficacy of the traps hence enormous effort was deployed to find efficient combination.

A blend of 3-n-propylphenol, 1-octen-3-ol, p-cresol and acetone enhance trap catches of tsetse flies of the morsitans group [21, 32]. Also, chemicals present in lizard odour can increase the numbers of *G. f. fuscipes* attracted to traps [33]. Despite of this enormous effort, we still lack an effective attractants or repellents for many riverine tsetse species, which are both medically and veterinary important vectors. Indeed, the main challenge in finding new or to improve the

tsetse attractant and repellents is that the target olfactory sensory neurons and the chemosensory proteins through which the attractants (and repellents operate are less investigated. However, recent progress in tsetse genomes has opened a new opportunity to investigate olfactory pathways in Glossina sp [5–7]

We believe that a better understanding of tsetse olfaction, the main sensory modality used to locate its hosts including human and livestock will contribute to the improvement of the intervention strategies used to control tsetse fly.

Insect OBPs contain an alpha helical barrel and are characterised by a highly conserved six-cysteine signature (C1-X20-35-C2-X3-C3 -X20-30-C4 -X8-12-C5-X8-C6) (Hekmat-Scafe *et al.*, 2002; Liu *et al.*, 2012; Sun *et al.*, 2017). The structural characteristics of insect OBPs make them suitable target for biosensor technology and to identify attractants which could help in the designing of an environmentally friendly tools for vectors control. From the model Drosophila, it is well established that OBPs contribute to the sensitivity of the olfactory system [16–19, 20–22, 23]. Their elicited a strong binding affinity with odorant compounds in insect like moth (Krieger *et al.*, 1996; Leal *et al.*, 2005; Khuhro *et al.*, 2017) OBPs may also be involved in gain control of olfactory sensory neurons (OSNs) response (Larter *et al.*, 2016; Scheuermann and Smith, 2019). Furthermore, OBPs play a role in social interactions (Bentzur *et al.*, 2018), essential amino acid detection (Rihani *et al.*, 2019) for taste and deactivation kinetics of signal transduction (Scheuermann and Smith, 2019). In insect vector like mosquitos, OBPs was found to be important for the detection of oviposition attractant (Pelletier *et al.*, 2010) and others general odour compounds (Biessmann *et al.*, 2010; Pelletier *et al.*, 2010; J. *et al.*, 2012). However, the function of OBPs in odor communication in insect in general is still elusive and particularly is not investigated in non-model insect like tsetse fly. For example, OBPs-ligand interaction, their molecular features, tissue-specific expression patterns and their role in odour detection and perception are yet to be studied in *Glossina sp*. Here, we investigated the olfactory function of OBPs using behaviourally well studied odorants. Hence, we aimed in this study to investigate the role of some selected OBPs in *Glossina fuscipes fuscipes.*

To decipher OBPs functional roles in olfaction, we first analysed the structural features of *G. f. fuscipes* odorant-binding proteins. Secondly, tissue-specific expression was done on nine selected OBPs, and then we targeted four OBPs that are expressed in the antenna (the main olfactory organ) of both male and female to study their olfactory function using RNA interference (RNAi) technology. Our multidisciplinary study demonstrated that OBPs expressed in the antennae are of critical importance for *G. f. fuscipes* olfaction.

4.2. Material and Method

4.2.1. Biological material

All the tsetse flies (*Glossina f. fuscipes*) used in this study were obtained from a colony maintained at the insectary of the International Centre of Insect Physiology and Ecology (*icipe*). The colony was maintained at 24±1ºC, 75–80% RH (relative humidity) under 12L:12D photoperiod and the flies were fed three times per week on silicon membrane with defibrinated bovine blood collected locally (Feldmann, 1994).

4.2.2. Chemicals

The chemicals (geranylacetone, δ-octalactone, guaiacol, pentanoic acid and 1-octen-3-ol) were purchased from Sigma-Aldrich at highest available purity. For the odorant exposure experiment, chemicals were diluted at 10-3 v/v. Pentanoic acid was di-luted in water. Geranyl acetone, δ-octalactone, guaiacol, and 1-octen-3-ol were diluted in absolute ethanol (99.8%) [42,43].

4.2.3. Odorants exposure and RNA extraction

Odorant exposure was done as previously described by (Weid *et al.*, 2015; Koerte *et al.*, 2018; Diallo *et al.*, 2020). Briefly, 25 teneral flies with 2-3 days old were exposed to different odorants volatiles (geranylacetone, δ-octalactone, guaiacol, pentanoic acid and 1-octen-3-ol) in a Plexiglas cage measuring 13.5 × 20 x 20 cm for 5 hours. The exposure room conditions were similar to the conditions at which the colony was maintained. Males and females were exposed in a separate cage to avoid mating and/or the release of potential sex pheromone, which might interfere with the experiments. After exposure, flies were snap-frozen for 5 min in a -80ºC freezer, and the antennae were harvested on ice. Antennae were manually removed from fly head and three replicates of 150 flies (male-female ratio 1:1) were used. Dissected antennae of male and female flies were pooled in 2.-mL microcentrifuge tubes. To conserve the integrity of the total RNA, the microcentrifuges were deep-frozen in liquid nitrogen during the antennae dissection. Tissues were homogenized in a bead mill (TissueLyser LT, Qiagen) for 10 min at 50 Hz and then centrifuged at 13000 g for 5 min, and 350 µl of the homogenate was used for total RNA isolation. TRIzol™ Reagent (Invitrogen, Thermo Scientific, Waltham, MA USA 02451) was used to isolate total RNA. RNA quality and quantity checked with a spectrophotometer (GeneQuant Pro RNA/DNA calculator, Amersham Biosciences, Cambridge, U.K.).

4.2.4. Quantitative real-time RT-PCR assay and data analysis

High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) was used for the cDNA synthesis according to the manufacturer's instructions. 500 ng of total RNA was reverse transcribed in a final volume of 20 µL reaction mixture. The cDNA was amplified with 5x HOT FIREPol® EvaGreen®qPCR Mix Plus (ROX) (Solis BioDyne Inc) according to the manufacturer's instructions. Real-time PCR was carried with QuantStudio 3 (Applied Biosystems 7500, USA) using the comparative ΔΔCT method as previously described (Rao *et al.*, 2014). The primer (Supplementary Table 3) sets were designed with Primer3 software http://bioinfo.ut.ee/primer3-0.4.0/ (Untergasser *et al.*, 2012) and optimized with gradient PCR (using Kyratec Thermal cycler). Additionally, tissue-specific expression was done using antennae and legs using real time quantitative PCR. The legs were chosen because several studies have demonstrated the abundance of OBPs in that part of the body (Sun *et al.*, 2017; Song, Sun and Du, 2018; Yang *et al.*, 2020). The tissue expression was used to validate the quality of the primers, also to identify a potential contact pheromone/Odour binding proteins. The PCR products were loaded on 1.5% ethidium bromide-stained agarose gel and visualized using ultraviolet light.

4.2.5. Structural analysis of *G. f. fuscipes* Odorant binding proteins

Sequences for *G. f. fuscipes* OBPs were retrieved from VectorBase (www.vectobase.org) and compared by multiple alignments performed by Multiple Sequence Comparison by Log-Expectation (MUSCLE) after removal of signal peptides. The resultant alignment was viewed and manually edited using Jalview (Waterhouse *et al.*, 2009). The signal peptide screening was performed using SignalP-5.0 webserver http:/www.cbs.dtu.dk/services/SignalP (Almagro Armenteros *et al.*, 2019). SignalP was chosen because of its reliability compared to the other available tools (Zhang, Li and Li, 2009; Almagro Armenteros *et al.*, 2019; Nielsen *et al.*, 2019). It combines deep learning and recurrent neural network to predict signal peptides (Almagro Armenteros *et al.*, 2019)

4.2.6. Homology modeling and binding pocket analysis

Homology modelling was done using Protein Homology/analogy, Recognition Engine V 2.0 (Phyre2) (Kelley *et al.*, 2015). Intensive mode and *ab-initio* techniques were used to perform complete modelling of the entire proteins. Swissmodel and I-tasser were used to generate comparative model but, Phyre2 offered better models. The quality of our predicted the models was checked using SAVES v5.0 (https://servicesn.mbi.ucla.edu/SAVES) tools, ProSA and

Qmean. The binding pocket was identified using DoGSite Scorer (**http://proteins.plus**) (Ahrrolfes *et al.*, 2017). The pocket size, shape and functional descriptors were compared and analysed.

4.2.7. Model optimization and molecular docking

The models were optimized and docked using ICM-Pro version 3.5 (Molsoft LLC). Five docking scores were generated which correspond to five different conformations. Best docking score for each odorant-binding protein was used for the statistical analysis and to build the heatmap graph. The heatmap graph was generated in R version 3.5.1 (Team, 2018). Ligplot+ was used to generate the 2D interaction diagram of different complexes.

4.2.8. Molecular Dynamic Simulations

The top-scoring four docked complexes were selected and subjected to MD simulations using GROMACS 2018-2 software package (Pronk *et al.*, 2013). Initially, the topologies of the protein structures in the docked complexes were generated using the GROMOS96 53a6 force field (Oostenbrink *et al.*, 2004) and the PRODRG server was used for the parameterization of complexes ligand (Schu, 2004). The latter server does not contain the functional module for the calculation of the partial charges. Consequently, the DFT method implemented in GAUSSIAN with B3LYP 6-31G (d,p) basis set in a combination of CHELPG program (Frisch, M. J., Trucks, G. W., Schlegel, H. B., Scuseria, G. E., Robb, M. A., Cheeseman, J. R., . . . Fox, 2009) was used for the charge correction. After parameterization, the solvation of docked complexes was performed using SPC/E water model (Zielkiewicz, 2005b) which was followed by neutralization in which countering of NA and CL were added to stabilize the systems. As a result, the solvated-neutralized systems were energetically minimized in the consecutive step using combined steepest descent and conjugate gradient algorithms, with a convergence criterion of 0.005 kcal/mol. Afterwards, the position restraints were applied to the structures of system ligands before the equilibration phase.

The equilibration step was carried out in combined NVT (constant volume) and NPT (constant pressure) ensemble stages, each at 100 ps time scale. The temperature of 300 K was maintained for the system using Berendsen weak coupling method, and pressure of 1 bar was maintained utilizing Parrinello-Rahman barostat in the equilibration stage. In the final production stage, the conformations were generated using the LINCS algorithm for 100 ns timescale, and trajectories were generated, which were analysed to understand the behaviour of each complex in the explicit water environment. The changes in the H-bonds, protein-

ligand distances, Gyration (Rg) as well as the Root Mean Square Deviations (RMSD) were analysed using the GROMACS utilities. Furthermore, the *g_mmpbsa* package was used for the calculation of the free energies of interaction between the complexed protein-ligand systems using the principles of Molecular mechanics Poisson–Boltzmann surface area (MM-PBSA) protocols (Kumari *et al.*, 2014b).

4.2.9. dsRNA preparation and its delivery to flies

dsRNAi targeting four OBPs genes (GffObp19a, GffObp83a1, GffObp83a2 and GffObp83a4) was prepared from PCR amplicons tailed with T7 promoter sequences using the Replicator RNAi kit (Finnzymes) according to manufacturer's instructions. The PCR amplification was done using gene-specific primers which were manually designed from the coding sequence (CDS) of each gene (supplementary table 3). To confirm the specificity of the primers, the PCR product was sent to Macrogen Europe B.V. Amsterdam, Netherlands for sequencing. For the PCR template, we used cDNA synthesized from total RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). The total RNA was extracted from the antennae and legs of 150 flies (male-female ratio 1:1). TRIzol™ Reagent (Invitrogen, Thermo Scientific) was used to isolate total RNA and RNA quality and quantity were assessed with a spectrophotometer (GeneQuant Pro RNA/DNA calculator, Amersham Biosciences, Cambridge, U.K.). Contaminating genomic DNA was removed from the transcription reaction by DNase treatment. dsRNAi was eluted in nuclease-free water and the concentrations were measured using a spectrophotometer (GeneQuant Pro RNA/DNA calculator, Amersham Biosciences, Cambridge, U.K.).

dsRNAi was delivered to flies by feeding through pre-warmed (at 37°C) bloodmeal containing the dsRNA (Figure 4A-B). The protocol of mixing the bloodmeal and the dsRNAi was adapted from (D. P. Walshe *et al.*, 2009). Twenty flies teneral were fed in a cage and kept under insectarium conditions for 72 h post dsRNAi feeding. Approximately, 10 µL of dsRNAi diluted to appropriate concentrations in nuclease-free water was added to 500 µL of bloodmeal. Unfed flies were automatically removed after the feeding. For each experimental group, 80 flies were used, 20 flies were used for the gene silencing efficiency checking after 96 h post-feeding and 60 flies used for the behavioural assay. Nuclease free water was used as the internal control.

4.2.10. Bioassay

To assess how the silencing of different OBPs affect the behaviour of the flies, attraction bioassay was performed in a plastic cage length=75cm, width=30cm, height=45cm) containing two sticky paper (13*10 cm) used as a trap (Figure 6D). The bioassay was done in same conditions as in the insectary with 24±1ºC, 75–80% RH (relative humidity) under 12L:12D photoperiod. On the sticky paper, we placed a cotton roll (100mmX15mm) which served as a dispenser. For attractive odour source, 100 µL of 10-3v/v 1-octen-3-ol diluted in mineral oil was loaded on the cotton roll dispenser and 100 µL of mineral oil only to serve as a control. In the cage, 20 flies (male and female in 1:1 sex ratio) starved for 3 days were introduced and each experiment was replicated three times. The flies were introduced into the cage 20 minutes before loading the attractive odour and the mineral oil. Flies were allowed to choose between the attractive source and the control for 24h afterwards the attraction was scored and the attraction index (AI) was calculated using:

$$AI = (N_{odour} - N_{control})/N_{total}$$

With N_{odour} corresponding to the number of flies trapped at the odour source, $N_{control}$ the number of flies trapped at the control and N_{total} the number of flies used for the assay (Retzke *et al.*, 2017). The significant difference between the attraction index were noted using the test of analysis of variance (ANOVA) followed by the Turkey's HSD posthoc test; owing to the normality of the data (shapiro test: $p>0.05$) and the homogeneity of the variance (Levene test: $p>0.05$).

4.3. Results

4.3.1. Structural analysis of *Glossina f. fuscipes* odorants binding proteins

To study the function of *G. f. fuscipes* OBPs, we retrieved from VectorBase all the putative OBPs that have been previously identified in *G. f. fuscipes* (Macharia *et al.*, 2016). In total, 23 odorant-binding proteins were analysed for their molecular structural features. The molecular weight of these OBPs ranged between 12-30 kDa, and the predicted OBP sequences encoded between 107 and 258 amino acids and less sequence similarity observed. The signal peptides screening showed three OBPs; GffObp44a, GffObp57c and GffObp84a lack a signal peptide sequence, but the other 19 OBPs had a signal peptide at N-termini. According to the number

of conserved cysteine motifs, Odorant Binding Proteins (OBPs) are divided into four groups (Classic OBPs, Minus-C OBPs, Plus-C OBPs, and atypical OBPs). Our structural analysis of G. *f. fuscipes* OBPs clustered in to three different classes: the classic OBPs, minus-C OBPs, and atypical OBPs as described in previous studies (Hekmat-Scafe *et al.*, 2002; Liu *et al.*, 2012). Twelve OBPs; GffObp19, GffObp19b, GffObp28a, Gffobp56d, Gffobp56e, Gffobp57c, Gffobp69a, Gffobp83a4, Gffobp83g, Gffobp84a, Gffobp99d and GffLush, showed six conserved cysteine (C1-C6) motifs and hence classified as classic OBPs. GffObp44a, GffObp8a, and Gffobp99b showed less than six conserved cysteine motifs and were classified as minus-C OBPs. Several OBPs (Gffobp19a, GffObp19c, GffObp56h, GffObp56i, GffObp83a1, GffObp83a2, GffObp83cd, and GffObp83ef) had more than six cysteine motifs and classified as atypical OBPs (Figure 1). We did not find any Plus-C OBPs having a proline residue next to the sixth conserved cysteine, from *G. f. fuscipes* OBPs analysed.

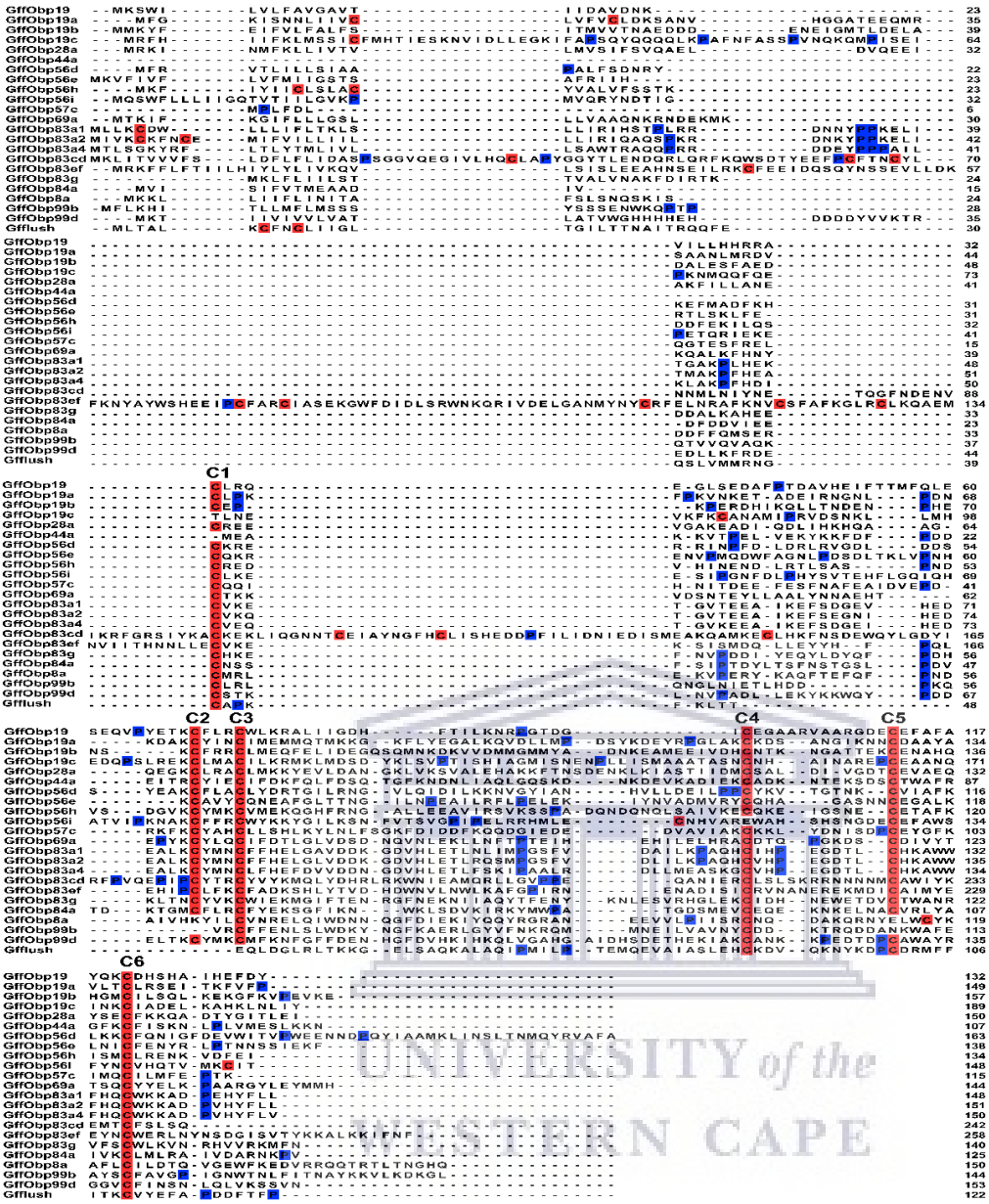

Figure1: Multiple alignments of *G. f. fuscipes* odorant-binding proteins genes. Conserved – cysteines are highlighted in red and proline residues in blue.

4.3.2. *In silico* homology modelling and binding pocket analysis *G. f. fuscipes* OBPs

To investigate the interactions between OBPs and our selected odorant compounds, we conducted molecular docking to predict the binding affinity and to further select best OBPs for functional analysis using RNAi silencing based techniques.

The homology modelling was done using Phyre2 web server. We found nine (9) models with template similarity of < 30% (supplementary table 1). The quality assessments of these 3D

models were further evaluated and validated for the molecular docking (Supplementary table 1). GffObp44a and Gffobp83a4 exhibited the smallest pocket size and volume (Supplementary table 2). Traditionally, OBPs contains six α-helices and three disulphide bridges with an internal binding cavity. All the analysed OBPs showed similar structural characteristics except GffObp44a (which had five α-helices and GffObp69a (7 α-helices) (Supplementary figure1). Their binding pocket did not possess any clear binding cavity on the surface and no subpocket was found in these OBPs (Figure 2). The biggest binding pocket was observed in GffLush, GffObp19a, GffObp69a, GffObp83a1, GffObp83a2, GffObp83g and GffObp99d (Figure 2) whereas GffObp83g had relatively small volume. GffLush, GffObp19a, GffObp83a1, GffObp83a2 and GffObp99d presented more than one binding cavity while GffOb69a and Gff83g had a unique and clear binding cavity on the surface.

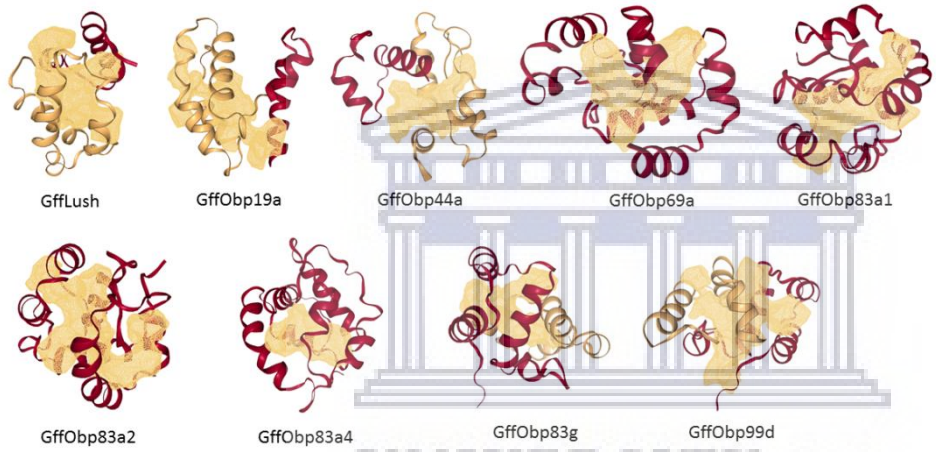

Figure 2: Structural features of 9 odorant-binding proteins of *Glossina f. fuscipes.* 3D structure showing the α-helices. In gold, the protein surface topology highlighting the binding pocket.

4.3.3. Tissue-specific expression of different OBPs

To assess a potential olfactory function of the selected OBPs, we performed a tissue-specific expression analysis in the antennal tissues and different legs (front, hind) of male and female *G. f. fuscipes* using quantitative real time Polymerase Chain Reaction (PCR). Except GffObp44a and GffObp69, all the studied OBPs were expression on the antenna. However, GffObp83a1, GffObp83a2, GffObp83a4, GffObp19a and GffLush were highly antennal enriched. GffObp19a is 50X expression on the antenna compared to the legs and GffObp83a2 was 100X expressed on the antenna. GffObp83a1 and GffOpb83a4 were highly expression in all the tissues. GffObp44a and GffObp69a were not expressed on the antennae but detected on

legs (Figure 3). GffObp99d was expressed in female and male legs as well as on the antenna. GffLush was expressed both in antennae and legs in both male and female.

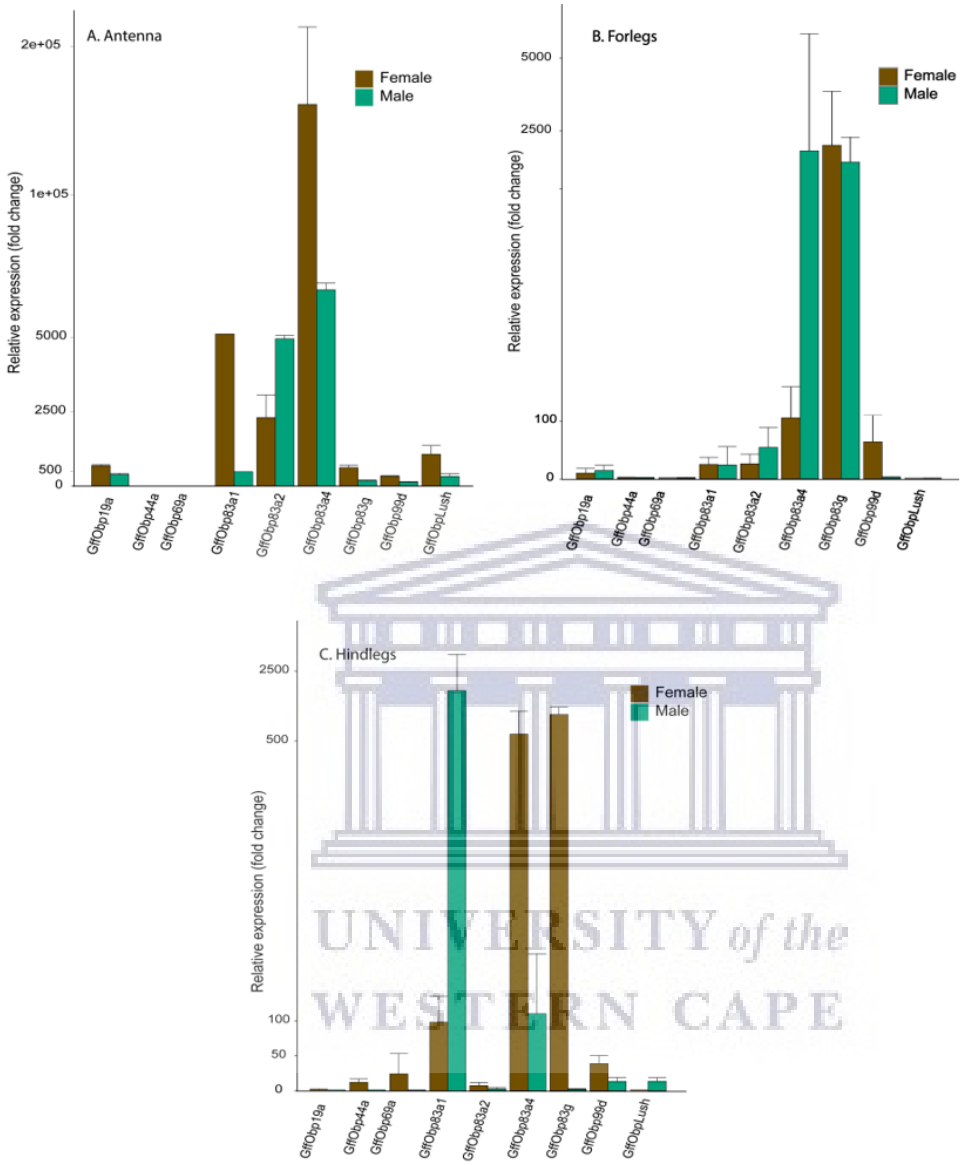

Figure 3. Tissue and sex-specific expressions of odorant-binding proteins in antenna and legs using RT-qPCR. B-actin was used as internal control to normalize the data and 2−ΔΔCT Method [81] was used to calculate to expression level. Bar represents the ±standard error. Given to the big variation of the expression in different tissue, the graphs were not presented with the same scale

4.3.4. The olfactory function of *Glossina f. fuscipes* OBPs expressed in the antenna

To investigate the olfactory role of OBPs that are expressed in the antennae in odour communication, we conducted behavioural response assays by comparing wild type flies response against flies where OBPs were individually silenced. Herein, we evaluated the

potential role of four OBPs (GffObp19a, GffObp83a1, GffObp83a2, and GffObp83a4) that are expressed in the antenna of both male and female. We silenced these OBPs using RNAi interference technique and evaluated the behavioural impact using a free flight bioassay. Our result showed that efficient silencing of OBPs can be achieved within 96h when flies were offered a blood meal containing dsRNAi of specific OBP (Figure 4A-C). Furthermore, OBPS gene silencing efficiency varies between OBPs, for example, the silencing of Gffobp83a1 was minimal as compared to other three OBPs (Figure 4C). The mortality of flies fed with dsRNAi was minimal (1/20 flies), which is the same as the control flies.

During the behavioural assay, the dsRNAi fed flies were flying normally as compared to the control flies, showing that OBPs silencing did not affect their flight ability. For the behavioural assay, the silencing of *Glossina f. fuscipes* Obp19a did not affect the attraction of *G. f. fuscipes* to 1-octen-3-ol as compared to the wild type (p=0.73); (Figure 4E, supplementary table 4). The attraction index (AI) was 0.55 which mean the flies were attracted. Whereas, the silencing of *Glossina f. fuscipes* GffObp83a1, GffObp83a2 and GffObp83a4 (Figure. 4C) has significantly reduced the flies attraction to 1-octen-3-ol as compared to wild type and + nuclease free water flies, (p=0.008) for Obpa83a1, (p=0.001) for GffObp83a2 and (p=0.003) for Obp83a4 (Figure 4D-E).However, the negative control flies fed on nuclease-free water had a similar attraction index as the wild type p > 0.05 (Figure. 4E. supplementary table 4).

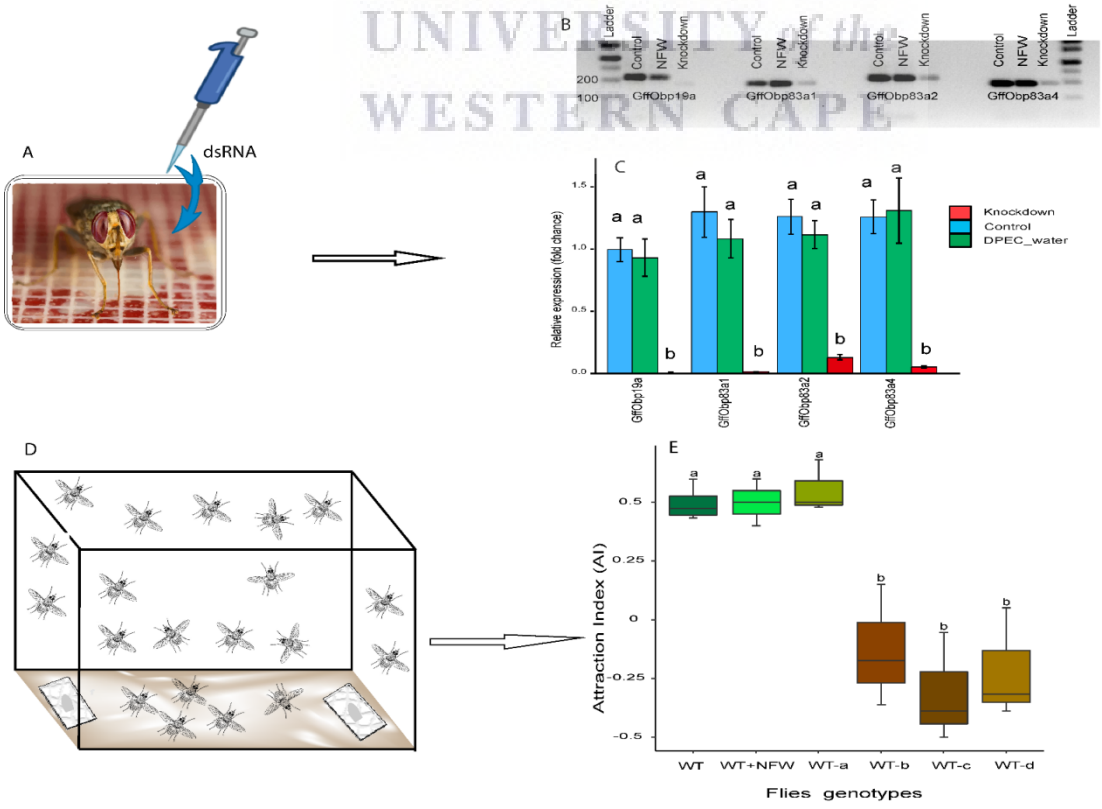

Figure 4: Schematic representation of RNAi experiment and its effect on OBPs expression and on the fly behaviour. **A**: dsRNAi delivery and fly conditioning after dsRNAi intake, B-**C**: comparative expression of different OBPs in wild type and knockdown using real qPCR, **D**: behavioural assay set up, **E**: Boxplots illustrating attraction index (AI) of various genotypes *G. f. fuscipes* (WT=wild type, WT + nfw= nuclease free water, WT-a= Obp19a silenced, WT-b= Obp83a1 silenced, WT-c= Obp83a2 silenced= and WT-d= Obp83a4 silenced. Box plots with different letters are significantly different from each other using ANOVA followed by Turkey test.

4.3.5. Physiochemical properties and molecular docking of *G. f. fuscipes* OBPs

To understand the dynamics of binding affinities of the OBPs, we analysed their physicochemical properties such as, hydrogen bond donors or acceptors and the number of hydrophobic interactions present in the binding pockets. All the analysed OBPs possessed more H bond acceptors than H bond donors (Table 1). GffLush, GffObp83a4, GffObp83a1, GffObp83a2, and GffObp44a have the smallest number of H bonds donors (13 or less). We noted a high number of H bond donors in GffObp83g, GffObp99d, GffObp19a, and GffObp69a (Supplementary table 2). The binding pockets with a high number of hydrophobic interactions were observed with GffObp19a, GffObp83a2, GffObp99d, GffObp69a and GffLush.

Having analysed the physicochemical properties of the OBPs, we then performed a molecular docking using Waterbuck Repellent Blend (WRB) compounds (pentanoic acid, δ-octalactone, geranyl acetone, and guaiacol) which inhibited blood-feeding of *G. f. fuscipes* behaviour and 1-octen-3-ol which enhanced blood feeding (Diallo *et al.*, 2020). Using the lowest docking score, we conducted unsupervised hierarchical clustering to identify ligand-OBPs interaction patterns (Figure 5). Three clusters were observed: the first cluster included Gffobp83g and GffObp69a. These OBPs strongly interacted with pentanoic acid, δ-octalactone, geranylacetone and guaiacol (Figure 5).

The second cluster consists of GffObp19a, GffLush and GffObp44a, highlighting a strong affinities to geranyl acetone, guaiacol and 1-octen-3-ol. Lastly, GffObp83a1, GffObp83a2, GffObp83a4 and GffObp99d were clustered together. Their best docking scores were observed with δ-octalactone, geranyl acetone, guaiacol, and 1-octen-3-ol whereas; GffObp83a4 and GffObp99d also interacted with pentanoic acid (Figure 5).

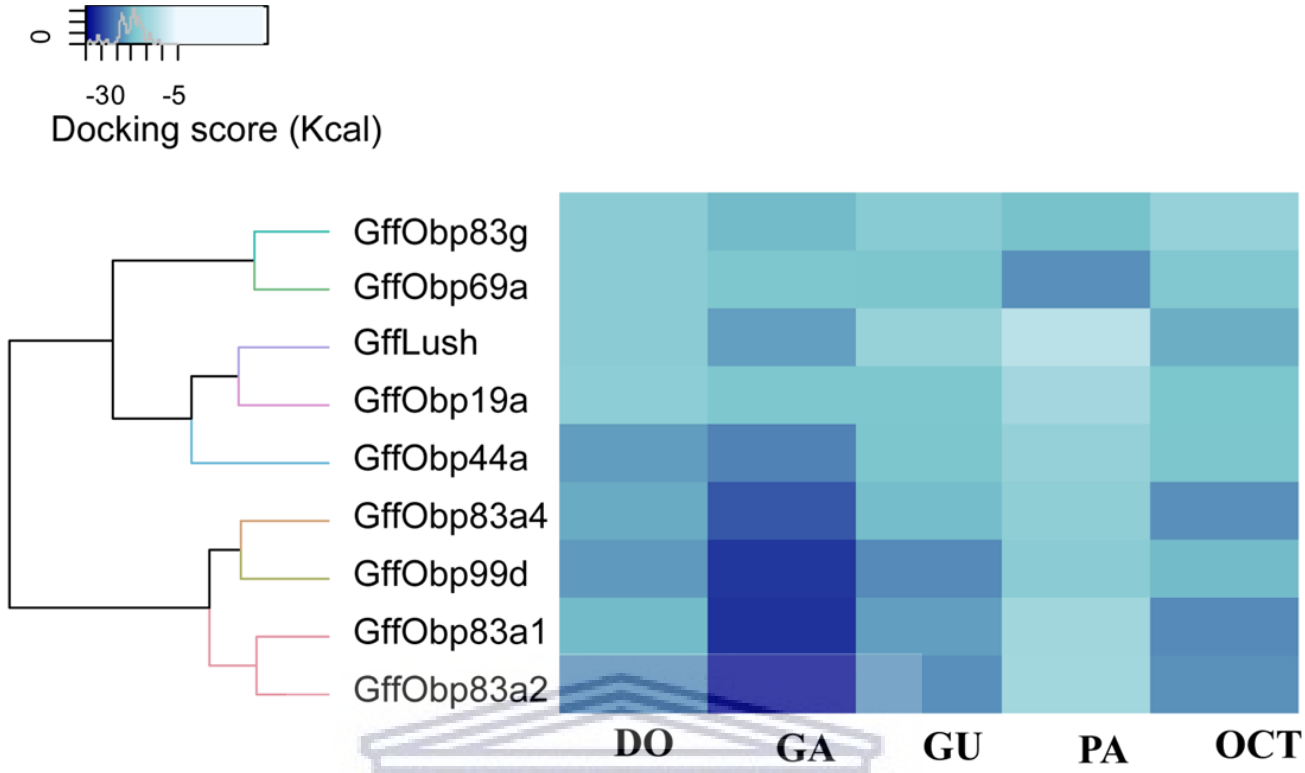

Figure 5: The molecular docking of different odorants binding proteins of *Glossina f. fuscipes* Heatmap showing the binding affinity of different OBPs to Waterbuck repellent components and 1-octen-3-ol. DO= δ-octalactone; GA= geranyl acetone; GU= guaiacol; PA= pentanoic acid; OCT= 1-octen-3-ol.

To understand the dynamics of the binding pockets, we analysed their physicochemical properties such as, hydrogen bond donors or acceptors and the number of the hydrophobic interactions present in the pocket. All the analysed odorant binding proteins possessed more H bonds acceptors than H bonds donors in number (Table 1). GffLush, GffObp83a4, GffObp83a1, GffObp83a2 and GffObp44a have the smallest number of H bonds donors (13 or less). We noted a high number of H bond donors in GffObp83g, GffObp99d, GffObp19a and GffObp69a (Table1). The binding pockets with high number of hydrophobic interactions were observed with GffObp19a, GffObp83a2, GffObp99d, GffObp69a and GffLush.

Table1. Summary of binding pocket dynamics and physicochemical characteristics of *Glossina f. fuscipes*

Obp	Class of Obp	Volume ($Å^3$)	Surface ($Å^2$)	H bonds donors	H bonds acceptors	Hydrophobic interactions
GffLush	Typical	1025.28	1417.17	13	41	67
GffObp69a	Typical	1008.64	1416.96	19	46	89
GffObp83a4	Typical	769.89	743.1	4	34	49

GffObp83g	Typical	922.69	1133.85	20	61	49
GffObp99d	Typical	1358.02	2058.23	31	74	96
GffObp19a	Plus-C	1426.18	2115.39	27	75	102
GffObp83a1	Plus-C	935.1	1116.77	7	47	54
GffObp83a2	Plus-C	1267.01	1558.91	12	59	87
GffObp44a	C-Minus	676.16	888.02	7	36	37

4.3.6. Conformational Dynamic of Docked Systems

To assess the efficiency of our docking and to understand the binding and its mechanism, we conducted molecular dynamics (MD) simulation using the functional characterised OBPs and 1-octen-3-ol, the odour we used for our behavioural assay.

The structural attributes of the docked systems were explored using the principles of MD simulations for the time scale of 100 ns (Diallo *et al.*, 2020). The structural compactness and stability of the docked systems were analysed in terms of the calculated radius of gyration (Rg) and Root Mean Square Deviation (RMSD) values (Supplementary Figure 3). The variations in the Rg values for all the systems were reported and it was observed that the GffObp19a_1_octen_3_ol system showed the highest level of structural compactness in which Rg values were fluctuating between 1.25 nm - 1.3 nm (Supplementary Figure 3A), while for the rest of the system the values were observed to be in the range higher than 1.3 nm highlighted the attainment of less compactness. Similarly, the RMSD values projections showed that the GffObp19a_1_octen_3_ol achieved the least stability among the studied systems for which the values were fluctuating between the 0.4 nm - 0.5 nm. All the other three systems showed relatively similar stability profile in which variations in the RMSD values were observed between 0.2 nm - 0.4 nm (Supplementary Figure 3B).

The closeness between the proteins and the docked ligands in the studied systems were understood in terms of calculated distances between the interacted molecules. The GffObp83a4_1_octen_3-ol followed by GffObp83a1_octen-3-ol showed the least distances in the respective systems which indicated that the higher degree of the interactions was observed in the respective systems as compared to the rest, with least calculated distances were observed in GffObp19a_1_octen_3_ol system (Supplementary Figure 3C).

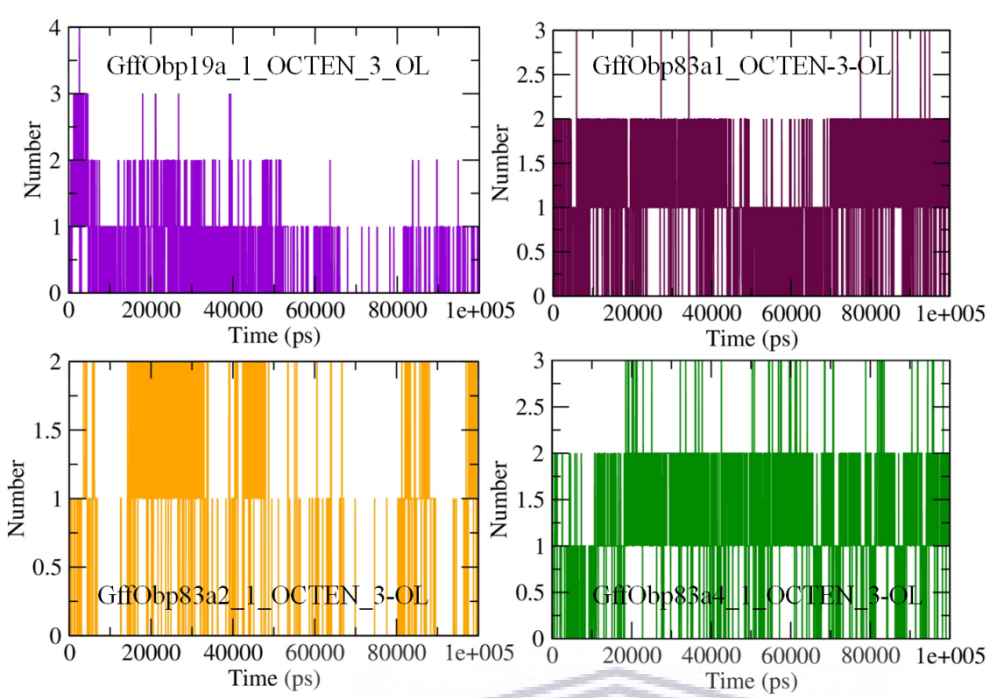

Figure 6: The graphs illustrating variations observed in the pattern of hydrogen bonding between the studied proteins and ligands during 100 ns MD simulations.

The hydrogen bond (H-bonds) patterns were further explored for understanding the nature of interactions between the proteins and docked ligands during MD simulations. The GffObp19a_1_octen_3_ol showed the presence of up to four H-bonds, while around three H-bonds were observed in GffObp83a1_octen-3-ol and GffObp83a4_1_octen_3-ol docked systems. The GffObp83a2_1_octen_3-ol showed the presence of only two H-bonds which indicated that comparatively lower interaction level was observed in the respective system (Figure 6).

Furthermore, the MM/PBSA based protocols were used for the calculation of free energies of interactions between interacting molecules of the docked systems (Figure 6). The GffObp83a1_octen-3-ol, GffObp83a2_1_octen_3-ol and GffObp83a4_1_octen_3-ol showed relatively similar pattern of the total interaction energy in which the values were observed between -100 kJ/mol - -150 kJ/mol. While GffObp19a_1_octen_3_ol showed relatively lower binding affinity which can be deduced from the calculated total free energy of binding calculated between -50 kJ/mol - -100 kJ/mol.

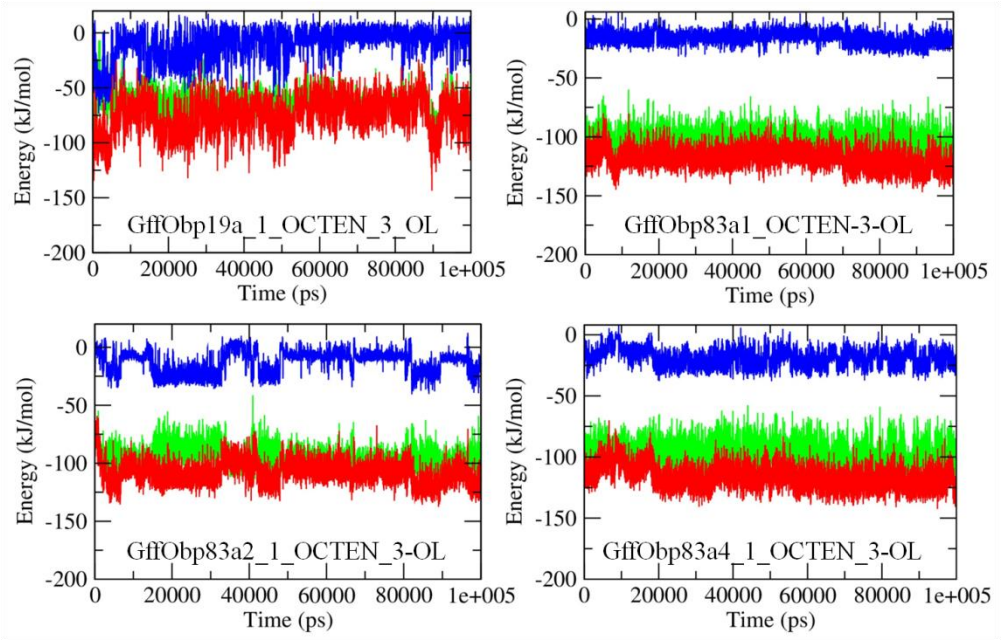

Figure 7: The MM/PBSA based calculated energy curves showing the variations in the interaction energies observed between the proteins and docked ligands during 100 ns MD simulations. (Light Green - Vander Waals energy, Blue- Electrostatic energy, Red - Total energy).

4.3.7. Evaluation of Deorphanization of Receptors based on Expression Alterations in mRNA levels (DREAM) on different OBPs

To assess the role of selected odorant-binding proteins in olfaction, we analysed the gene expression alteration patterns in the antenna after exposure to the different odorants compounds. The gene expression analysis showed that mRNA transcripts level of GffObp19a, GffObp83a1, GffObp83a2, GffOb83a4 and GffObp99d were upregulated in the antennae when the *G. f. fuscipes* were exposed to δ-octalactone. Furthermore, the mRNA transcripts levels of GffLush and GffObp44a were not affected when the flies were exposed to δ-octalactone (Figure 7A). For geranyl acetone exposure, GffObp19a, GffObp83a4 and GffObp83g were upregulated; GffObp83a1, GffObp83a2 and GffObp99d were downregulated (Figure 7B). Meanwhile, GffObp44a and GffLush did not show any change in the mRNA transcripts level. The exposure to guaiacol affected five odorant-binding proteins; GffObp83a1, GffObp83a2 were downregulated while GffObp83a4 and GffObp99d were upregulated (Figure 7C). The mRNA transcript levels of GffObp83g, GffObp69a and GffObp83a4 did not change on exposure to pentanoic acid. On the other hand, GffObp83a1, and GffObp99d were upregulated while GffObp44a, GffObp83a2 and GffLush were downregulated (Figure 7D).

The exposure to 1-octen-3-ol did not affect GffLush, GffObp44a and GffObp69a. However, GffObp19a, GffObp83a1, GffObp83a4 and GffObp99d were significantly upregulated while GffObp83g and GffObp83a2 were significantly downregulated when exposed to 1-octen-3-ol (Figure 7E).

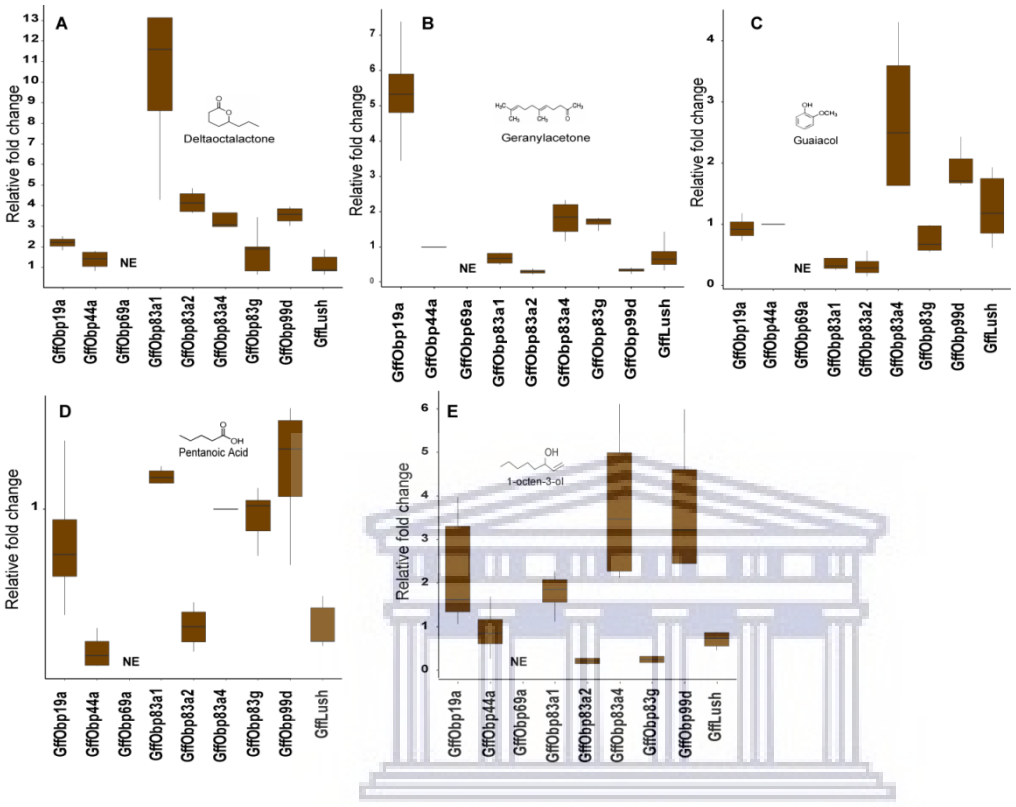

Figure 7. mRNA transcript changes patterns when the flies were exposed to different chemicals.

(A-E) Boxplots illustrating relative fold change with (A). Flies were exposed to δ-octalactone; (B). Flies were exposed to geranyl acetone; (C). Flies were exposed to guaiacol; (D). Flies were exposed to pentanoic acid; (E). Flies were exposed to 1-octen-3-ol. NE means that OBP was not expressed in the antenna.

4.4. Discussion

In this study by employing a multiple approaches i.e., tissue expression, structural, ligand interaction, molecular dynamics and silencing we demonstrated the essential olfactory function of OBPs expressed in the antenna of tsetse fly. Our results show that *Glossina f. fuscipes* odorant-binding proteins are subdivided into three subfamilies (minus-C, classical and atypical OBPs) (Xu, Cornel and Leal, 2010; Zhou, 2010; Pelosi *et al.*, 2014; Missbach *et*

al., 2015; Jiang *et al.*, 2017). Minus-C OBPs present an intermediate structure in the functional evolution of OBPs (Zheng *et al.*, 2016). In the present study, three Minus-C OBPs; were found, GffObp44a, GffObp8a, and Gffobp99b in *G. f. fuscipes*. GffObp44a which was expressed only in the female front leg is an OBP without signal peptides (SPs) and showed a small binding pocket with a smaller number of hydrophobic interactions compared to the OBPs with signal peptides. The function of such OBPs need further investigation(Hekmat-Scafe *et al.*, 2002).

OBPs without SPs have been also identified in other insects (Hekmat-Scafe *et al.*, 2002) however, the role of signal peptides in the interactions between OBPs and ligands remains unclear. The physio-chemical properties of these OBPs from the structural analysis suggest that they could be important for the General Odorant binding proteins (GOBPs) as they contribute to rendering the binding pocket more hydrophobic, thus allowing higher flexibility of the pocket towards general odorants (Hajjar E, Perahia D, Débat H, Nespoulous C, 2006). However, OBPs without signal peptides are considered as mature proteins and their binding function could be limited to small ligands because of the shape and dynamics of their binding pocket. Otherwise, signal peptides were also found to play a role in the protein stability (Szabady *et al.*, 2004); more recently it has been suggested that the signal peptide at the N-terminal end could be used for designing highly specific primers and probes to detect the expression patterns of odorant-binding protein genes in the main olfactory and gustatory organs (Ghavami, Khoeini and Djadid, 2020).

Classical and atypical OBPs have been extensively studied in different insects and considered as key players in olfactory processing (Pelosi, 1996; Kim, Repp and Smith, 1998; Biessmann, Le and Walter, 2005; De Bruyne and Baker, 2008; Leal, 2013; Larter *et al.*, 2016; Gonzalez *et al.*, 2019). Similarly, classical and atypical OBP, showed binding pockets that are suitable for binding diverse odorants. This was supported by our mRNA transcriptome alteration which is a proxy of ligand OBPs interaction results, whereby a given classical and atypical OBPs OBP interacted with more odorants and a given odorant activated more classical and atypical OBPs. It is well established that the dynamics of protein binding pockets are crucial for their interaction efficiency and specificity (Liang, Edelsbrunner and Woodward, 1998; Hajjar E, Perahia D, Débat H, Nespoulous C, 2006; Kahraman *et al.*, 2007; Stank *et al.*, 2016). The shape and the volume of the classical (739Å^3 to 1389 Å$^{3)}$ and atypical (935 Å^3 to 1429 Å^3) binding pocket cavities and their structural flexibility allowed us to postulate that they are suitable for various ligand binding. This is also supported by the physicochemical properties of the binding cavities, where we observed several hydrophobic interactions and hydrogen

bonding in the binding cavities as previously reported for other insects (Pace *et al.*, 2011; Li *et al.*, 2015). Hydrophobic interactions reduce considerably the undesirable interactions with water molecules which increase the efficiency of receptor-ligand interactions. The molecular docking result showed that pentanoic acid interacts with GffLush, GffObp44a, GffObp83a1 and GffObp83a2 while δ-octalactone, geranylacetone, guaiacol and 1-octen-3-ol interacted with Gffobp19a, GffObp83a1, GffObp83a2 and GffObp99d. The binding pockets of these four OBPs were also found to have large volume, area and better physicochemical characteristics such as hydrogen bonding and hydrophobic interactions. These observations are in line with previous studies (Tsitsanou *et al.*, 2013; Li *et al.*, 2015; Stank *et al.*, 2016) on the correlation between the binding pocket dynamics and the flexibility of the proteins to adapt their binding affinity to different molecules The structural basis for such flexible chemical recognition remains unknown. A given OBP interacted with more than one odorant with diverse chemistry. For example in odorant receptors (ORs), recent study (Mármol et al., 2021) showed at odour binding is mediated by hydrophobic non-directional interactions with residues distributed throughout the binding pocket on the ORs, enabling the flexible recognition of structurally distinct odorants. Similarly, these OBPS have a high hydrophobic interaction (supplementary Table 1). GffObp19a, GffObp83a1, GffObp83a2, which are expressed in both male and female antenna, presented the best physicochemical features such as hydrogen acceptor/donors and hydrophobic interactions and they interacted efficiently with ligands (Figure.7). Where we further investigated the function of GffObp19a, GffObp83a1, and GffObp83a2 by RNAi based silencing. The studied OBPs showed variations in their expression between tissues (antenna and legs) and sexual dimorphism, For instance, except for GffObp69a and GffObp44a, all other OBPs were expressed in the antennae, whereas GffObp69a was only expressed in the female front legs. This finding is neither unique nor surprising as there are sex specific ecological and physiological behaviours. In *Drosophila*, DmObp69a has been shown to be involved in social interactions (Bentzur *et al.*, 2018), probably involved in the detection of contact sex pheromones. The selective expression of GffObp69a and Gffobp44a in the female leg is not clear but might be associated with the female-specific behaviour such as larviposition that need further investigation. Almost all the other OBPs are expressed in the *G. f. fuscipes* legs indicating that they might also have a role in social interaction, such as sexual behaviour (Xiao, Sun and Carlson, 2019).

We found the same as ORs, the OBPs mRNA expression were altered by up and down-regulation and others not affected when flies were exposed to WRB and 1-octen-3-ol, showing that OBPs are also selective the same as odorant receptors (Diallo *et al.*, 2020).

GffLush, that was conserved between sex in the tissues in its expression, which is considered as a pheromone binding protein in *Drosophila melanogaster*, did not exhibit a strong interaction with any of the WRB components and 1-octen-3-ol. Four antennal enriched OBPs were selected for their functional study using RNAi mediated gene silencing. The RNAi silencing via dsRNAi feeding indicate that it is possible to silence OBPs in non-model insects such as tsetse flies and investigate their function. The target gene interference was efficient, which indicates the effectiveness of studying insect OBPs using dsRNAi silencing (Bento *et al.*, 2020) (D. P. Walshe *et al.*, 2009). We successfully silenced 4 OBPs expressed in the antenna of both sexes in *Glossina f. fuscipes* and which have demonstrated favouring physiochemical properties and three of the four OBPs reduced *G. f. fuscipes* behavioural response to 1-octen-3-ol as compared to control and wild type flies. Our behavioural assay demonstrated that GffObp83a1, GffObp83a2 and Gff83a4 play an important role in the detection and perception of 1-octen-3-ol. Their silencing significantly reduced the attraction of the fly to 1-octen-3-ol which is known attractant in tsetse fly. GffObp19a was found to have less effect in the perception of 1-octen-3-ol.

To understand the dynamic of the binding poses of 1-octen-3-ol to the four OBPs, we conducted molecular dynamics and ligand-obp interactions patterns. We found that the gene silencing results are in line with the *in-silico* prediction of the interactions of GffObp83a1, GffObp83a2 and Gff83a4 with 1-octen-3-ol. The molecular dynamics showed similar patterns. The lack of GffObp19a silencing in response to 1-octen-3-ol supported by our molecular dynamics studies that did not elicit good stability during the molecular dynamics simulation compared to the complexes formed by GffObp83a1, GffObp83a2 and GffObp83a4. Few hydrogen bonding were observed in the molecular dynamics simulation while hydrophobic interactions (Van der Waals) were elicited in the 2D interaction diagram (Figure 6). Furthermore, the change in mRNA expression in GffObp19a when exposed to 1-Octen-3-ol might be a false positive effect from the experiment (Koerte *et al.*, 2018).

In the binding cavities, GffObp83a1 (Tyr145, Phe146 and His144), GffObp83a2 (Tryp140, Phe149 and Tyr148) and Gff83a4 (Try139, His146, Tyr147 and Phe148) that have affected the behavioural response showed good hydrophobic interactions compared to GffObp19a (supplementary figure 4). Similar binding patterns were observed by (Li *et al.*, 2015), where they found hydrogen bonding less important than hydrophobic interactions. The feasibility of OBPs silenced flies supports the hypothesis that OBPs might be evolved with terrestrial life (Missbach *et al.*, 2014). Similarly, reducing the expression of DmelOBP59a affects the

detection of attractant odorants in drosophila (Swarup, Williams and Anholt, 2011; Swarup *et al.*, 2014).

Cumulatively, our study shows a clear evidence of the role of GffObp83a1, GffObp83a2 and GffObp83a4 in the detection and perception of 1-octen-3-ol by *G.f. fuscipes*. Similarly, reduced expression of DmelOBP59a in Drosophila affects the detection of 1-hexanol, 2-heptanone, and propanal and a decrease in bitter taste consumption(Swarup, Williams and Anholt, 2011; Swarup *et al.*, 2014). We have successfully silenced OBPs, which enabled us to study the function of certain OBPs in non-model insect like tsetse fly using dsRNAi feeding approach. However, it could be interesting to study how long the dsRNAi could stay stable in the blood.

In summary the olfactory tissue expression, the selective mRNA alteration when exposed to odorants and their significant effect when silenced on the behavioural response, demonstrates OBPs are directly involved in odorant detection and perception. Furthermore, these OBPs vary in their physiochemical structures that might affect their ligand interaction, selectivity and their various potential roles in olfactory function. Furthermore, the sexual dimorphism and tissue-specific expression indicates their involvement in various sensory modalities such as olfaction including sexual interaction, and taste. We believe that a better understanding of OBPs in chemical communication will contribute to the more efficient development of olfactory-based tools, such as sensors as well as control tools such as attractant and repellent for tsetse fly, a vector of sleeping sickness and nagana.

Chapter five

General summary, conclusions and furthers perspectives

5.1. General summary

The olfactory system represents an immense importance in insect interactions with their natural world. In insect vectors such as tsetse flies, olfactory system mediates host locations and thus underlies the transmission of disease which can affect millions of livestock and people each year around the world. Hence, to develop olfactory based tools for vectors control, understanding the sense of smell in insect vectors has become an area of investigation for the last decade. In this thesis, we first analysed the diversity and the abundance of the antennal sensilla using scanning electron microscopy. We characterised the sensillar types on the dorsal side of the *G. f. fuscipes*, we found that *G. f. fuscipes* is equipped with diverse and rich olfactory sensillar types including coeloconic, basiconic and trichoid sensilla. Basiconic sensilla are highly multiparous compared to the other types. There is at least one sensory pit per antenna housing club shaped basiconic sensilla, very distinct in shape as compared to the basiconic sensilla on the surface of the flagellum. The basiconic sensilla were located almost everywhere on the third antennal segment, with higher density at the base and middle of the flagellum, but their number decrease at the tip. However, trichoid sensilla are present all over the flagellum almost in equal number. Coeloconic sensillum has restricted distribution especially at the basal and middle section of the flagellum. Furthermore, we demonstrated that there is slight numerical difference between male and female, there are more basiconic and trichoid sensillar in male than female. But the number of coeloconic sensilla is the same between male and female. The difference in number, distribution and types of olfactory sensilla might induce both physiological and behavioural difference between male and female flies.

Secondly, we performed a functional study of odorant receptors in *Glossina fuscipes fuscipes* by combining several approaches. We demonstrated that WRB inhibits blood feeding in both *Glossina pallidipes*, Austen, 1903 and *G. f. fuscipes*, Newstead, 1910. This finding could suggest tsetse repellent could be used as pour-on for the control of African trypanosomiasis. But the toxicity of the repellent compounds should be evaluated before any field application. Using the DREAM (Deorphanization of receptors based on expression alterations in ORs mRNA levels) technique, combined with ortholog comparison and molecular docking, we predicted the signature putative odorant receptors for the WRB in *G. f. fuscipes*, a non-model

insect. We show that exposure of *G. f. fuscipes* in vivo to WRB odorant resulted in up- and down-regulation of mRNA transcript of several odorant receptors (ORs). WRB component with strong feeding inhibition altered mRNA transcript differently as compared to an attractant odour, showing these two odours of opposing valence already segregate at the cellular and molecular level. Furthermore, molecular dynamics simulations demonstrated that the predicted ligand-OR binding pockets consisted mostly of hydrophobic residues with a few hydrogen bonds but a stable interaction. Our electrophysiological response shows the olfactory sensory neurons of *G. f. fuscipes* tuned to the tsetse repellent components in different sensitivity and selectivity. These findings open an alternative method in the functional analysis of odorant receptors in non-model insect. Also, the predicted receptor can used to screen a large library of chemicals in order to identify a novel repellent using reverse chemical ecology.

Lastly, we analysed the structural features of *Glossina fuscipes fuscipes* odorant binding proteins. Using molecular-docking simulations coupled with gene expression studies the functional role of nine *G. f. fuscipes* OBPs were analysed. We found a structural variability between the different OBPs that impacted the binding affinity to Waterbuck repellent compounds (WRC) (δ-octalactone, geranyl acetone, guaiacol and pentanoic acid) and 1-octen-3-ol. Also, we identified some of the putative OBPs for the Waterbuck repellent compounds (WRC) and 1-octen -3-ol. We demonstrated sexual dimorphism in the expression of OBPs pattern. Our results suggested that the tissue specific expression might contribute to enhance olfactory and taste functions in the antenna and legs. Thus, their physiochemical properties, their mRNA alteration combined with expression in olfactory tissues suggest they play a role in the olfactory processing of WRC compounds. The RNAi silencing via dsRNA feeding indicated that it is possible to silence OBPs in non-model insects such as tsetse flies and investigate their function. The silencing of 3 OBPs clearly reduced the behavioural response of the flies as compared to control. Our behavioural finding demonstrated that GffObp83a1, GffObp83a2 and Gff83a4 play an important role for the detection and perception of 1-octen-3-ol.

5.2. Implications and recommendations

This PhD project highlighted that *G. f. fuscipes* olfactory sensilla are closely similar to *Drosophila melanogaster* and other tsetse species. Olfactory sensilla are considered as the "nose" of insects, hence knowing their abundance and diversity on the antenna could help to

understand the olfactory mechanism governing the behavioural variability between different tsetse species and sexes. It will be interesting to do a comparative analysis of the olfactory sensilla abundance and diversity between tsetses from different ecological niches. Also, an olfactory receptor and OBPs map per olfactory sensillum can be further investigated. Our study has also shown the antifeedant effect of the Waterbuck Repellent compound which opens the possibility of the use of WRC as pour-on treatment in the management of African trypanosomiasis. However, the toxicity of these compounds should be first checked. It will be also interesting to investigate if the WRC repellent can also be used as footbath insecticides for vector control. The combination of several approaches has allowed us to identify the putative receptors of the WRC and 1-octen-3-ol. This finding has two major implications: (i) it can be applicable for other non-model insect which will help in the functional characterisation of olfactory proteins, such as ORs and OBPs. However, to clearly confirm our approaches, the identified receptor for WRC and 1-octen-3-ol can be validated using the conventional empty neuron system through electrophysiological characterization. Furthermore, DREAM is a validated technique with all its limitation to deorphanise receptors however, we can minimize the limitation by doing comparative DREAM experiments between non model insects such as tsetse flies against model drosophila using the same odour. It might also be important to expose flies individually if that makes a difference in the gene expression. Furthermore, in the prediction of tertiary structure of ORs and OBPs it is nice to use various comparative homology modeling.

We also characterised some OBPs using the same combinatorial approach. The laboratory bioassay after gene silencing assessed the importance of certain OBPs in odour detection and perception in *G. f. fuscipes*. It might be interesting to conduct a semi-field or field assay to evaluate the field efficacy of RNAi mutant flies in the line of developing an efficient vectors control tools for *Glossina sp.* Furthermore, we show they have sexual dimorphism and tissue specific expression showing their involvement in various sensory modalities such as olfaction, and taste. We believe that a better understanding of OBPs on chemical communication will contribute to more efficient development of olfactory-based tools, such as sensors as well as olfactory based tools, attractants, repellents for tsetse fly, vector of African Trypanosomiasis (AT). We found a given odorant activated more than one ORs or OBPs with unknown mechanism. We also know that Odours are not specific, they are conserved between organism, i.e., flies, plants, and animals it is very difficult to give signature odours to a specific organism. Similarly, almost all ORs with a few exceptions are broadly tuned. The broad tuning of olfactory receptors is essential for detecting wide range of odorant and

discrimination of important odour in insect and giving the entire olfactory system a great plasticity. Although, Some ORs have already been shown to be specific but recent structural and mechanistic study demonstrated that ligand-receptor binding is mediated by hydrophobic, non-directional interactions with residues distributed throughout the binding pocket, enabling the flexible recognition of structurally distinct odorants by the same receptor (Del Mármol, Yedlin and Ruta, 2021)**.**

We also believe that CPS might have a functional role in tsetse olfaction, there functional characterisation will help address some unknown questions in the olfactory processing.

The mechanism of the WRC repellency especially the contribution of olfaction and taste need to be dissected which will enable us to find other odours with similar behavioural effect, to manage repellent resistant.

The great advances in the past decade in defining basic mechanisms and principles of insect olfaction have provided an exciting opportunity. The molecular and cellular insight has laid a foundation for the development of olfactory-based insect control technology. The timing is auspicious: there has been renewed interest in controlling the insect vectors of disease, because other approaches, including vaccine and drug development, continue to encounter major challenges. There is added urgency to vector control efforts because of the predicted effects of climate change on the geographical distribution of many of these insects. Olfactory behaviour, particularly host seeking and oviposition, offers opportunities to disrupt the disease-transmission process. In this section, we consider how recent advances can be applied to the problem of vector control and how some limitations might be overcome through basic research.

A number of ORs, Grs, and IRs are promising targets for manipulating the olfactory-guided behaviour of insects. Compounds that excite or inhibit these receptors and that are inexpensive, stable, and nontoxic could provide effective and environmentally friendly means of controlling insect vectors and pests.

The identification of molecular targets may greatly increase the efficiency of screens for activators of either attraction or avoidance circuits; high-throughput cell-based expression systems can be used to screen large chemical libraries and rapidly identify candidate compounds. If the cognate receptor for this OSN can be identified, the development of new repellents could be significantly advanced.

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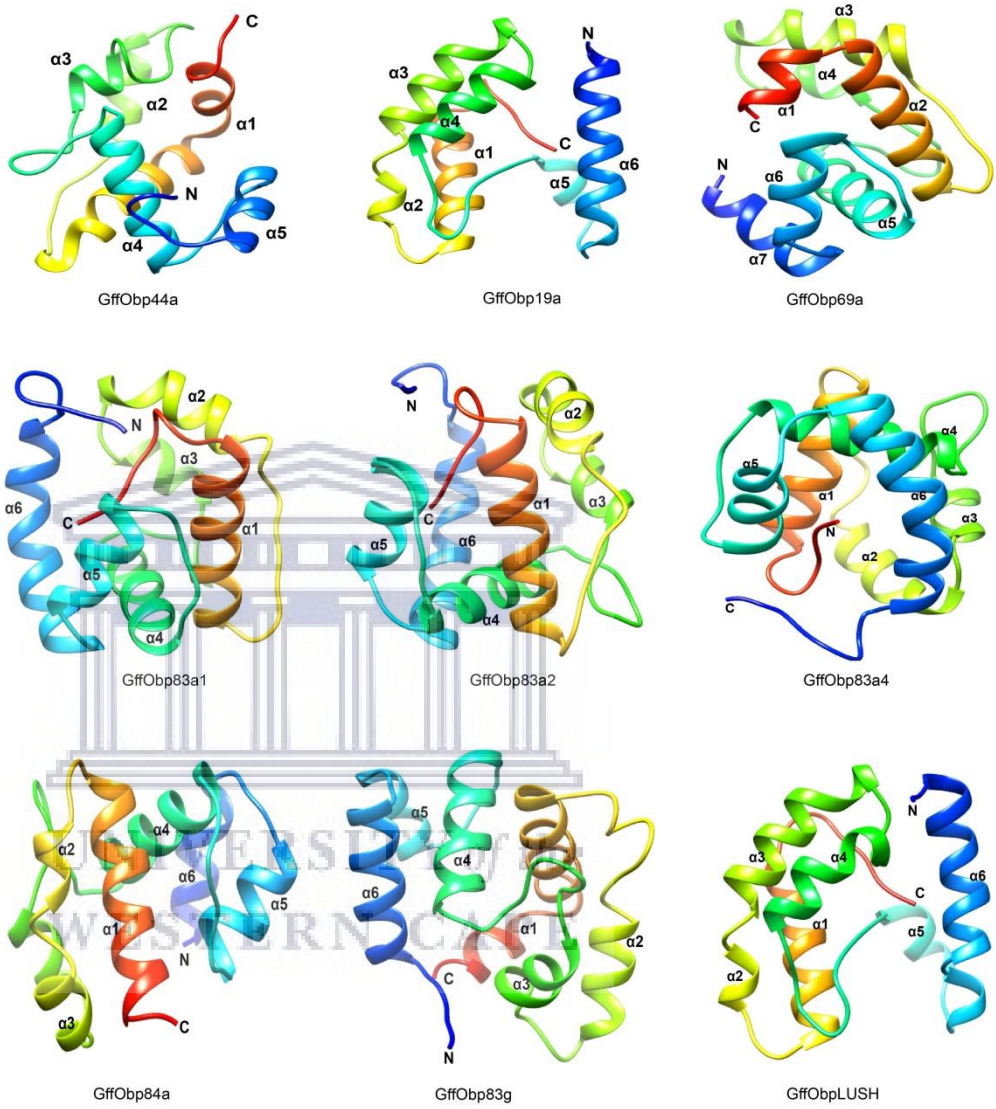
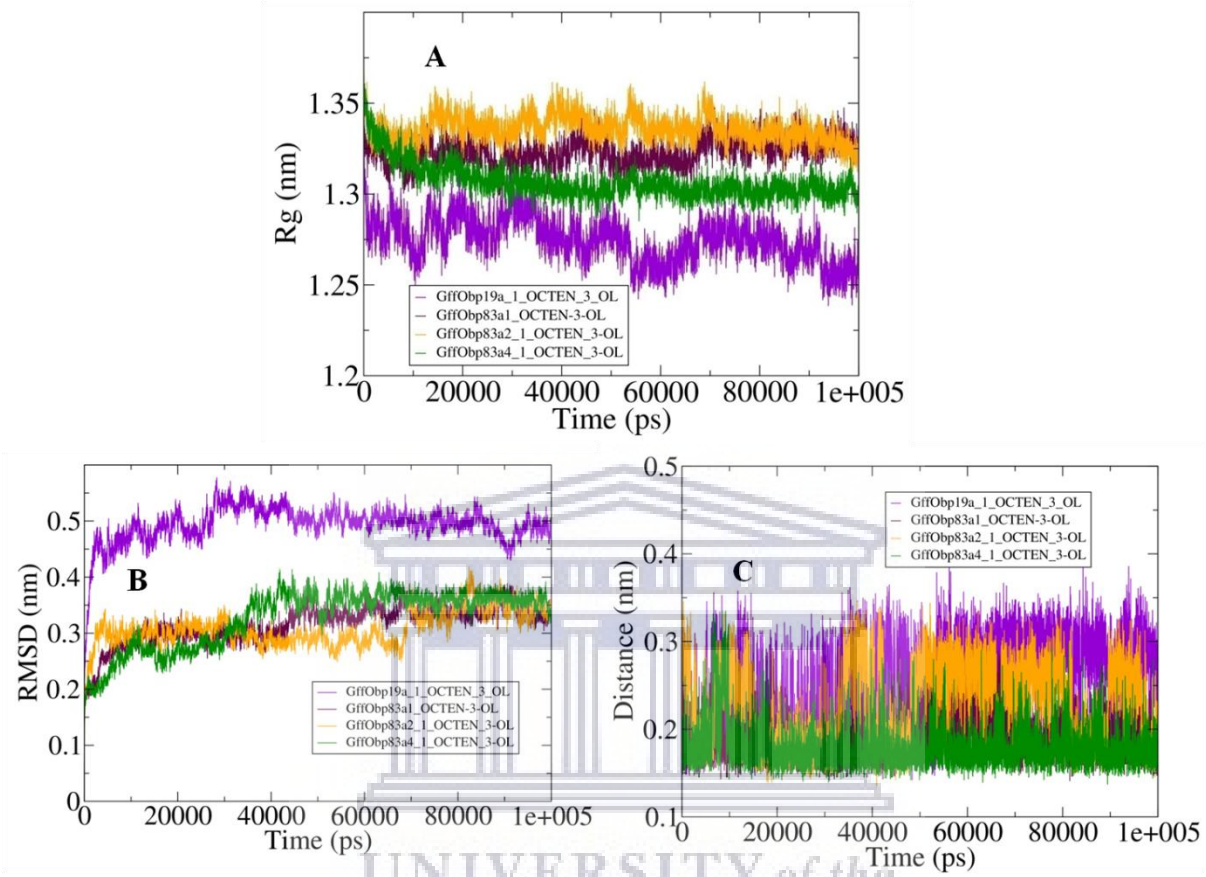
Supplementary figures and Tables

Supplementary figure 1:



3D structure of 9 odorant binding proteins in *Glossina f. fuscipes* highlighting the number of α-helices.

Supplementary figure 3



The MD simulations based parameters' curves (A) showing the changes observed in the compactness of the studied systems in terms of Radius of Gyration (Rg) (B) highlighting the changes observed in the Root Mean Square Deviation (RMSD) values (C) illustrating the variation in the calculated distances between protein and ligands in the studied systems. (Purple - GffObp19a_1_OCTEN_3_OL, Maroon - GffObp83a1_OCTEN-3-OL, Orange - GffObp83a2_1_OCTEN_3-OL, Dark Green - GffObp83a4_1_OCTEN_3-OL)

Supplementary table 3: Primers for RT-qPCR and RNAi experiments

Name	Sequence
Gfflush_F	ATGTTAACTGCGTTAAAATG
Gfflush_R	GGACTCAGTCCCGACCCAAT
Gffobp19a_F	ATGTTTGGCAAAATATCGTA
Gffobp19a_R	AAGGGAAAACGAATTTTGTG
Gffobp83a1_F	ATGCTTTTAAAGTGTGATTG
Gffobp83a1_R	TCCCACACAAAACGATTATC
Gffobp83a2_F	GCAAGCAATGATTGTTAAGT
Gffobp83a2_R	CACTATGCACGCACTTGTCA
Gffobp83a4_F	AGGATGACTTTGTCCGGTAA
Gffobp83a4_R	TTGCAGTAGATGATCGATCT
T7Gfflush_F	TAATACGACTCACTATAGATGTTAACTGCGTTAAAATG
T7Gfflush_R	TAATACGACTCACTATAGGGACTCAGTCCCGACCCAAT
T7Gffobp19a_F	TAATACGACTCACTATAGATGTTTGGCAAAATATCGTA
T7Gffobp19a_R	TAATACGACTCACTATAGAAGGGAAAACGAATTTTGTG
T7Gffobp83a1_F	TAATACGACTCACTATAGATGCTTTTAAAGTGTGATTG
T7Gffobp83a1_R	TAATACGACTCACTATAGTCCCACACAAAACGATTATC
T7Gffobp83a2_F	TAATACGACTCACTATAGGCAAGCAATGATTGTTAAGT
T7Gffobp83a2_R	TAATACGACTCACTATAGCACTATGCACGCACTTGTCA
T7Gffobp83a4_F	TAATACGACTCACTATAGAGGATGACTTTGTCCGGTAA
T7Gffobp83a4_R	TAATACGACTCACTATAGTTGCAGTAGATGATCGATCT

Supplementary table 4

Species	Fly type	Replicate	Number release	Flies in Control	Flies in Treatment	No response	Dead flies	Attraction Index
Gff	Obp83a1-/-	1	20	7	3	10	0	-0.173913043
Gff	Obp83a1-/-	2	20	2	5	11	2	0.15
Gff	Obp83a1-/-	3	20	8	0	12	0	-0.363636364
Gff	Obp83a2-/-	1	20	2	1	15	2	-0.055555556
Gff	Obp83a2-/-	2	20	11	2	6	1	-0.5
Gff	Obp83a2-/-	3	20	10	3	4	3	-0.388888889
Gff	Obp83a4--/	1	20	7	1	9	3	-0.315789474
Gff	Obp83a4-/-	2	20	5	6	7	2	0.05
Gff	Obp83a4-/-	3	20	8	1	9	2	-0.388888889
Gff	Op19a-/-	1	20	0	10	10	0	0.5
Gff	Op19a-/-	2	20	1	12	7	0	0.47826087
Gff	Op19a-/-	3	20	1	16	3	0	0.681818182
Gff	Wild type	1	20	1	11	8	0	0.5
Gff	Wild type	2	20	4	13	3	0	0.45
Gff	Wild type	3	20	4	14	3	0	0.434782609

Gff	Wild type	4	20	3	15	2	0	0.6
Gff	Wild type - NFW	1	20	4	8	5	3	0.2
Gff	Wild type - NFW	2	20	2	14	2	2	0.6
Gff	Wild type - NFW	3	20	5	9	3	3	0.2

Odorant binding proteins homology modelling quality check

Protein	VB_Acc_N	Uniprot_N	Template	ID %	Mod_cor	Resolution (Å)	Z-sore (ProS	Z-Score ramachadran	Qmean	G-facto	Ramachadran
Gfflush	GFUI025618-PA	A0A1A9XY57	c3q8i_A	53	99.9	1.5	-4.78	-0.66	0.70 ± 0.08	-0.2	94.4
GffObp19	GFUI007906-PA	A0A1A9XEM7	c6hhe_A	25	93.8	1.5	-1.72	-1.531	0.44 ± 0.09	-0.2	88.6
GffObp19a	GFUI000760-PA	A0A1A9X703	C3vb1_A	49	100	1.5	-4.75	-1.114	0.75 ± 0.08	-0.2	95.3
GffObp19b	GFUI000759-PA	A0A1A9X702	d1dqe_A	15	99.9	1.5	-4.68	-1.104	0.57 ± 0.08	-0.2	88.7
GffObp19c	GFUI000757-PA	A0A1A9X700	d1ooh_A	17	99.8	1.5	-3.76	-1.393	0.53 ± 0.08	-0.2	85.3
GffObp28a	GFUI048313-PA	A0A1A9YMD6	c2wcl_A	23	99.9	1.5	-4.59	-0.757	0.55 ± 0.07	-0.19	93.3
GffObp44a	GFUI004675-PA	A0A1A9XB75	c6nbn_A	46	99.9	1.5	-6.42	-2.135	0.68 ± 0.08	0.05	87.9
GffObp56d	GFUI008988-PA	A0A1A9XFU7	c3r1p_F	25	99.9	1.5	-4.16	-2.063	0.56 ± 0.09	0.03	89.5
GffObp56e	GFUI008564-PA	A0A1A9XFC6	c3s0b_A	23	95.9	1.5	-3.77	-1.592	0.56 ± 0.09	-0.56	82.7
GffObp56h	GFUI009068-PA	A0A1A9XFX1	C3vb1_A	23	99.9	1.5	-5.29	0.386	0.59 ± 0.08	0.34	92.7
GffObp56i	GFUI007894-PA	A0A1A9XEM0	c5dic_A	23	98.1	1.5	-3.34	-2.057	0.42 ± 0.08	-0.35	82.1
GffObp57c	GFUI026749-PA	A0A1A9XZG5	c3l47A	26	99.8	1.5	-4.93	-0.5	0.49 ± 0.08	0.1	88.6
GffObp69a	GFUI040667-PA	A0A1A9YE07	c6hhe_A	33	99.9	1.5	-3.92	-0.152	0.70 ± 0.08	0.17	88.8
GffObp83a1	GFUI048612-PA	A0A1A9YMN8	c6hhe_A	33	99.9	1.5	-7.61	-0.224	0.78 ± 0.08	0.38	92.5
GffObp83a2	GFUI048613-PA	A0A1A9YMN9	c2erb_A	59	100	1.5	-7.27	-0.289	0.77 ± 0.07	0.39	92.5
GffObp83a4	GFUI048614-PA	A0A1A9Y943	c2erb_A	66	100	1.5	-6.84	0.102	0.80 ± 0.07	0.39	93.5
GffObp83cd	GFUI049167-PA	A0A1A9YN81	c3l47A	24	99.5	1.5	-4.35	-4.125	0.46 ± 0.08	-0.42	80.7
GffObp83ef	GFUI004156-PA	A0A1A9XAN4	c6nbn_A	19	98.4	1.5	-3.11	-2.138	0.36 ± 0.06	-0.44	85.1
GffObp83g	GFUI004155-PA	A0A1A9XAN3	c6nbn_A	30	100	1.5	-5.71	-2.228	0.60 ± 0.07	0.21	87.1
GffObp84a	GFUI027466-PA	A0A1A9Y077	c6nbn_A	25	99.8	1.5	-4	-2.087	0.57 ± 0.08	-0.13	88
GffObp8a	GFUI045274-PA	A0A1A9YIU1	c4pt1_B	14	99.9	1.5	-5.4	-1.919	0.56 ± 0.08	0.35	88.3
GffObp99b	GFUI035804-PA	A0A1A9Y960	c6nbn_A	19	99.6	1.5	-0.95	-0.822	0.52 ± 0.08	0.22	92.5
GffObp99d	GFUI035776-PA	A0A1A9Y943	c6nbn_A	42	100	1.5	-6.17	-2.227	0.66 ± 0.07	0.07	87.6