ORGAN DISTRIBUTION AND EXPRESSION PROFILE OF *GLOSSINA MORSITANS MORSITANS* ODORANT BINDING PROTEIN HOMOLOG GENES SEQUENCES IN *GLOSSINA FUSCIPES FUSCIPES*

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DECLARATION

This research thesis is my original work and has not been presented to any other institution for a degree award.

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DEDICATION

I dedicate this research thesis to mother Judith Atieno, father Saul Pepela and late Christopher Simiyu for helping me understand the value of education at a tender age. Uncles Samuel Simiyu, Prof. Godfrey Netondo, Tom Mboya and Silas Walumbe for encouraging me to pursue this course. My brothers Willis Odhiambo, Don Omondi and Elvis Oduor; My sisters Evelyn Adoyo, Caro Achieng and Brenda Akinyi for inspiring me at the times I felt low.

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TABLE OF CONTENT

DECLARATION ..... **ii**
DEDICATION ..... **iii**
ACKNOWLEDGEMENT ..... **iv**
TABLE OF CONTENT ..... **v**
LIST OF TABLES ..... **vii**
LIST OF FIGURES ..... **viii**
LIST OF PLATES ..... **ix**
LIST OF APPENDICES ..... **x**
ABBREVIATIONS AND ACRONYMS ..... **xi**
ABSTRACT ..... **xiii**
CHAPTER ONE **1**
1.0 INTRODUCTION **1**
1.2 Problem statement 3
1.3 Justification 4
1.4 Null hypothesis 5
1.5 Objectives 5
1.5.1General Objective 5
1.5.2 Specific Objectives 5
CHAPTER TWO **6**
2.0 LITERATURE REVIEW **6**
2.1 African Trypanosomiasis 6
2.2 Tsetse fly systematic and distribution 9
2.3 Tsetse fly Host Preference 11
2.4 Tsetse fly control strategies 12
2.5 Olfaction in Insects 14
2.5.1 Chemosensory proteins 18
2.5.2 Odorant degrading enzymes 19
2.5.3 Odorant receptor 19
2.5.5 Odorant Binding Proteins and Pheromone binding proteins 20
2.6 Quantitative real time PCR for Gene Expression 22
CHAPTER THREE **23**
3.0 MATERIALS AND METHODS **23**

3.1 Study Design 23
3.2 Study site and Tsetse fly collection.....23
3.3 DNA extraction from males and females *Glossina fuscipes fuscipes* tissues 27
3.4 Amplification of *Glossina fuscipes fuscipes* Tissues 28
3.5 Total RNA extraction from *Glossina fuscipes fuscipes* antennae and leg.....33
3.6 Preparation of First strand cDNA for *Glossina fuscipes fuscipes* antennae and leg tissues 34
3.7 Design of *Glossina morsitans morsitans* OBP primers for Quantitative Real Time PCR 35
3.8 Validation of Primers Used for Quantitative Real Time PCR.....37
3.9 Quantitative Real Time PCR of *Glossina fuscipes fuscipes* antennal and leg cDNA.....38
CHAPTER FOUR.....39
4.0 RESULTS.....39
4.1 Tsetse fly collection 39
4.2 Presence of *G. morsitans morsitans* Odorant Binding Proteins genes in *G. fuscipes fuscipes*.....39
4.3 Distribution of *Glossina morsitans morsitans* Odorant Binding Proteins in *Glossina fuscipes fuscipes* body parts 41
4.4 Expression of *Glossina morsitans morsitans* OBP genes in male and female *Glossina fuscipes fuscipes* antennae and leg.....46
4.5 Primer efficiency (E) and Regression Coefficient (R^2).....50
4.6 Quantitative Polymerase chain reaction (qRT-PCR) data Analysis.....51
4.7 Bioinformatics Analysis.....54
CHAPTER FIVE.....58
5.0 DISCUSSION, RECOMMENDATIONS AND CONCLUSION.....58
5.1 Discussion 58
5.3 Conclusion.....61
5.2 Recommendation 62
REFERENCES.....63
APPENDICES 80

LIST OF TABLES

Table 3.1: Tsetse trap position as determined by the global positioning system (GPS) 24

Table 3.2: *Glossina morsitans morsitans* Odorant Binding Proteins Sequences and characteristics…….……29

Table 3.3: Primers designed for *Glossina morsitans morsitans* Odorant Binding Proteins…….….32

Table 3.4: Quantitative Real Time Primers designed for *Glossina morsitans morsitans* Odorant Binding Proteins……...……35

Table 4.1: *Glossina morsitans morsitans* Odorant Binding Proteins Present or absent in *Glossina fuscipes fuscipes*…….……..…..40

Table 4.2: Summary of *Glossina morsitans morsitans* present or absent in female, males or both female and male *Glossina fuscipes fuscipes*…….……..43

Table 4.3: Summary of *Glossina morsitans morsitans* odorant binding proteins in *Glossina fuscipes fuscipes* organs……..…..45

Table 4.4: Bioinformatics Analysis……..…….55

LIST OF FIGURES

Figure 2.1: Life cycle of African trypanosomiasis…….….7

Figure 2.2: Distribution of *Typanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* in Africa …….…..…..8

Figure2.3: Tseste fly habitats in Kenya, (Zones 1–7) and *Glossina* species, Kenya…….10

Figure 2.4: The insect antenna……..……...….17

Figure 2.5: Hypothetical model incorporating recent insights about molecular interactions in the lumen and at the dendritic membrane of an insect ORN……..……...…18

Figure 3.1: The biconical trap (Trap 4) being set up at Sori B at the shores of Lake Victoria……..25

Figure 3.2: Mapping of ordinates after setting up the biconical trap……..26

Figure 4.1: General proportional *G. morsitans morsitans* distribution in female and male *G. fuscipes fuscipes*…….……..……...44

Figure 4.2: Percentage *G. m. morsitans* OBP distribution in different tissues of *G.f.fuscipes*……..…….…..…51

Figure 4.3: *Glossina morsitans morsitans* OBP genes relative expression rates in the female and male *Glossina fuscipes fuscipes* antennae……...52

Figure 4.4: *Glossina morsitans morsitans* OBP genes relative expression rates in the male *Glossina fuscipes fuscipes* antennal and leg organs..……..…...….52

Figure 4.5: *Glossina morsitans morsitans* OBP genes relative expression rates in the male *Glossina fuscipes fuscipes* antennal and leg organ…….....53

LIST OF PLATES

Plate 4.1: 1% agarose gel depicting *G. m. morsitans* OBP genes present in male *G. f. fuscipes*……41

Plate 4.2: 1% agarose gel depicting *G. m. morsitans* OBP genes present in male *G. f. fuscipes*……...41

Plate 4.3: Distribution of OBP genes in different body parts of male *G. f fuscipes* PCR products amplified with OBP primers……..42

Plate 4.4: Formaldehyde denaturing gel of total RNA extracted from the antennal and leg tissues of male and female of *Glossina fuscipes fuscipes*..……42

Plate 4.5: 1% Agarose gel of cDNA library constructed from female and male *G. f. fuscipes* antennae and leg…..……..……46

Plate 4.6: 1% agarose gel of GAPDH on first strand cDNA of male and female *Glossina fuscipes fuscipe*s antennae and leg…….47

Plate 4.7: 1% agarose gel primer validation for female *G. fuscipes fuscipes* genomic DNA……...……47

Plate 4.8: 1% agarose gel primer validation in male *Glossina fuscipes fuscipes*, primers for GAPDH (+) and OBP1-OBP12……48

Plate 4.9: 1% agarose denaturing gel-electrophoresis primer validation in male *Glossina fuscipes fuscipes*, primers for GAPDH (+) and, OBP13-OBP27……..49

Plate 4.10: 1% agarose denaturing gel-electrophoresis primer validation in male *Glossina fuscipes fuscipes*, only GAPDH (+), OBP2B, OBP28-OBP30…..……..49

LIST OF APPENDICES

Appendix 1: Number of *Glossina fuscipes fuscipes* collected per trap at the sampling sites in Sori, Homa Bay County……..…...80

Appendix 2: Estimation of DNA concentration and purity for samples extracted from male and female *Glossina fuscipes fuscipes* body parts……..….81

Appendix 3: Total RNA quantification and purity ratio……..82

Appendix 4: Quantification and purity ratios for first strand cDNA from male female *G. f. fuscipes* antennae and legs……..……83

Appendix 5: Quantitative RT-PCR primers standard curve values for Y-intercept, slope and coefficient of Regression……84

Appendix 6: Standard curve dilution curve values…….....85

Appendix 7: Primer percentage efficiency and the respective tissues in which the OBP primers amplified……..…….86

Appendix 8: The standard curve slope for 24 *G. morsitans morsitans* OBP primers that amplified in *G. fuscipes fuscipes* tissues and the respective regression co-efficients……...…….87

Appendix 9: Reagent Preparation…….88

Appendix 10: Treatment of plastic and glassware for use…….……..91

Appendix 11: Field photos……...92

Appendix 12: Laboratory Equipments used……94

ABBREVIATIONS AND ACRONYMS

AAT: Animal African Trypanosomiasis

BHC: Benzene Hexa Chloride

CATT: Card Agglutination Trypanosomiasis Test

cDNA: Complementary Deoxyribonucleic Acid

CSF: Cerebrospinal Fluid

CSPs: Chemosensory Proteins

DDT: Dichlorodiphenyltrichloroethane

DEPC: Diethylpyrocarbonate

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

GOBP: General Odorant Binding Protein

GPS: Global Positioning Systems

HAT: Human African Trypanosomiasis

HCH: Hexachlorocyclohexane

MOPS: 3-[N-Morpholino]propanesulfonic acid

NaOH: Sodium Hydroxide

OBPs: Odorant Binding Proteins

ODEs: Odorant Degrading Enzymes

ORN: Olfactory Receptor Neurons

ORs: Odorant Receptors

PBPs: Pheromone Binding Proteins

PCR: Polymerase Chain Reaction

RNA: Ribonucleic acid

SAT: Sequential Aerial Technique

SIT: Sterile Insect Technique

ABSTRACT

The tsetse fly, *Glossina fuscipes fuscipes*, is a riverine species and major vector of Human African Trypanosomiasis (HAT). Olfaction plays critical role in determining tsetse fly behavior. Odorant Binding Proteins (OBPs) are expressed abundantly in insect olfactory tissues and are postulated to be involved in the first step of odorant reception. A total of 33 OBPs have been reported from *G. m. morsitans* genome sequence while antennal transcriptome analysis of *G. morsitans morsitans* identified 22 OBPs. In this study, detection of *G. m. morsitans* OBPs was determined in male and female *G. f. fuscipes* head, thorax, abdomen and legs by polymerase chain reaction while expression levels were quantified in male and female *G. f. fuscipes* antennae and legs by quantitative real time polymerase chain reaction (qRT-PCR). A total of 31.8% of the OBPs identified were detected in the female head while 22.7% were detected in male head. From the thorax 31.8% of the OBPs were identified in males and 18.2 in females; 18.2% were identified in male abdomen tissues while 27% were identified in female leg tissues and then 22.7% of the tissues were identified in male leg tissues against 18.2% in females. Relative expression rate revealed that majority of *G. m. morsitans* OBP genes are highly transcribed in female antennae than in males while the male legs had the least expression levels. This study confirms the presence and expression of *G. m. morsitans* OBP genes in both male and female *G. f. fuscipes* tissues implying, that *G. m. morsitans* (Savannah tsetse) and *G. f. fuscipes* (Riverine tsetse) OBPs could be playing similar roles in the two species and that the antennae is the main olfactory organ. Future functional characterization of *Glossina fuscipes fuscipes* OBPs will go a long way in elucidating the function of these genes in the riverine species.

CHAPTER ONE

1.0 INTRODUCTION

Tsetse flies (Diptera: Glossinidae) are vectors of trypanosomes, the causative agents of Human African Trypanosomiasis (HAT) (sleeping sickness in humans) and African Animal Trypanosomiasis (AAT) (Nagana in animals) (Cox, 2004). The disease, African trypanosomiasis is re-emerging and has considerable impact on public health and economic development in sub-Saharan Africa (van Hove, 1997, Shaw, 2004). Tsetse flies contain about 30 living taxa, 22 species and 8 subspecies divided into three main groups namely; *Morsitans* (savannah), *Fusca* (forest) and *Palpalis* (riverine) which inhabit different ecological niches. *Glossina morsitans morsitans, Glossina austeni, G. longipalpis*, *G. swynnertoni* and *G. pallidipes* are savannah flies; *G. fuscipes fuscipes*, *G. pallicera pallicera*, *G. palpalis palpalis*, *G. palpalis gambiensis* and *G. tachinoides* are riverine flies while *G. brevipalpis*, *G. fusca congolensis*, *G. fusca fusca*, *G. fuscipleuris*, *G. frezili*, *G. haningtoni*, *G. longipennis*, *G. medicorum*, *G. nashi*, *G. nigrofusca hopkinsi*, *G. nigrofusca nigrofusca*, *G. severini*, *G. schwetzi*, are forest flies (Jordan, 1993).The three *Glossina* groups are distributed discontinuously with the *morsitans* group occupying much of the savannah (grassy woodland) of Africa. The distribution patterns of *fusca* group is confined to dry parts of Africa, from Ethiopia and Somalia in the north, to Mozambique and South Africa in the south, Uganda to the east and limited to thickly forested areas of Africa (Abila *et al*., 2008; Beadell *et al.*, 2010) while *Palpalis* group are found in similar forest habitats throughout very humid areas of Africa and extend into the mangrove swamps, riverine and lakeside forests (Jordan, 1993).

Glossina morsitans is a vector of *Trypanosome brucei* and *Trypanosoma congolense* responsible for HAT and AAT respectively (Van den Bossche, 2004). *Glossina fuscipes fuscipes* is a major vector of *T. brucei* causing sleeping sickness (Abila *et al*., 2008) while *G. pallidipes* is known to be vectors of *T. congolense* and *T. simiae* responsible for nagana (Simo *et al*., 2006).

The control of Trypanosomiasis includes use of chemotherapy. However, there has been reported increase in parasite resistance to developed drugs coupled with the ability of the parasite to change its surface glycoprotein poses challenges to effective treatment and vaccine development respectively (Akhoon *et al.,* 2011). This has shifted disease management to the control of the vector which offers potential avenues to controlling the disease. The vector control techniques include: bush clearing, Sterile Insect techniques (SITs), spraying with insecticides, use of traps and targets technologies that rely on tsetse olfactory, visual and gustatory cues to find their hosts (Gikonyo *et al*., 2002).

Tsetse flies from different ecosystems have unique feeding behavior. The savannah species for example do not prefer feeding on riverine hosts and vice versa (Gikonyo *et al*., 2002; 2003; Omolo *et al*., 2009). *G. morsitans* and *G. pallidipes* which are both savanna species do not feed on monitor lizard but feed on *Phacochoerus africanus* (warthog), *Ovibos moschatus* (Ox), *Syncerus caffer* (buffalo), *Tragelaphus strepsiceros* (kudu) while *G. fuscipes* hardly feed on buffalo but mainly feed on monitor lizard and crocodile (Clausen *et al*., 1998; Gikonyo *et al.,* 2002; Omolo *et al*., 2009). It is believed that the preferential feeding behavior of tsetse flies could be mediated by the different olfactory proteins found within the antennae (Rutzler & Zwiebel, 2005).

In tsetse, the antenna is the main olfactory organ and is perforated by sensilla that house olfactory proteins which are responsible for the detection, transport and transduction of olfactory signal (Voskamp *et al*., 1998; Rutzler & Zwiebel, 2005). The antenna comprises of three segments, with the largest segment containing the arista, which mainly function to trap the volatile odors from host (Rutzler & Zwiebel, 2005). Olfactory proteins that have been identified in *G. m. morsitans* antennae include odorant binding proteins (OBPs), pheromone binding proteins (PBPS), chemosensory binding proteins (CSPs), odorant degrading enzymes (ODEs), odorant receptors (Ors), ionotropic receptors (IRs) and ionotropic glutamate receptors (IgluR) (Liu *et al*., 2010a; Liu *et al*., 2012c; Obiero *et al*., 2014). It is believed that OBPs, PBPs and CSPs found within the sensillium bind odors entering through the antennal pores and are transported

to Ors located within the olfactory receptor neurons (ORNs). The sequencing of *G. m. morsitans* genome opens new avenues for control of tsetse flies based on its unique biology (IGGI, 2014). Genes related to olfaction can be used to screen and synthesize new potent tsetse attractants and repellents that can improve the efficacy of trapping technologies based on their specific functions in tsetse flies. Odorant binding proteins are critical in modulating tsetse olfactory behavior. It is not known whether the savannah, riverine and forest tsetse flies use the similar or different OBPs in discriminating the various hosts they feed on. This study examined presence and expression levels of *G. m. morsitans* OBPs (savannah fly) in *G. fuscipes fuscipes* (riverine tsetse fly).

1.2 Problem statement

More than 60 million people are at risk of contracting Human African Trypanosomiasis disease, with an estimated 50,000 to 70,000 persons newly infected cases annually (WHO, 2014). The economical impact of the animal disease, Nagana, is much greater as it deprives farmer's milk, meat, oxen for ploughing and transport (Shaw, 2004). It is estimated that the cost to these problems is more than US$ 4.5 billion every year including losses to agricultural production and perennial expenditure on trypanosoidal drugs and other social intervention schemes in an attempt to cope with trypanosomiasis (Shaw, 2004; Garber *et al*., 2010). While there have been some efforts geared towards treatment of trypanosomiasis, the vector (tsetse) and host reservoir poses the greatest challenge to this efforts (Maudlin, 2006). It's therefore emerging that the control of the disease still remains a pipe dream as there is no vaccine yet and the traditional way of control which included killing of the host animal and clearing of bushes has been considered environmental friendly despite registering considerable success. Additionally, despite studies showing the importance of odorant binding protein in tsetse fly olfaction and identification of suitable host's scanty information is available on OBPs diversity across different species occupying different ecological niches. Despite the annotation of odorant binding proteins in *Glossina morsitans morsitans,* no information has been generated so far on their homolog expression levels in *G. fuscipes*

fuscipes. This information could be very critical in developing better control measures that will target cross species. It's against this background that this study intended to study the presence of odorant binding proteins homolog genes sequences of *Glossina morsitans morsitans* in *Glossina fuscipes fuscipes*.

1.3 Justification

The long term control of trypanosomiasis is centered on the control of the parasite vector, tsetse fly. Many insect genomes have been sequenced as it is hoped that genomic studies will open new avenues that can be exploited to control tsetse flies (Aksoy *et al.,* 2014). For example, genes that play a critical role in the insect physiology and behavior will be easily identified and their role determined. Genes related to olfaction are important in determining the tsetse fly host, larviposition sites, mates for reproduction and resting sites. Odorant binding proteins are potential targets in controlling the tsetse vectors because it is involved in the first step of identification, binding and transport of odors from the external environment through the sensillium lymph to Ors upon which is manifested into behavioral response. Different tsetse flies feed on different host species and though the role of OBPs in tsetse flies in discriminating the hosts has not been established. It is not known if tsetse flies use the same or different OBPs in locating their hosts based on the unique ecological zones they occupy. The expression profile will provides insight on highly expressed *G. m. morsitans* OBP genes homologs in *G. f. fuscipes* which could further provide more interest in studying specific olfactory roles played by them. This study therefore sort to establish the presence and distribution of OBPs previously identified in *G. m. morsitans* in the male and female of *G. f. fuscipes.*

1.4 Null hypothesis

Glossina fuscipes fuscipes do not have homologous OBP genes sequences to those in *Glossina morsitans morsitans* and are not distributed across different organs.

1.5 Objectives

1.5.1General Objective

To determine the organ distribution and expression profile of Odorant Binding Proteins homolog genes in *Glossina f. fuscipes* tissues

1.5.2 Specific Objectives

1. To determine the presence and organ distribution of *G. morsitans morsitans* Odorant Binding Proteins homolog genes sequences in *G. fuscipes fuscipes*
2. To determine the expression of Odorant Binding Proteins homolog genes sequences in males and females *G. f. fuscipes*
3. To profile the relative expression levels of *G. morsitans morsitans* OBP homolog genes sequences in *G. fuscipes fuscipes* antennae and legs

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 African Trypanosomiasis

African Trypanosomiasis is a neglected tropical disease transmitted by tsetse flies, vectors of trypanosome parasites (Lundkvist *et al*., 2004). There are two forms of African Trypanosomiasis namely Human African Trypanosomiasis (HAT) (or sleeping sickness) in humans and African Animal Trypanosomiasis (AAT) (or Nagana) in animals (Garner *et al.,* 2009). Both HAT and AAT are restricted to sub-Saharan Africa where the disease is a major cause of rural under development (Robays *et al.,* 2004). HAT is caused by the species *Trypanosoma brucei,* which is composed of *three* subspecies: *T. b. gambiense,* which causes endemic disease in central and west Africa; *T. b. rhodesiense,* which causes more acute disease in east and southern Africa (Figure 2.2) and *T. b. brucei,* which usually infects domestic and wild animals but not man (Picozzi *et al*., 2005; Simarro *et al.,* 2011). It has been reported that *T. vivax* and *T. congolense* are major pathogens of cattle. *T. simiae* causes high deaths in domestic pigs while *T. b. brucei* affect all livestock (Mugasa *et al.,* 2008). The parasite is transmitted by infected tsetse fly during feeding or mechanically by other blood suckling arthropods such as mosquitoes (Steverding, 2008).

During a blood meal on the mammalian host, an infected tsetse fly (genus *Glossina*) injects metacyclic trypomastigotes into skin tissue. The parasites enter the lymphatic system and pass into the bloodstream. Inside the host, they transform into bloodstream trypomastigotes, which are carried to other sites throughout the body, reaching other blood fluids which include lymph, spinal fluid, and continue the replication by binary fission. The entire life cycle of African Trypanosomes is represented by extracellular stages. The tsetse fly becomes infected with bloodstream trypomastigotes when taking a blood meal on an infected mammalian host. In the fly's midgut, the parasites transform into procyclic trypomastigotes, multiply by binary fission, leave the midgut, and transform into epimastigotes. The epimastigotes reach the fly's salivary glands and

continue multiplication by binary fission (Figure 2.1). The cycle in the fly takes approximately 3 weeks. Humans are the main reservoir for *Trypanosoma brucei gambiense*, though this species can also be found in animals. Wild game animals are the main reservoir of *T. b. rhodesiense*. (Rotureau & Van Den Abbeele, 2013; Haines, 2013; Wang *et al*., 2013; Geiger *et al*., 2013).

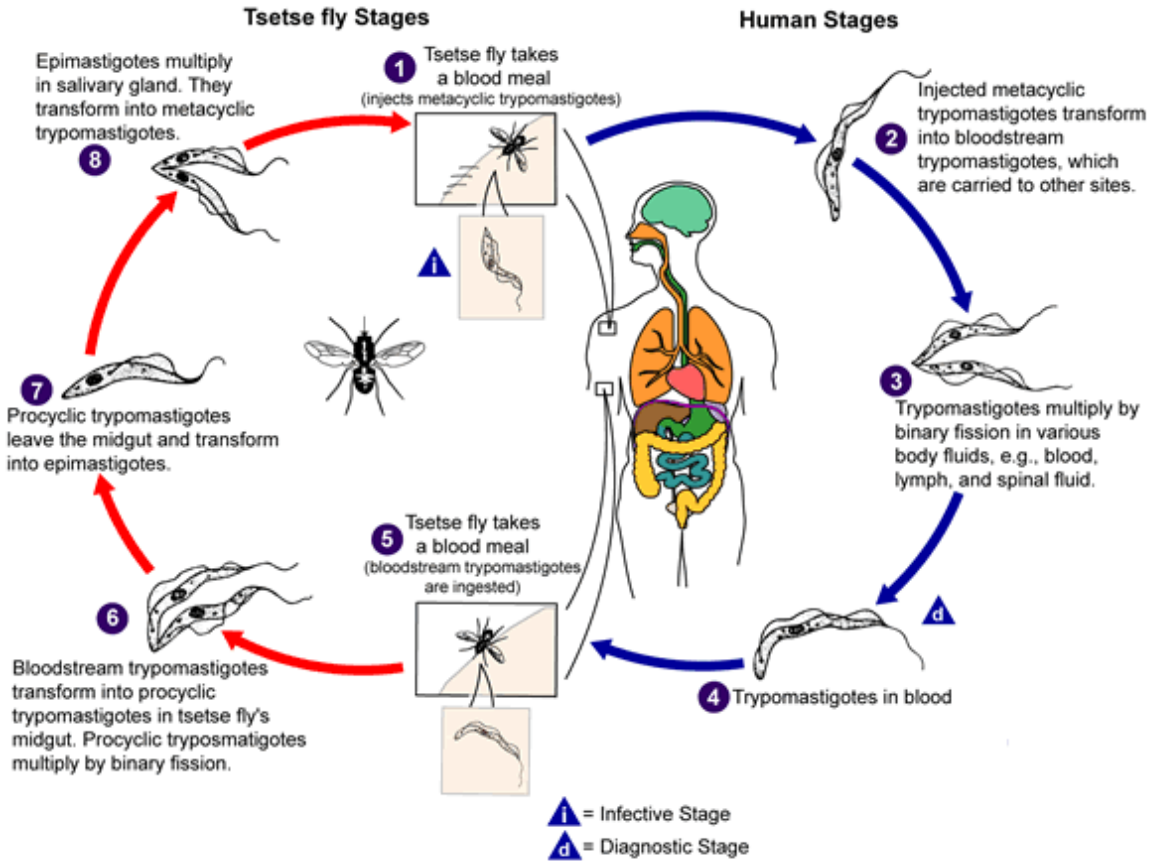

Figure 2.1: Lifecycle of African trypanosomiasis, adapted from CDC (http://www.cdc.gov/parasites/sleepingsickness/biology.html)

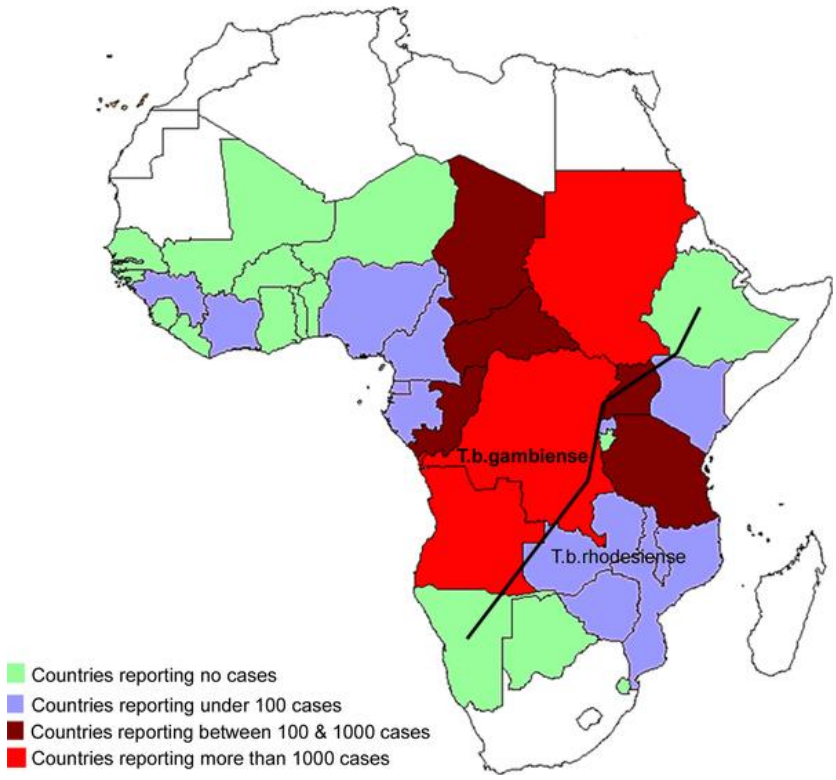

Figure 2.2: Distribution of *Typanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* in Africa (Simarro *et al.*, 2010)

The drugs used for early-stage sleeping sickness treatment include pentamidine for gambiense infection and suramin for rhodesiense infection and for late-stage sleeping sickness treatment, melarsoprol for both gambiense and rhodesiense infection and eflornithine for gambiense infection (Kennedy, 2013). Diagnosis and treatment of HAT is by no means an easy task, even without the logistical and infrastructural difficulties of patient access in remote rural areas.

In the early haemolymphatic stage, there are no specific symptoms and the infection is readily confused with other febrile illnesses, especially malaria. The later stage of infection (meningo-encephalitic stage) is easier to diagnose but can have irreversible consequences and requires treatment with drugs with adverse side-effects (WHO, 2014).

Diagnosis of the disease currently relies on parasitological examination of blood using various concentration techniques and serological tests such as the card agglutination trypanosomiasis test (CATT) (Kennedy, 2013). Determination of the stage of disease must be made by parasitological and biological examination of cerebrospinal fluid (CSF) obtained by lumbar puncture. The later is particularly important for determining both treatment regime and prognosis (Kennedy, 2013).

2.2 Tsetse fly systematic and distribution

There are three *Glossina* groups which are distributed discontinuously; *morsitan*s are the savannah tsetse flies and occupy the savannah grassy woodland of Africa. They are mostly found in the South, especially Zimbabwe and Botswana, North of West and Central Africa. Examples include G*lossina austeni*, *Glossina morsitans morsitans, Glossina pallidipes* and *Glossina swynnertoni* (Rodgers & Robinson, 2004). *Fusca* group are the forest flies and their distribution takes three main patterns. *G. longipennis* is found mainly in South East Sudan, southern border of Ethiopia and North Eastern part of Somalia, Kenya and Tanzania. *Glossina brevipalpalis* is scattered widely in most of Eastern parts of Africa, from Ethiopia and Somalia in the North, to South Africa and Mozambique in the South. Some *fusca* species are limited to thick forest areas of Africa where *G. tabaniformis, G. nashi* and *G. hanningtoni* restricted to rain forest. The other *fusca* flies include *G. fusca, G. medicorum, G. fuscipleuris* and *G. schetzi* while *G. medicorummay* is found in riverine ecosystems (FAO, 2000). *Fusca* are favored by dry conditions while *morsitans* are favored by cold winter conditions.

Flies of the *palpalis* group generally occupy riverine and lacustrine habitats and are opportunistic feeders (Leak, 1999). *Glossina fuscipes fuscipes* are found within a large area of central Africa, from Cameroon, Gabon, Congo, Zaire and the Central African Republic eastwards to Uganda and western Kenya. Highly localized patches exist on the margins of Lake Victoria in Tanzania and small, isolated patches of *G. f. fuscipes* are also found in southwestern Ethiopia and southern Sudan (Rogers & Robinson, 2004). *Glossina f. fuscipes* are vectors of *Trypanosoma brucei gambiense* and *Trypanosoma*

bruce rhodesiense that cause HAT particularly in Uganda and western Kenya (Waiswa *et al.*, 2006). Uganda is an active focus of HAT (Welburn *et al.*, 2006), although *G. f. fuscipes* is principally zoophagic (Wamwiri *et al.*, 2007). The main trypanosome reservoirs include cattle and other domestic livestock, as well as numerous wild mammals (Waiswa *et al.*, 2003). In Kenya, tsetse flies are distributed discontinuously ranging from Western region through to the coastal region (Figure 2.3).

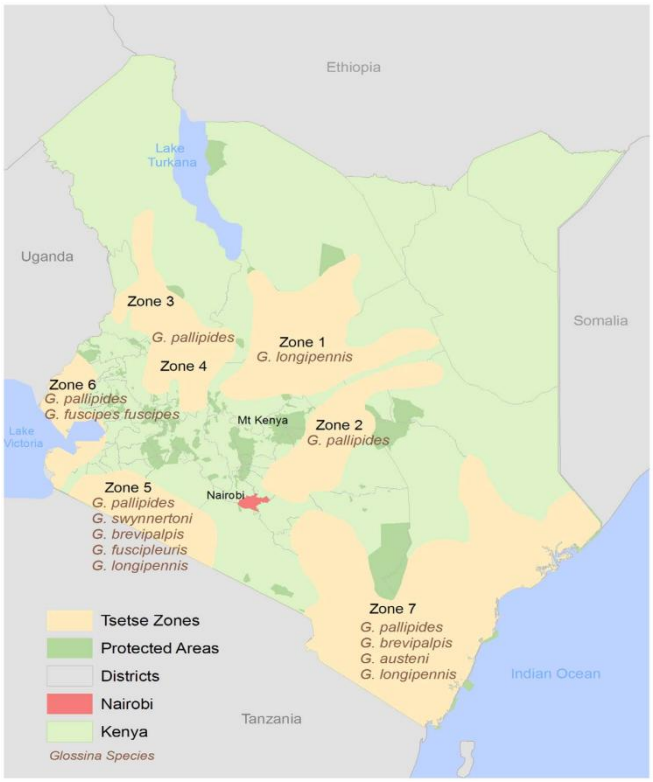

Figure 2.3: Tseste fly habitats in Kenya, (Zones 1–7) and *Glossina* species, Kenya. Data Source: International Livestock Research Institute, Kenya 2009. doi:10.1371/journal.pntd.0000957.g002

2.3 Tsetse fly Host Preference

Tsetse flies exhibit different feeding patterns on specific groups of vertebrate hosts. Animals like warthog, bushpig, bushbuck, cattle, elephant and buffalo are frequently fed on by tsetse flies while others like Zebra, giraffe, black rhinoceros, waterbuck, hartebeest and Impala are rarely fed on (Clausen *et al.,* 1998). Tsetse locates and recognizes preferred host through olfactory and visual cues. The host produces odors which activate the fly beyond the visual range and at close vicinity to the host; the fly is stimulated by visual cues such as shape, color, size and movement (Leak, 1998). Excretory products (Urine, dung, breath and glandular secretions) emit attractive odors to tsetse which are detected by the antennae (Omolo *et al*., 2009).

Savanna tsetse flies are attracted by buffalo urine which has been reported to contain 7 phenolic compounds (Hassenali *et al.,* 1986; Owaga *et al.,* 1988). Body and breaths are other sources of tsetse attractants (Vale & Hall, 1985) while body odors from Waterbucks repel tsetse flies (Gikonyo *et al.,* 2000; 2002; 2003). Of the known tsetse fly attractants 4-cresol (4-methoxyphenol) and 3-n-propylphenol are common in buffalos and ox (Saini *et al*., 1993). Guaicol (2-methoxyphenol), 3-isopropyl-6-methylphenol and a series of (C_8-C_{13}) methylalkenes in Waterbuck are moderate attractants (Gikonyo *et al*., 2002).

Rivarine tsetse flies on the other hand have been reported to be opportunistic in their feeding habits (Gikonyo *et al*., 2003; Omolo *et al*., 2009) so that mammals are not their main hosts. They have been known to feed on reptiles, their blood meal have been seen to account to between 14-26% reptilian (Gouteux *et al*, 1994). Since the reptiles are small in sizes with low population density and their discreet way of life, it has been suggested that these reptiles attract riverine tsetse flies with their odors (Gouteux *et al*, 1994). Feeding responses of *G. pallidipes, G. m. morsitans* and *G. f. fuscipes* to their referred hosts have been studied with a range of natural odors identified (Gikonyo *et al*., 2002; Omolo *et al*., 2009). The identified volatile odors (attractants and repellants) from tsetse hosts find application in providing alternative means that can be exploited to

control the fly by developing more potent odors through structural studies (Saini & Hassenali, 2007).

2.4 Tsetse fly control strategies

Early attempts to control tsetse flies included bush clearance (designed to eliminate the shaded places where tsetse fly rest and lays their larvae) and shooting of wild game animals (designed to eliminate the host fed on by the tsetse flies). Although widely effective, such methods can no longer be recommended because they are not environmental friendly (Bourne *et al*., 2001). Spraying with insecticide involved using dichlorodiphenyltrichloroethane (DDT), Benzene Hexa Chloride (BHC) and Hexachlorocyclohexane (HCH) which were the only synthetic compounds available (Bourne *et al*., 2001). The application of residual deposits of persistent insecticides to tsetse resting sites was widely used, but is now discouraged due to concerns about effects on non-target organisms (Bourne *et al*., 2001).

Since tsetse flies had exquisite susceptibility to modern insecticides, high levels of tsetse control can be achieved by sequential aerial spraying of ultra low dosages of biodegradable products. Using modern global positioning systems (GPS), Sequential aerial technique (SAT) can now be applied accurately along pre-planned flight lines. In the past, SAT involved the use of endosulfan, an organophosphate that can no longer be recommended on environmental grounds (Kgori *et al.,* 2006). More recently, pyrethroids such as deltamethrin have been used at low doses that hardly provoke significant effects on other fauna. For example, deltamethrin have been applied to control *G. m. centralis* in Botswana were approximately 10 000 times less than those typically applied against Chagas disease vectors in Latin America (Kgori *et al.,* 2006).

Other tsetse control strategies involve treating cattle with appropriate insecticide formulations, usually by means of cattle dips, or as pour-on, spot-on, or spray-on veterinary formulations (Ndeledje *et al*., 2013). These are highly effective against tsetse

and have the additional advantage of controlling other flies and cattle ticks (Ndeledje *et al*., 2013).

Sterile insect technique (SIT) exploits mating biology of tsetse. Male flies are mass reared in the laboratory, sterilized by irradiation, and released to mate with wild females. Females mated with sterile males are unable to produce viable offspring and these results in reduction in tsetse population (Vreysen, 2001). Unlike all other tsetse control techniques, SIT has no effect on non-target organisms; it is more efficient at lower fly densities and is ideally suited to the final phase of local tsetse eradication (Vreysen, 2001).

Traps and targets are currently being used to control tsetse flies and a reduction in transmission rate has been reported in areas where the traps have been used (Vale *et al*., 1988; Hargrove *et al.,* 1995). Tsetse fly have high metabolic rate and feed exclusively on vertebrate blood hence their survival depends on detecting and encountering suitable hosts on which to feed. This principle has been exploited in the design of traps and targets which mimic key features of host animals, attracting tsetse in such a way that they are captured (Kuzoe & Schofiel, 2004). The captured tsetse flies in traps can be identified and counted, providing an immediate use in sampling the existing tsetse population(s). In the early 1900s, sticky traps worn by plantation workers were successfully deployed on the Island of Principe to eradicate *G. palpalis* (Vreysen, 2001). The traps generally use blue and black cloth in a shape that attracts the tsetse flies and then funnels them upwards into a netting trap, usually in the form of a monoconical (pyramidal) or biconical shape. Example of traps that have been designed and used for tsetse control includes Ngu and biconical traps (Belete *et al*., 2004). A simpler and cheaper device for controlling tsetse flies involves a suspended screen of blue and black cloth (often known as a tsetse target) impregnated with a biodegradable pyrethroid insecticide such as deltamethrin. Flies are attracted by the blue segments and land on the black segment, quickly succumbing to the insecticide (Kaba *et al*., 2013). The effectiveness of traps and targets can be greatly enhanced by addition of appropriate

odor bait which are either attractants or repellants and have been isolated from host animals (Gikonyo *et al*., 2003; Omolo *et al*., 2009).

In addition, synthetic odor analogs have been synthesized and used to improve trap technologies resulting in further reduction of tsetse population and cases of trypanosomiasis (Saini & Hasanali, 2007). Recent technologies have also been innovated such as "tsetse collars" that is hanged on the neck of cows in a herd. The collar has a dispenser that emits volatile repellant at periodic interval thereby pushing the tsetse fly away from feeding onto the herd (Saini *et al*., 1993). If used in an integrated push-pull system with traps, the repellant act to push the tsetse fly from the grazing herd while the traps act to pull the tsetse vector into the trap (Esterhuizen *et al.,* 2006). The technology has reported success and reduction in trypanosomiasis incidence in areas that it has been used though hardly sustainable by the local pastoralists because of the high cost of maintenance (Esterhuizen *et al.,* 2006). Trapping and repellant technologies depend on tsetse flies olfaction and visual cues to respond to the odors and colors respectively.

2.5 Olfaction in Insects

An antenna is the main olfactory organ in insects which is responsible for translating the chemical odorant messages into neuro electrical activities to elicit behavioral-physiological responses. Its surface is innervated by sensilla which is also distributed to other parts of the insect's body and are often used for purposes unrelated to feeding including mechano-, hygro-, and thermo reception (Boeckh *et al*., 1987; de Bruyne *et al.*, 2001). There occur three morphological and functionally different auxiliary cells per sensillium. These are thecogen, tricogen and tomogen cell. The thecogen encloses the neurons from the axons up to the outer dentritic segment; tricogen cell is the largest and secretes the cuticle of hair shaft during development while tomogen cell secretes part of the hair base during development (Hansson, 1999). The cuticular forms of sensilla are very variable. For example in locust and *Drosophila* there are distinct types, two of basiconic and one of coeloconic sensilla (Stocker, 1994). The long hairs with thick walls

are trachoid sensilla, short finger like projections (basiconic pegs), flat plates level with the general surface of the cuticle (plate or placoid sensilla) and short pegs sunk in depressions of the cuticle and opening to the exterior via a relatively restricting opening (coeloconic sensilla) (Wegener *et al*., 1997).

The axons from the neurons extend without synapses to the antennal lobes encoding in arborizations that form the olfactory glomeruli (Figure 2.4). Generally each neuron ends in one glomeruli and all neurons responding to the same or group of compounds ends in the same glomerulus and all nervous responding to the same or group of compounds end in the same glomerulus as is the case of sensilla on the palps and antennae (Vosshall *et al.,* 2000) (Figure 2.4).

Tsetse flies, just like other insects depend on olfactory or visual cues to communicate with its external environment (Pelosi, 2006). Olfaction is an important sensory modality in insects as it plays a critical role is identification of mate, larviposition/oviposition sites and location of hosts (Kaissling, 2001). The antenna is the main olfactory organ that house olfactory proteins that detect volatile chemicals from the environment **(**Shanbhag *et al.,* 1999). Other sensory organs like maxillary palps also detect odors in many insect species (Jonathan *et al*., 2014). The majority of olfactory sensilla can be found on the antennae, but smaller sets are also located on other head appendages such as maxillary palps (de Bruyne *et al*. 1999) and labial palps (Stange 1992; Kwon *et al*. 2006). Olfactory sensilla fall into two ultra structural categories, double walled and single walled. While the former are usually small and uniformly peg shaped, the latter can take on many different shapes (Steinbrecht, 1997). In moths, single walled sensilla can be up to 100 µm long thick walled pointed hairs (trichoid sensilla), but in most insects (e.g., *Drosophila*), they are much smaller. Trichoid sensilla typically house neurons with unbranched dendrites (Shanbhag *et al.,* 2000). Hymenoptera (e.g. honeybees) and certain Coleoptera have pored plates (placoid sensilla), some with large numbers of branched dendrites (Nikonov & Leal 2002). Most insects including tsetse

flies have thin-walled short (5–30 µm) blunt hairs basiconic sensilla (Shanbhag *et al*., 2000).

Olfactory proteins that have been identified in insects include soluble proteins (odorant binding proteins, pheromone binding proteins, chemosensory proteins and odorant degrading enzymes) and receptor proteins (odorant receptors, ionotropic receptors and ionotropic glutamate receptors (Walter, 2013). It is postulated that volatile odors from the environment gets through the antennal pores into sensillum lymph. The odors are picked by the soluble proteins (either odorant binding proteins (OBPs) (Liu *et al*., 2010b), pheromone binding proteins (PBPs) or chemosensory proteins (CSPs) (Liu *et al*., 2012c) and are transported through sensillium lymph to the odorant receptors (Ors) located within the olfactory receptor neurons (ORNs) in the dendrites (Obiero *et al*., 2014). The signal is then propagated through axons to higher brain centers which are then translated to behavioral responses (Walter, 2013) (Figure 2.4).

The perireceptor events involved in odor coding range from odorant capture to activation of ORN with generation of electrical messages trough signal transduction process. The OBPs, PBPs,CSPs, ODEs and Ors are expressed in the support cells and secreted into the sensilla lumen (Steinbrecht *et al*., 1992; Sandler *et al*., 2000) while ORNs express one or a few types of Ors (Clyne *et al.*, 1999; Benton, 2006). The odor enters through cuticular pores and are loaded into the OBPs that transport chemicals conventional Ors and also protect them from degradation by ODEs. Odorants transportation is directed by specific OBP receptor that is either constituted by (1) the conventional Or or (2) by different molecules interacting with the odorant which may physically interact with highly conserved 83b family of Or which is expressed in majority of ORNs. Or83b family protein and possibly additional molecules maybe required to fully activate heretomeric G-protein (Rutzler & Zwiebel, 2005) (Figure 2.5).

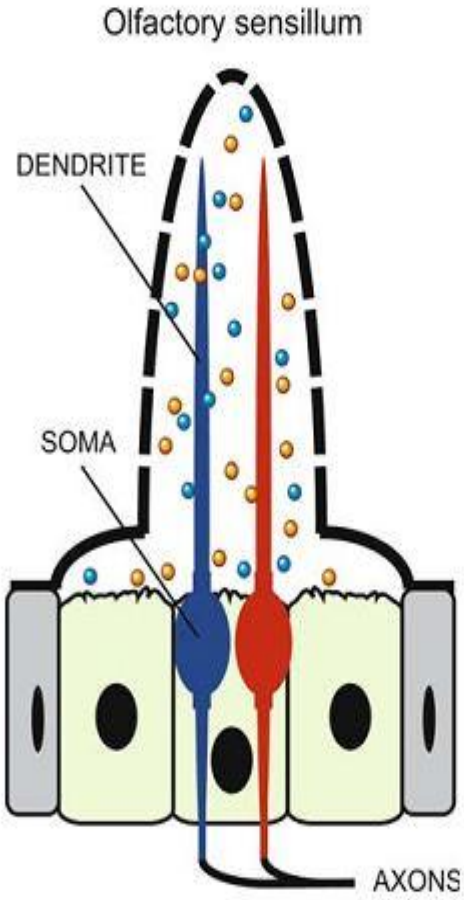

Figure 2.4: The insect antenna typically bears between several hundred and some thousand sensillar hairs, depending on the species, which are depicted on the left. (Rutzler & Zwiebel, 2005)

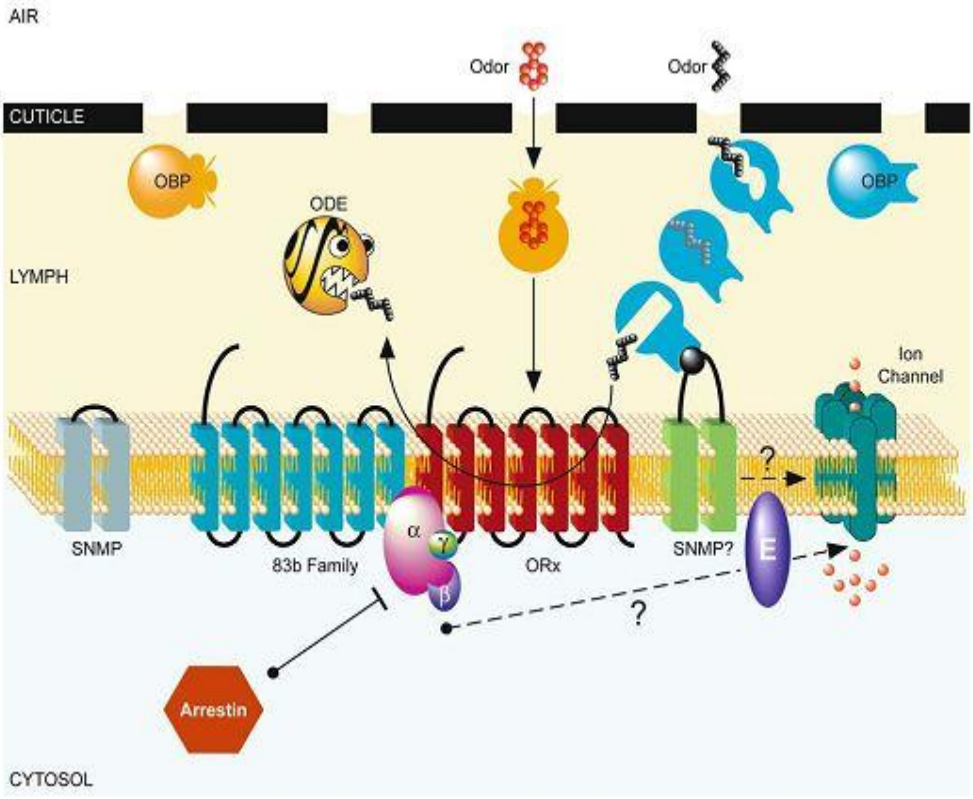

Figure 2.5: Hypothetical model incorporating recent insights about molecular interactions in the lumen and at the dendritic membrane of an insect ORN (Rutzler & Zwiebel, 2005).

2.5.1 Chemosensory proteins

Chemosensory proteins are soluble proteins found in the sensillum lymph of insect antenna (Tomaselli *et al.*, 2006). They have conserved four-cysteine signature (C1-X6-C2-X6-18-C3-X2-C4) and are secreted into the sensillum lymph of insect chemosensory sensilla. They are involved in CO_2 detection, chemical signal transmission, regenerating legs and chemoperception (olfaction and taste), based on whether they are present in antennae, tarsi or the labarum (Picimbon *et al.*, 2001). Although many are expressed in the antennae, others are expressed in other tissues including legs (Picimbon *et al.*, 2001), labial palps (Maleszka & Stange, 1997), tarsi (Angeli *et al.*, 1999), brain (Whitfield *et al.*, 2002), proboscis (Nagnan-Le Meillour *et al.*, 2000), pheromone gland (Dani *et al.*,

2011) and wings (Ban *et al*., 2003). In *Apis mellifera* CSPs have been reported to be involved in larval development and brood pheromone transportation (Briand *et al*., 2002). In the cockroach *Blatta germanica* a CSP is involved in leg regeneration (Kitabayashi *et al*., 1998). One CSP of the diamond-back moth, *Plutella xylostella*, is able to bind nonvolatile oviposition deterrents (Liu *et al*., 2010d). Several CSPs are highly expressed in the lymph of chemosensilla and exhibit binding activity towards odorants and pheromones (Pelosi *et al*., 2006), but there is little evidence of a role in olfaction. In tsetse flies, five CSPs have been identified in *G. morsitans morsitans* and established to be related to female host seeking behavior (Liu *et al.,* 2012c).

2.5.2 Odorant degrading enzymes

Odorant Degrading proteins Enzymes are found in the sensillum lymph of antennae. They are thought to be involved in odor degradation and clearance thereby terminating the olfactory signal excitation. Odorant receptors are embedded in the cell membrane of dendrites housed within the sensillum (Xu *et al*., 2005). Insect odorant receptors (ORs) are ligand-gated ion channels formed by a pair of proteins: a canonical receptor protein that gives the protein complex its specificity and an obligatory co-receptor named Orco (Nakagawa *et al.,* 2005; Sato *et al*., 2008; Wicher *et al*., 2008). Intracellular ODEs, such as glutathione-transferase or Cytochrome P450 have been reported to degrade odors entering the support cells of the sensilla (Maibeche-Coisne *et al*., 2004). While in *Phyllopertha diversa,* cytochrome P450 had been characterized and reported to degrade pheromones localized in the plasma membrane of the dendrite (Wojtasek & Leal, 1999).

2.5.3 Odorant receptor

Insect's odorant receptors (Ors) are membrane receptors having seven transmembrane domains quite diverse in amino acid sequence and unrelated to odorant receptors of mammals, fish, or *Caenorhabditis elegans (*Vosshall *et al.,* 1999*)*. Insect Ors are a heteromultimeric complex of unknown stoichiometry of two seven transmembrane domain proteins with no sequence similarity to and the opposite membrane topology of

G-protein-coupled receptor with conserved Glu, Asp and Tyr residues (Nakagawa *et al*., 2012)

The insect genomes characterized to date contain from 60 to 341 Or genes with *D. melanogaster* having 60 Or genes encoding 62 gene products through alternative splicing (Robertson *et al*., 2003) while 79 Or genes have been identified in *An. gambiae* whereas the red flour beetle, *Tribolium castaneum,* has 341 predicted Ors (Touhara &Vosshall, 2009). The genes that encode sex-pheromone-component sensitive Ors of *B. mori* have been characterized and shown to respond to the two pheromone components *bombykol* and *bombykal* (Sakurai *et al.,* 2004; Krieger *et al.,* 2005; Nakagawa *et al*., 2005; Syed *et al.,* 2006). These receptors are expressed in adjacent neurons in a single trichoid sensillum. In *H. virescens,* six related Ors have been characterized, and three were shown to respond to its pheromone components (Krieger *et al*., 2002, 2004; Grosse-Wilde *et al.,* 2007). One of these is expressed in the most abundant type of trichoid sensillum, while the other two co-inhabit another type. It is likely that the choice of an Or for placement in a moth-pheromone-sensitive ORN relies upon the same genetic processes as described for *Drosophila* ORs for general odorants (Ray *et al*., 2007). In Tsetse fly 46 putative Or genes have been identified in *G. m. morsitans* which could be very important in its olfactory and gastutory responses (Obiero *et al*., 2014).

2.5.5 Odorant Binding Proteins and Pheromone binding proteins

Odorant binding Proteins (OBPs) and Pheromone Binding Proteins (PBPs) are highly diverse group of olfactory proteins even in insects of the same species. They are antennal specific soluble proteins containing about 130 - 150 amino acids residues. They are small (14-20 kda) proteins with a signal peptide at the N-terminal and six conserved cysteines (Prestwich, 1993; Biessmann *et al.,* 2002). OBPs bind general odors while PBPs bind pheromones (Pelosi, 2006). There are three types of chemically identified insect released pheromones. These include: those which cause sexual attraction, alarm behavior and recruitment. Sex pheromones are released by the female insect to cause response to sexual behavior. This makes the male insect to be attracted to the female and

attempt to copulate with the female that has released the sex pheromone. As a way of survival and continuity this explains why insects are rather sensitive and selective to sex pheromone of their species. However the insects show far less sensitivity and chemo specificity for alarm pheromones (Fred & John, 1968).

In *Drosophila,* 35 OBP genes have been identified in complete genome sequence (Vogt *et al.,* 1991). *An. gambiae* has 60 putative OBP genes (Biessmann *et al*., 2002), *Culex* mosquito has 2 identified OBP genes (Ishida *et al.,* 2002**,** Sengul & Tu, 2008**),** *A. mellifera* has 21 OBPs **(**Sylvain & Ryszard, 2006) while *Aedes aegypti* has 22 OBPs (Sha *et al*., 2008). In *Glossina morsitans morsitans* a total of 22 OBPs genes have been reported (Liu *et al.,* 2010b) while the genome sequencing of *G. m. morsitans* revealed 33 OBPs (IGGI, 2014). Clearly, OBPs are essential components of the chemosensory system based on the high number reported in different insect species (Plettner *et al*., 2000).

The olfactory defects associated with loss of an OBP shows that these proteins are required for normal olfactory behavior at the point of odor detection. The potential roles for these proteins has been reported to include solubilizing or concentrating odorants in the sensillum lymph, mediating odorant removal or acting as co-ligand at the receptor (Pelosi, 1994; Sengul & zhijian 2009). In mosquitoes the OBPs are used to identify and discriminate hosts by temperature and chemical sensation (Dekker *et al*., 2002).Volatile compounds released from human skin attract female *An. gambie* (Dekker & Takken, 1998).

Glossina species use OBPs to find their suitable hosts (Liu *et al*., 2010b). The uniqueness of their ability to detect their suitable hosts makes them not necessarily to feed on the hosts that happen to be in the same habitat. This phenomenon was observed by Clausen *et al*., (1998) who noted that common animals, such as zebra and wildebeest, are not hosts to riverine species (*G. f. fuscipes*) but to savanna species (*G. m. morsitans*) and therefore each *Glossina* species has a specialist range of host animals. The preferred

host for *G. m. morsitans* is the warthog. Other animals fed on by *G. m. morsitans* include ox, buffalo, kudu and human (Den Otter *et al.,* 1992).

2.6 Quantitative real time PCR for Gene Expression

Gene expression analysis provides insight into complex regulatory networks which will lead to the identification of genes involved in new biological processes or implicated in disease development. Quantitative real-time polymerase chain reaction (qPCR) is often used as a tool to determine the expression of target genes, which are normalized against a stable reference gene. qPCR has increasingly gained importance due to its high sensitivity, accuracy, reproducibility, and its potential for high throughput (Bustin, 2002). Obtaining accurate and reliable quantitative gene expression results is difficult, this is due to experimental variation, such as differences in amount and quality of starting material, quantity and quality of RNA, and enzymatic efficiencies during reverse transcription. However with the choice of a good housekeeping gene robust results in real time PCR can be achieved, because reference genes undergo the same preparation steps as the target gene, by selecting one or more reference genes used for normalization in real-time PCR analysis this issue can be avoided. These reference genes under selection could potentially stabilize the experimental variability that typically occurs as a result of the various steps of the experimental procedure (Tricarico *et al*., 2002). Certainly, the expression levels of reference genes should remain constant between the cells of different varieties, tissues, lifecycle phases and experimental conditions, otherwise, it can lead to erroneous results in quantification of the interesting gene (Huggett *et al*., 2005)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Design

The design of this study involved tsetse fly collection from Mbita, Homa Bay County, Down loading of *Glossina morsitan morsitans* OBP gene sequences from NCBI database which were used to design primers for both conventional and real time PCR analysis. DNA was extracted from first whole insect both male and female to screen for the presence of *G. m. morsitans* OBP genes then screen different organs (Head, Thorax, Abdomen leg) to determine the distribution of the OBPs in this organs. Total RNA was then extracted from the antennae and leg of both male and female tsetse flies from which first strand cDNA was synthesized for quantitative real time PCR. The data was finally analyzed at JKUAT biochemistry laboratory followed by manuscripts and thesis development.

3.2 Study site and Tsetse fly collection

Tsetse flies (*Glossina fuscipes fuscipes*) were collected from Sori in Homa Bay County, Kenya situated at 0° 25' 0" south and 34° 12' 0" north, on the shores of Lake Victoria. This site was chosen because it's predominantly infested with *Glossina fuscipes fuscipes*. The purposive sampling method was used to determine the sample size required for the studies which were collected using biconical traps placed randomly at strategic locations along the lake shore (Figure 3.1). The study site was divided into Sori A and Sori B with eight (8) traps set at different coordinates (Table 3.1). The biconical traps, colored blue and black which are known colors that attract tsetse flies, were baited with acetone and cow urine to attract tsetse flies to the trap. The cages were emptied after every 12 hours catch for three days according to Brightwell *et al.,* (1991). The samples were then preserved and transported to Biochemistry laboratory at Jomo Kenyatta University of Agriculture and technology for analysis.

Table 3.1: Tsetse trap position as determined by the global positioning system (GPS)

Sub-site A			**Sub-site B**		
Trap No.	**Coordinate**	**Elevation (above sea level)**	**Trap No.**	**Coordinate**	**Elevation (above sea level)**
1	S00.81682º E034.11965º	1143m	1	S00.84289 º E034.16889 º	1150m
2	S00.81667º E034.12054º	1132m	2	S00.84269 º E039.16925 º	1139m
3	S00.81711º E00.12181 º	1137m	3	S00.84238 º E039.16559 º	1132m
4	S00.81744 º E034.12196 º	1133m	4	S00.84238 º E034.16993 º	1136m
5	S00.81700 º E034.12202 º	1139m	5	S00.84228 º E039.1704 º	1136m
6	S00.81762 º E034.12243 º	1138m	6	S00.84230 º E039.17168 º	1135m
7	S00.81789 º E034.12306 º	1134m	7	S00.84244 º E034.17220 º	1132m
8	S00.8148 º	1136m	8	S00.84249 º	1133m

	E034.12349 º			E034.1721 º	

Number of traps set up in study site (Sori A and B) indicating the trap number, coordinates and elevation above sea level.

Figure 3.1: The biconical trap (Trap 4) being set up at Sori B at the shores of Lake Victoria.

Figure 3.2: Mapping of ordinates after setting up the biconical trap.

Glossina f. fuscipes were identified based on morphological features including dark fore leg, three bristles in scutum (thorax) and abdomen. The males and females *G. f. fuscipes* were separated based on presence of hypopigium in males and large abdominal segments in females (FAO, 1982a). The antennae and legs were dissected from 100 males and 100 females *G. f. fuscipes*, stored in liquid nitrogen and transported to the Biochemistry laboratory of JKUAT in Juja and stored till use.

3.3 DNA extraction from males and females *Glossina fuscipes fuscipes* tissues

DNA was extracted from head, thorax, abdomen and legs of 10 males and 10 females of *G. f. fuscipes* using phenol chloroform as described by Abdel-Hamid *et al.*, (1999). Briefly, the tissues (head, thorax, abdomen and legs) were separated and placed into different sterile eppendorf tubes. The tissues were washed in 200µl of 20 mM Tris EDTA (T.E) buffer of pH 8.5 and centrifuged at 17000 x *g* for 5 minutes at room temperature. These tissues were homogenized in the eppendorf using mortar pestle, vortexed, 25µl of 10% SDS added to the mixture and centrifuged at 17000 x *g* for 5 minutes at room temperature. A total volume of 15µl of Proteinase K (10mg/mL) and RNase A (10mg/mL) were added to the homogenate and mixed thoroughly. The contents were incubated in a water bath at 37ºC for 2 hours. Equal volumes of phenol/chloroform was added and centrifuged at 17000x*g* for 5 minutes. An equal volume of isoamylalcohol/chloroform was added and centrifugation carried out at 17000x*g* for 5 minutes. A tenth of the sample volume of 3M potassium acetate of pH 7.4 was then added followed by 2.5 times of ice cold absolute ethanol. Centrifugation was done at 17000x*g* for 10 minutes to precipitate the DNA. The pellet formed was washed with 100% ice cold ethanol and centrifuged briefly for 10 seconds. After drying, the pellet was dissolved in 50µl of Tris EDTA buffer at pH 7.4.

UV spectrophotometer technique was used to quantify extracted DNA. Briefly, 900µl of Tris EDTA (T.E) buffer at pH 8 was used to calibrate UV spectrophotometer at 260nm and 280nm. A volume of 10µl DNA samples (from head, thorax, abdomen, and legs) was added to the cuvettes and absorbance reading taken at 260nm and 280nm (Gaurav & Reject, 2012).

To estimate molecular weight of the DNA samples, 1g agarose was dissolved in 100µl of X1 Tris-borate EDTA (TBE) and boiled for 4 minutes in microwave. The contents were cooled to 45ºC and 3µl of ethidium bromide added. The gel was allowed to polymerize, after which immersed in the gel tank. DNA samples (5µl) were mixed with 5µl bromophenol blue and loaded onto the gel and run for 40 minutes at 250V. The gel

was visualized under Syngene InGenius3 Gel documentation (Syngene, USA). DNA marker (1kb, Fischer thermo Scientific) was used to determine the band sizes of DNA extracted from different tissues.

3.4 Amplification of *Glossina fuscipes fuscipes* Tissues

The extracted DNA was used as template for PCR amplification of the target *G. m. morsitans* OBP genes. Primers were designed at JKUAT Biochemistry department laboratory for 22 *G. m. morsitans* OBPs gene sequences downloaded from NCBI (Table 3.2) (Liu *et al*., 2010) using Primer3 (v0.4.0) software (Table 3.3)**.** The total reaction volume was 20µl containing 8µl of PCR master mix, 8µl of nuclease free water, 1µl each of reverse and forward primer (20µM) and 2µl of DNA template (50 ng/µl). The reaction was carried out in a DNA thermal cycler (MiniCycler, MJ Research, Watertown, MA) with initial heating at 95°C for 1 min and followed by 33 cycles at 95°C for 1 minute, 55ºC for 1 minute and 72°C for 3 minutes with final elongation step at 72°C for 8 minutes. The amplicons were then confirmed by 1% agarose gel electrophoresis run alongside 1kb ladder at 70V for 40 minutes.

Table 3.2: *Glossina morsitans morsitans* Odorant Binding Proteins sequences and characteristics

OBP	Number of amino acids	Accession Number	EMBL Accession Number
GmmOBP1	141 aa	CBA11305.1	FN432779.1
GmmOBP2	153 aa	CBA11306.1	FN432780.1
GmmOBP3	227 aa	CBA11307.1	FN432781.1
GmmOBP4	178 aa	CBA11308.1	FN432782.1
GmmOBP5	184 aa	CBA11309.1	FN432783.1
GmmOBP6	145 aa	CBA11310.1	FN432784.1
GmmOBP7	240 aa	CBA11311.1	FN432785.1
GmmOBP8	150 aa	CBA11312.1	FN432786.1
GmmOBP9	150 aa	CBA11313.1	FN432787.1
GmmOBP10	94 aa	CBA11314.1	FN432788.1

GmmOBP11	118 aa	CBA11315.1	FN432789.1
GmmOBP12	95 aa	CBA11316.1	FN432790.1
GmmOBP13	113 aa	CBA11317.1	FN432791.1
GmmOBP14	121 aa	CBA11318.1	FN432792.1
GmmOBP15	111 aa	CBA11319.1	FN432793.1
GmmOBP16	88 aa	CBA11320.1	FN432794.1

Continuation of table 3.2

OBP	Number of amino acids	Accession Number	EMBL Accession Number
GmmOBP17	102 aa	CBA11321.1	FN432795.1
GmmOBP18	109 aa	CBA11322.1	FN432796.1
GmmOBP19	109 aa	CBA11323.1	FN432797.1
GmmOBP20	88 aa	CBA11324.1	FN432798.1
GmmOBP21	137 aa	CBA11325.1	FN432799.1
GmmOBP22	126 aa	CBA11326.1	FN432800.1

Glossina morsitan morsitans OBPs amino acid number and accession numbers as obtained from NCBI database for conventional PCR primer design.

Table 3.3: Primers designed for *Glossina morsitans morsitans* Odorant Binding Proteins

Gene	Primer Sequence	
	Sequence (5'►3')	
	Forward primer	**Reverse primer**
GmmOBP1	ATGAAGACTACCGCCGTTAT	TTAGTTCTTTTTCAAACTTT
GmmOBP2	ATGAAAACAATTATCGTGAT	TTAATTGACACTGGACTTAA
GmmOBP3	AACAGTGAAATATTACGCAA	TTAAAAGTTAAAGATTTTTT
GmmOBP4	ATGAAATCGAAAATTATTTT	TTAAGCAAAAGCAACTCTAT
GmmOBP5	ATGAGATTTCATATCATACT	TTAATAAATCAAATTCAATT
GmmOBP6	ATGTTTAAATTATTACTCGT	TTAAATTTCCAACGTGATAC
GmmOBP7	ATGAAATTAATTACCGTTAT	TCATTGAGACAAACTGAAAC
GmmOBP8	ATGAAAAAGTATCATATTTA	TTACGGCAAAAAGTAAAGCA
GmmOBP9	ATGACTTTGTCCGGTAAATA	TCCATTACTTTTTGGTGTAA
GmmOBP10	GAGGCAATTAAGGAGTTCAG	TCAAGTTTCTTCCATTAAAT
GmmOBP11	ACTAAAGACGATGCTTTGAA	CTAAGTAAACATTTTACGCA
GmmOBP12	GAGGCNATTAGAGAATTTAG	TTATAATGATTCTTGTGCTA
GmmOBP13	ACTAAAGACGACTTTGAAAA	TTATATTTCAAAGTCAACCT
GmmOBP14	GCTACCGAAGAACAGATGAG	TTAAGGGAAAACGAATTGTG
GmmOBP15	ACCAGAGAAACTTTGCAAAA	TTACTTGGCTAAGCGATTGT
GmmOBP16	GAAAATTTTAATGCTTTTCA	TTATTCAGTTGGCTCAAACA
GmmOBP17	AACATTCCGGGCAGATTTAA	CTAAGTTAAACACTTTACCA
GmmOBP18	CATATGCGTTACGCAGAATA	TTAATGCATCATATATTCTA
GmmOBP19	CATATGCGTTACGCAGAATA	TTAATGCATCATATATTCTA
GmmOBP20	ATGCTTTTCACACTATTTTT	AATTTTTATTAAGTATACTA
GmmOBP21	GCAGAGGACGAGGACTGGCA	TTAAGCATTTTTCGCTGCAT
GmmOBP22	GATGATTTTTTTCAAATGTC	CTACTGATGTCCATTTGTTA

Conventional PCR Primers designed from *Glossina morsitans morsitans* OBP Sequences. Annealing temperature of 55ºC and band size range of between 200bp and 400bp.

3.5 Total RNA extraction from *Glossina fuscipes fuscipes* antennae and leg

Total RNA was extracted from antennae and legs of 100 males and 100 females *G. f. fuscipes*. The tissues were homogenized in 1.5ml eppendorf tubes containing 550µl PBS (pH 7.4). The lysate was centrifuged at 17000x*g* at 4ºC for 5 minutes. The supernatant (250µl) was transferred to a clean micro centrifuge tube containing 750µl TRIzol (Invitrogen, Carlsbad, CA, USA) then incubated at room temperature for 10 minutes to completely dissociate nucleoprotein complexes.

During phase separation, 200µl of chloroform was added to the supernatant and vortexed vigorously for 5 seconds. The samples were incubated at room temperature for 10 minutes and centrifuged at 17000x*g* at 4ºC for 10 minutes. The clear aqueous solution (containing the RNA) was pippeted carefully without disturbing the interphase into a clean RNase free 1.5ml eppendorf tube.

To precipitate RNA, 1µl of glycogen and 500µl of Iso-propanol were added to the sample and incubated at -80ºC overnight. The samples were centrifuged at 17000x*g* at 4ºC for 20 minutes. A white pellet formed at the bottom of the tube and was air-dried under a clean bench. To wash RNA, 50µl of ice cold 75% ethanol was added, the tube capped and inverted gently to wash the sides of the tube. The content was centrifuged at 17000x*g* at 4ºC for 10 minutes, supernatant discarded followed by a further centrifugation at 17000x*g* for 30 seconds. The excess liquid was removed using a pipette making sure that the pelleted RNA was not disturbed.

To redissolve the RNA, 12µl of nuclease free water was added to resuspend the pellet and left to stand at room temperature for 5 minutes thereafter vortexed to mix. This was followed by DNase treatment to degrade any genomic DNA. Briefly, 2µl of 10X reaction buffer was added to the dissolved RNA sample followed by 2µl DNase I (100mM) and 2µl of DEPC (0.01v/v) treated water. The content was incubated at 37ºC for 30 minutes after which 1µl of 50 mM EDTA was added and incubated at 65ºC for 10 minutes.

Quantification of extracted RNA was done using Nanodrop reader (Thermo Scientific, Thermo Scientific, UV-VIS Spectrophotometer, California, USA). The machine was blanked with 1µl nuclease free water before 1µl of RNA sample loaded. After

quantification, data was exported on excel sheet and stored on a computer for further analysis.

The integrity of the extracted RNA was determined by preparing 1.2% formaldehyde gel. This was carried out by taking 10ml of x10 MOPS buffer, 90ml of nuclease free water and 1.8ml of 37% formaldehyde added to a conical flask containing 1.2g of agarose. The contents were heated in a microwave for 4 minutes and left to cool to about 40ºC before 3µl of ethidium bromide added and allowed to polymerize. 5µl of RNA sample was mixed with 2µl of RNA loading dye and loaded in the respective well for both *G. f. fuscipes* male and female leg and antennae along with 0.5-10Kb RNA ladder and left to run at 60V for 2hours.

3.6 Preparation of First strand cDNA for *Glossina fuscipes fuscipes* antennae and leg tissues

First strand cDNA for male and female *G. f. fuscipes* antennae and leg tissues was prepared using RevertAid cDNA kit (Fermentas, Glen Burnie, MD). Negative and positive control samples were prepared by adding 4µl RNA template, 1µl oligo $(dT)_{18}$ primer and 7 µl of nuclease free water. The contents were mixed gently and centrifuged at 17000x*g* for 1 minute at 4ºC followed by incubation at 65ºC for 5 minutes. The content was chilled on ice, spun slowly and placed back on ice. A volume of 4µl 5X reaction buffer was added followed by 1µl ribolock RNase inhibitor and 2µl of dNTP (10mM) mix. For the positive control sample 1µl RevertAid^{TM} H M-MuLV reverse transcriptase enzyme was added to the reaction mix while the negative control lacked the reverse transcriptase enzyme. The contents were mixed gently and centrifuged at 17000x*g* for 1 minute at 4ºC, incubated at 42ºC for 1 hour followed by heating at 70ºC for 5 minutes to terminate the reaction.

The newly prepared cDNA was quantified by placing 1µl of the cDNA sample on a Nanodrop reader (Thermo Scientific, Thermo Scientific, UV-VIS Spectrophotometer, California, USA) after blanking the machine with 1µl of nuclease free water. The data generated was exported on excel sheet and saved on the computer.

The prepared first strand cDNA and PCR amplification product with GAPDH from male and female *G. f. fuscipes* antennae and leg were visualized on a 1% agarose gel. The gel

was run for 40 minutes at 70V and visualized under Syngene InGenius3 Gel documentation (Syngene, USA). PCR amplification (MiniCycler, MJ Research, Watertown, MA) of positive and negative 1st strand cDNA with initial heating at 95°C for 1 min and followed by 33 cycles at 95°C for 1 minute, 55ºC for 1 minute and 72°C for 3 minutes with final elongation step at 72°C for 8 minutes.

3.7 Design of *Glossina morsitans morsitans* OBP primers for Quantitative Real Time PCR

Primers were designed for quantitative real time PCR from 33 *G. m. morsitans* OBPs downloaded from Vectorbase (VB-2014-04) (Megy *et al*., 2012) using Primer3 plus (v0.4.0) software (Koressaar & Remm, 2007). The primer sequence length was set at a minimum of 18bp to a maximum of 27 bp with the primer temperature at a minimum of 57ºC and a maximum of 63ºC. The GC content was set at 20% with the product size range of 150 to 200bp. The primers were searched and the best hit selected and sent for synthesis at Inqaba Biotec, South Africa. To reconstitute the primers, the stock concentration of 100µM was diluted to 10mM based on the formulae $C_1V_1=C_2V_2$. The final working volume of 200µl was prepared by aliquoting 20µl of the 100mM stock with 180µl of nuclease free water (Table 3.4). Primer efficiencies of between 90-110% is considered significant for RT-PCR (Fisher Thermo scientific).

Table 3.4: Quantitative Real Time Primers designed for *Glossina morsitans morsitans* Odorant Binding Proteins

Gene	Primer Sequence	
	Sequence (5'►3')	
	Forward primer	**Reverse primer**
GmmOBP1	GATCACGCGCTGTTACATTG	CTTGTCTGCGCATTTTTCAA
GmmOBP2a	TGAAAATTGGAAACGGCCTA	CGTTTGTTGAAAACGTGAGC
GmmOBP2b	TTGGTCACATTGGCAACAGT	ACCTCATCGTCGGGATATTG
GmmOBP3	AGTGAGAAGGGCTGGTTTGA	TCATTTCGGCCTGTTTAAGG
GmmOBP4	GCTGGATCGGCTTAGAGTTG	CTTCATCCAGAAGCACACGA
GmmOBP5a	TAAGCCTGCGGATAATTTCG	TAAGCCTGCGGATAATTTCG

GmmOBP5b	TAAGCCTGCGGATAATTTCG	AGCGAAGGCTGATCTTCGTA
GmmOBP6	TGCTAATGGCAAATTGGTGA	CCGTACGTATCGGCTTGTTT
GmmOBP7	ACGCAGGGCTTTAATGAAGA	TAGCCCGCTTAGCTTCCATA
GmmOBP8a	GTGAATGTTGTCGACGATGC	GAAGCAAACATGCCACGAC
GmmOBP8b	GCATGTGTAAGGCACACTGG	GATCCGGGCATACTTTGATG
GmmOBP9	CGATGACAATGGTGACGTTC	CAGGGTCTGCTTTCTTCCAG
GmmOBP10	CATGAATTGGGTTTGGTTGA	TTCCAGCATTGATGAAACCA
GmmOBP11	TGTAAAATGCTGGGTGGAAA	TCTGTTTCCCATTCGTTGTG
GmmOBP12	CGATGACAATGGTGACGTTC	CAGGGTCTGCTTTCTTCCAG
GmmOBP13	TGAAGTGCGTCATGGAGAAA	CGCAAGCACATGGATACCTT
GmmOBP14	CTGCAAATTTGATGCGTGAT	CCCTTCTTCATCGTTTGCAT
GmmOBP15	TCATGGCTTGGAATTTTTCG	TAATACGAACTCCGCCGTTT
GmmOBP16	AACCGAATCCTTCTGGGAAT	ATCGAATTTACCCGAAAACG
GmmOBP17	GCTGAAATTGCTCCCAAAAA	ATTTTGAGCCCATTCTGTCG
GmmOBP18	GCTCGTTGCAGCTCAAAATA	TCCACCAATCCGAGAGAATC
GmmOBP19	TTCCAACTTGAGTCCGAGGT	CATTCATCACCACGAGATGC
GmmOBP20	GTGGCCGTTGTCAGGACTAT	GCATACAATCCATTGGCACA
GmmOBP21	ATGATTAGGGCAGAGGACGA	TGATGTGCGCAGAAAACTTC
GmmOBP22	GCTCAATTCACGGAATTTCAA	TTGATTGCATTGCGATATGA
GmmOBP23	TTCCACTGGCTCCTTACCAG	CGCAAGAATTGGGTTCTTTG
GmmOBP24	TGCGTCATGAAAAAGTTTGG	TCTTTCAAGCAAGCACCGTA
GmmOBP25	GGTAGCACTTCGGCTTTCAG	TGGTCAATCCAAATGCTTCA
GmmOBP26	CAATGGATGCGCTCCTAAAT	TTGTGCAAGAGCCTTTTGTG
GmmOBP27	CATCGGAAGCCGAATTAAAG	ATCGCCACCATTATCTGCAT
GmmOBP28	CTGCAAATTTGATGCGTGAT	CCCTTCTTCATCGTTTGCAT
GmmOBP29	AGAGCTTTTGCGGAAGATTG	TTTGAGTGACGCGAAACTTG
GmmOBP30	TTTGGCCCCATGATATGAAT	ATACGCTTGTCCTCGAATGG

Quantitative Real-Time primers designed from *G. m. morsitans* OBP genes using Primer3 plus (v0.4.0) (Koressaar and Remm, 2007) with annealing temperature of 55ºC and band size range of between 200bp and 500bp.

3.8 Validation of Primers Used for Quantitative Real Time PCR

Conventional PCR was optimized and used to test for the amplification of newly designed primers designed for *G. m. morsitans* OBPs to determine which primers amplified the *G. f. fuscipes* genomic DNA before using them for qRT-PCR. The primers were diluted to 5 µM and 25µl PCR master mix reaction constituted as follows: Template 5µl (50ng/ml), 10 x PCR buffer 2.5µl, 50 mM MgCl 0.5µl, 10mM dNTP 0.5µl, 2µl of 5µM forward and reverse primer (20ng/µl), 1µl containing 10 units of Taq polymerase and 11.5µl of nuclease free water. The PCR program used was initial denaturation temperature of 95 ºC for 5 minutes, subsequent denaturation temperature of 95ºC for 1 minute, annealing temperature of 58ºC for 1 minute, extension temperature of 72ºC for 1 minute and Final extension temperature of 72ºC for 8 minutes for 33 cycles. The PCR was carried out on ABI GeneAMP 9700 (Applied Biosystem, USA). The amplified PCR product was visualized using 1% agarose gel under UV transilluminator (Thermo Scientific, Thermo Scientific, UV-VIS Spectrophotometer, California, USA).

The primer efficiency (E) and regression coefficient (R^2) was determined for qRT-PCR primers that amplified *G. f. fuscipes* genomic DNA from conventional PCR was determined. Primer efficiency of between 90 to 110 % were considered significant.

Genomic DNA was extracted from *G. f. fuscipes* whole body, quantified using Nanodrop (Thermo Scientific, UV-VIS Spectrophotometer) and serial diluted to 81 times with a dilution factor of 3. For every dilution 1µl of the template was loaded in the qRT-PCR tube in duplicate and 7µl of SYBR Green/ROX qPCR Master Mix (Fermentas, USA) and 1µl each of forward and reverse primers added. The qRT-PCR tubes were sealed with an optical cover and placed in PikoReal time machine (Thermo Fisher Scientific, Vantaa, Finland) with program set at 95ºC for initial denaturation for 5 minutes, 95ºC subsequent denaturation for 1 minute, 58ºC annealing temperature for 1 minute, initial extension temperature of 72ºC for 1 minute, final extension temperature of 72ºC for 8 minutes and final holding temperature of 4ºC. The data generated was exported to excel sheet for analysis. Quantification cycle of the amplified fragment was plotted against dilution ratios to generate the standard curve. Efficiency of 95% to 110% and regression coefficient of 0.95 to 0.999 were considered acceptable (Asuero *et al.,* 2006).

3.9 Quantitative Real Time PCR of *Glossina fuscipes fuscipes* antennal and leg cDNA

Integrity of male and female *G. f. fuscipes* antennae and leg cDNA was validated through PCR amplification with GAPDH specific primers; forward (5'-TAAAATGGGTGGATGGTGAGAGTC-3') and reverse (5'-CTACGATGAAATTAAGGCAAAAGT-3') (product size 377bp) (Attardo *et al*., 2006). Briefly, 1µl cDNA products were amplified with 0.2ul DNA polymerase (0.02µg/µl), 4µl 5X PCR buffer, 0.5µl dNTPs (10mM) and 13ul PCR grade water, in the presence of 0.5µl of each primer (10pmol). Reactions were carried out in a ABI GeneAMP 9700 (Applied Biosystem,) with the following conditions: initial denaturation at 98°C for 1 minute; 33 cycles of 98°C for 1 minute, 55°C for 1 minutes, 72°C for 1 minutes; final extension at 72°C for 8 minutes. To visualize the amplicons 1% agarose gel was prepared and the samples ran alongside 1kb (Fermentas, USA) ladder for 40 minutes at 60V. The gel was then visualized under UV transilluminator (Syngene, USA).

The relative expression rate of *G. m. morsitans* OBPs in male and female *G. f. fuscipes* antennae and legs was optimized and determined using RT-PCR (Thermo Fisher Scientific, Vantaa, Finland) with the 33 *G. m. morsitans* OBP real time primers. The samples were prepared in triplicate and loaded on a 24 well plate along with the GAPDH as the reference gene (7µl SYBR Green/ROX qPCR Master Mix (Fermentas, USA), 1µl template cDNA, 1µl forward primer and 1µl reverse primer) and negative control [7µl SYBR Green/ROX qPCR Master Mix (Fermentas, USA) and 3µl of nuclease free water]. The program was set at 95ºC for the initial denaturation for 5 minutes, 95ºC subsequent denaturation for 1 minute, 58ºC annealing temperature for 1 minute, initial extension temperature of 72ºC for 1 minute, final extension temperature of 72ºC for 8 minutes and final holding temperature of 4ºC. The data generated was exported and analyzed in an excel work sheet.

Bioinformatics analysis was done to compare the *G. m. morsistans* OBP genes transcribed in *G. f. fuscipes* antennae and legs by performing basic local alignment search (BLAST) in flybase (St. Pierre *et al.*, 2014) using nucleotide query sequences from Vectorbase (Megy *et al*., 2012).

CHAPTER FOUR

4.0 RESULTS

4.1 Tsetse fly collection

A total of 796 *G. f. fuscipes* flies were collected for two days which included 241 flies collected on day one and 555 flies collected on day two. On day one, the total collection in sub-site A was 140 flies which included 76 females and 64 males, while in sub-site B a total of 101 flies were collected comprising of 46 females and 55 males. On day two a total of 120 flies were collected in sub-site A and 435 flies in sub-site B, this comprised of 66 females and 54 males in sub-site A and 231 females and 204 males (Appendix 1). Proportionally the percentage of females and male flies collected in two days was 52.6% females and 47.4% males.

4.2 Presence of *G. morsitans morsitans* Odorant Binding Proteins genes in *G. fuscipes fuscipes*

From the DNA extracted from male and females *G. f. fuscipes*, amplification of each 22 *G. m. morsitans* OBPs using conventional PCR revealed that 15 OBP genes (GmmOBP1, GmmOBP2, GmmOBP3, GmmOBP4, GmmOBP5, GmmOBP6, GmmOBP8, GmmOBP9, GmmOBP11, GmmOBP12, GmmOBP15, GmmOBP17, GmmOBP18 and GmmOBP19) were present. This could indicate that 15 *G. m. morsitans* OBP genes were conserved on *G. f. fuscipes* with molecular weight of between 350bp to 213 bp. (Table 4.1, Plates 4.1; 4.2).

Table 4.1: *Glossina morsitans morsitans* Odorant Binding Proteins Present or absent in *Glossina fuscipes fuscipes*

***G. morsitans morsitans* OBPs**	***G. m. morsitans* OBP status in *G. f. fuscipes***
GmmOBP1	√
GmmOBP2	√
GmmOBP3	√
GmmOBP4	√
GmmOBP5	√
GmmOBP6	√
GmmOBP7	**X**
GmmOBP8	√
GmmOBP9	√
GmmOBP10	**X**
GmmOBP11	√
GmmOBP12	√
GmmOBP13	√
GmmOBP14	**X**
GmmOBP15	√
GmmOBP16	**X**
GmmOBP17	√
GmmOBP18	√
GmmOBP19	√
GmmOBP20	**X**
GmmOBP21	**X**
GmmOBP22	**X**

Glossina morsitans morsitans OBP genes present and absent in *Glossina fuscipes fuscipes*. √=present and X=absent.

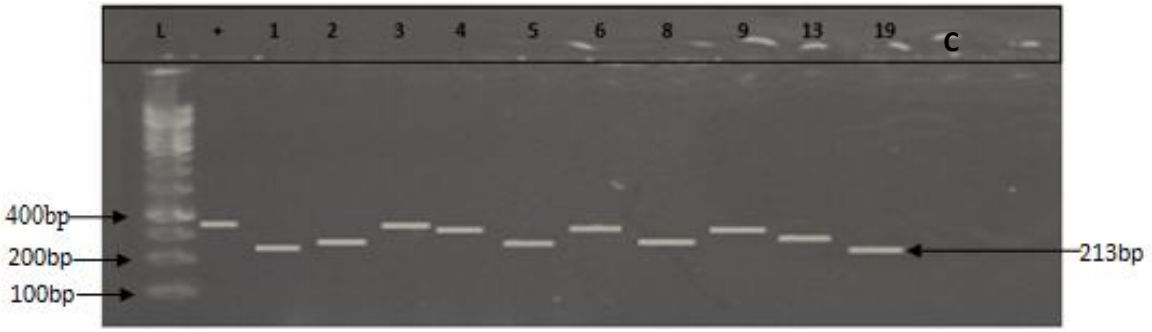

Plate 4.1: 1% agarose gel depicting *G. m. morsitans* OBP genes present in female *G. f. fuscipes*. L=1kb ladder, += internal control gene (GAPDH), C= negative control, 1=GmmOBP1, 2=GmmOBP2, =GmmOBP3, 4=GmmOBP4, 5=GmmOBP5, 6=GmmOBP6, 9=GmmOBP9 11=GmmOBP11, 2=GmmOBP12, 15=GmmOBP15, 17=GmmOBP17, 18=GmmOBP18 and 19=GmmOBP19.

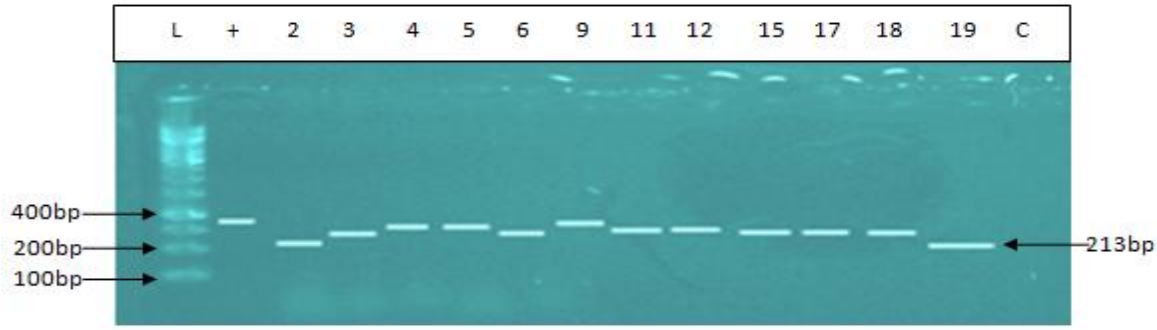

Plate 4.2: 1% agarose gel depicting *G. m. morsitans* OBP genes present in male *G. f. fuscipes*. L=1kb ladder, += internal control gene (GAPDH), C= negative control, 1=GmmOBP1, 2=GmmOBP2, 3=GmmOBP3, 4=GmmOBP4, 5=GmmOBP5, 6=GmmOBP6, 9=GmmOBP9, 13=GmmOBP13, and 19=GmmOBP19.

4.3 Distribution of *Glossina morsitans morsitans* Odorant Binding Proteins in *Glossina fuscipes fuscipes* body parts

The DNA concentration and purity ranged from 20 to 80ng/µl and 1.83 to 2.02 for females and 70 to 97ng/µl and 1.81 to 1.98 for males (Appendix 2).

The female and male head, thorax, abdomen and leg tissues were amplified by the internal control GAPDH primer (377bp). Ten OBP genes amplified *G. f. fuscipes* head, thorax, abdomen and leg with band size range between 213bp to 345bp (Figure 4.3). OBP19 and OBP8 amplified leg (213bp) and thorax (243bp) respectively. Two OBPs

(OBP6 and OBP9) were identified in the thorax and abdomen while three OBPs (OBP1, OBP2 and OBP13) were localized in the legs and head. OBP3 was detected in the thorax, abdomen and leg with band size of 345bp and two other OBPs (OBP4 and OBP5) were found to be present in the head, thorax and abdomen (Plate 4.3).

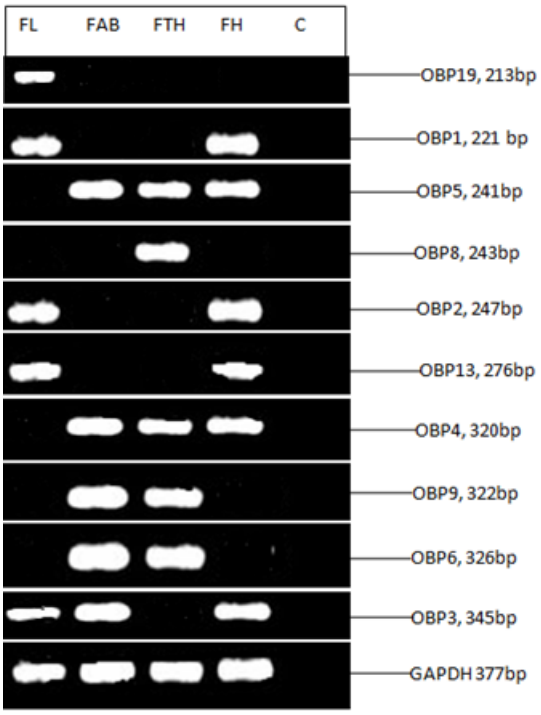

Plate 4.3

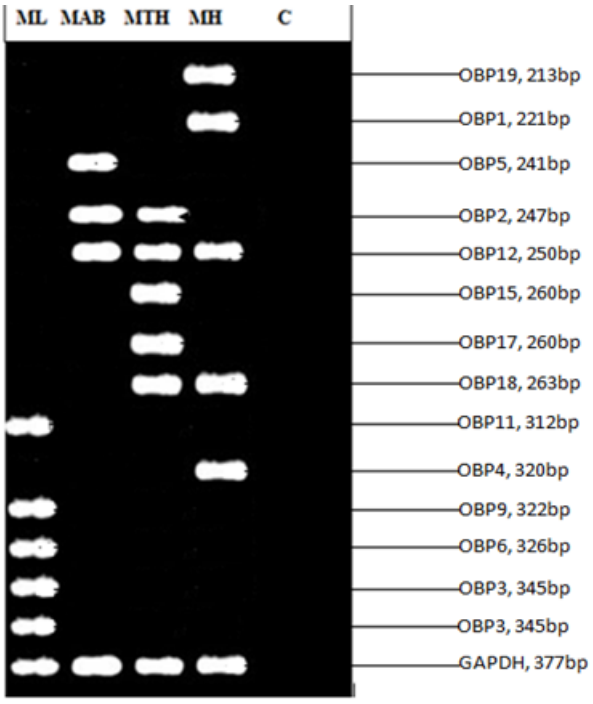

Plate 4.4

Plate 4.3: Distribution of OBP genes in different body parts of female *G. f fuscipes* PCR products amplified with OBP primers. L = 1 kb ladder; FL = female legs; FAB = female abdomen; FTH = female thorax; FH = female head –C = negative control. **Plate 4.4:** Distribution of OBP genes in different body parts of male *G. f fuscipes* PCR products amplified with OBP primers. L = 1 kb ladder; FL = female legs; FAB = female abdomen; FTH = female thorax; C = negative control.

Table 4.2: Distribution of odorant binding protein genes in female and male *Glossina fuscipes fuscipes*

Present in females	**Present in males**	**Present in females only**	**Present in males only**	**Present in both males and females**	**Absent in both males and females**
GmmOBP1	GmmOBP1	GmmOBP8	GmmOBP11	GmmOBP1	GmmOBP7
GmmOBP2	GmmOBP2	GmmOBP9	GmmOBP12	GmmOBP2	GmmOBP10
GmmOBP3	GmmOBP3	GmmOBP13	GmmOBP15	GmmOBP3	GmmOBP14
GmmOBP4	GmmOBP4		GmmOBP17	GmmOBP4	GmmOBP16
GmmOBP5	GmmOBP5		GmmOBP18	GmmOBP5	GmmOBP20
GmmOBP6	GmmOBP6			GmmOBP6	GmmOBP21
GmmOBP8	GmmOBP9			GmmOBP9	GmmOBP22
GmmOBP9	GmmOBP11			GmmOBP19	
GmmOBP13	GmmOBP12				
GmmOBP19	GmmOBP15				
	GmmOBP17				
	GmmOBP18				
	GmmOBP19				

Distribution of *Glossina morsitans morsitans* OBP genes in *Glossina fuscipes fuscipes*.

More OBP genes were identified in female head, abdomen and legs than in the same organs in male. However, more OBP genes were identified in male thorax compared to the female thorax (Figure 4.1).

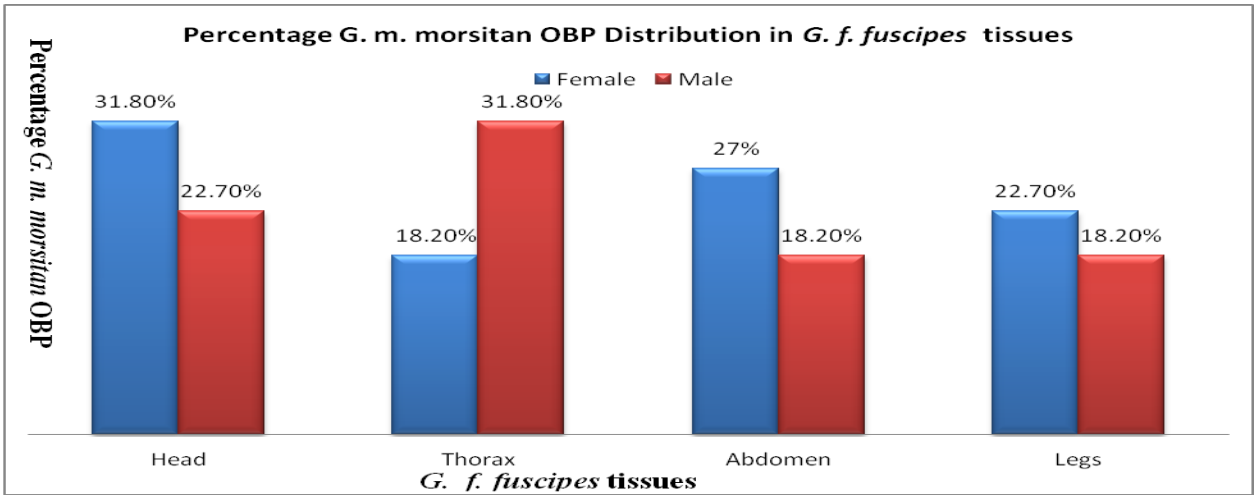

Figure 4.1: Percentage *G. m. morsitans* OBP distribution in different tissues of *G. f. fuscipes*

Similarly, thirteen OBP genes together with the internal control (GAPDH gene) amplified all male *G. f. fuscipes* organs (Plate 4.4). Majority of the OBP genes were detected in only the head tissue (OBP1, OBP3, OBP4, OBP6, OBP9, OBP11, OBP15 and OBP19) with band size range between 221bp and 320bp. OBP2 was identified in male abdomen and thorax while OBP18 was identified in thorax and head with molecular weight of 250bp and 260bp respectively OBP19 was detected in the head, thorax and abdomen with band size of 256bp (Plate 4.4).

Comparatively, eight OBP genes (OBP1, OBP2, OBP3, OBP4, OBP5, OBP6, OBP9 and OBP19) were identified in both males and female *G. f. fuscipes*, three OBP genes (OBP8, OBP9 and OBP13) were only found in the females while nine OBPs (OBP11, OBP12, OBP15, OBP17 and OBP18) were identified only in the males *G. f. fuscipes* (Table 4.3).

Table 4.3: Summary of *Glossina morsitans morsitans* odorant binding proteins in *Glossina fuscipes fuscipes* organs

Present in Female Head	**Present in Male Head**	**Present in Female Thorax**	**Present in Male Thorax**	**Present in Female Abdomen**	**Present in Male Abdomen**	**Present in female legs**	**Present in Male legs**
GmmOBP1	GmmOBP1	GmmOBP4	GmmOBP2	GmmOBP3	GmmOBP2	GmmOBP1	GmmOBP3
GmmOBP2	GmmOBP4	GmmOBP5	GmmOBP12	GmmOBP4	GmmOBP5	GmmOBP2	GmmOBP6
GmmOBP3	GmmOBP12	GmmOBP6	GmmOBP15	GmmOBP5	GmmOBP12	GmmOBP3	GmmOBP9
GmmOBP4	GmmOBP18	GmmOBP8	GmmOBP17	GmmOBP6		GmmOBP13	GmmOBP11
GmmOBP5	GmmOBP19	GmmOBP9	GmmOBP18	GmmOBP9		GmmOBP19	
GmmOBP13							

Summary of *Glossina morsitans morsitans* present in *Glossina fuscipes fuscipes* female head, thorax, abdomen and legs and Male head, thorax, abdomen and legs.

4.4 Expression of *Glossina morsitans morsitans* OBP genes in male and female *Glossina fuscipes fuscipes* antennae and leg

Formaldehyde denaturing gel used to visualize RNA extracted from antennae and legs of both male and female *G. f. fuscipes* revealed similar molecular weight for 28s and 18S subunits of approximately 1.8kb and 1.3kb respectively (Plate 4.5).

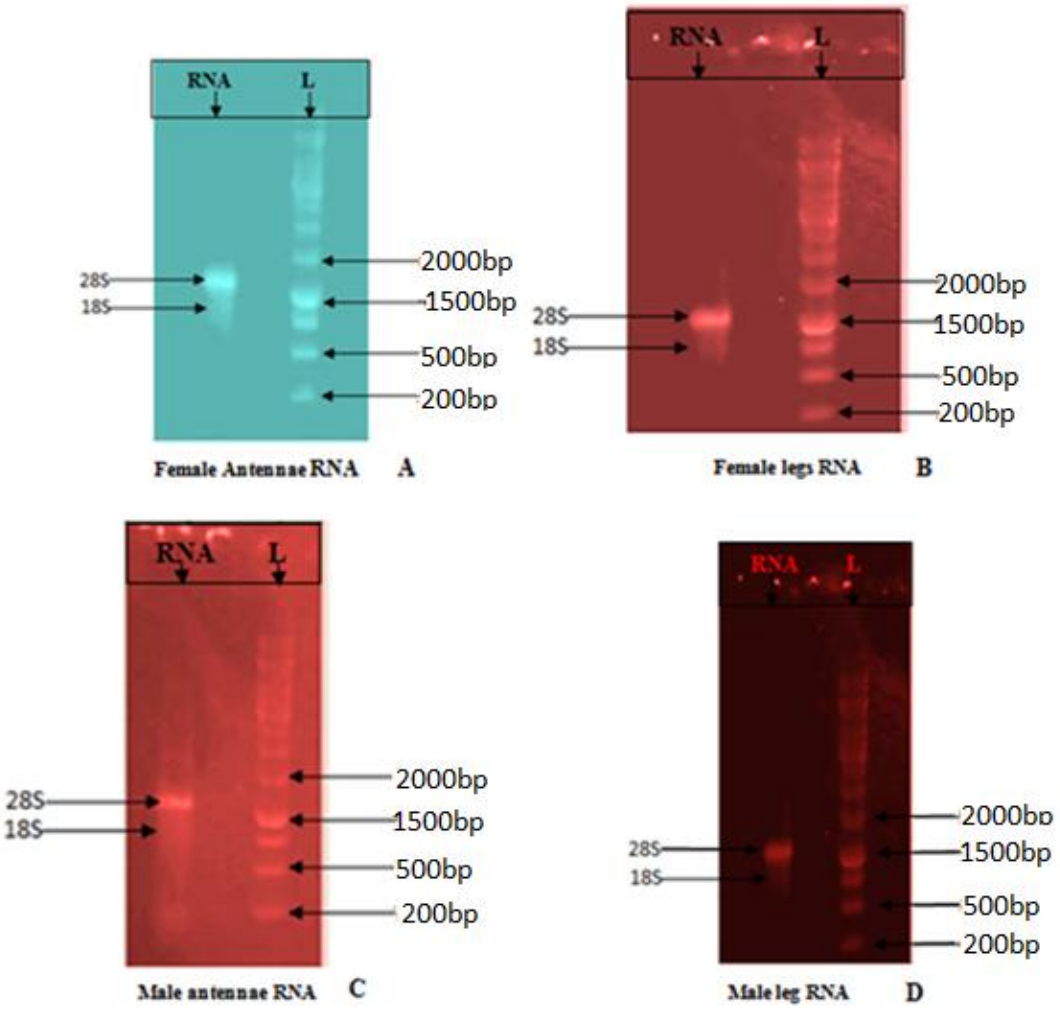

Plate 4.5: Formaldehyde denaturing gel of total RNA extracted from antennae and leg tissues of male and female of *Glossina fuscipes fuscipes*. Ladder (L) of 0.5-10kb RNA ladder (Fermentas); Ant RNA= Total RNA extracted from antenna leg RNA=Total RNA extracted from the leg tissue. A= total RNA from female antennae, B=Total RNA from female leg, C= Total RNA from male antennae while D=Total RNA from male leg.

The synthesized cDNA from the female and male *G. f. fuscipes* antennae and legs had a smear between the ranges 250bp to 3kb (Plate 4.6). The first strand cDNA was quantified as shown in Appendix 4.

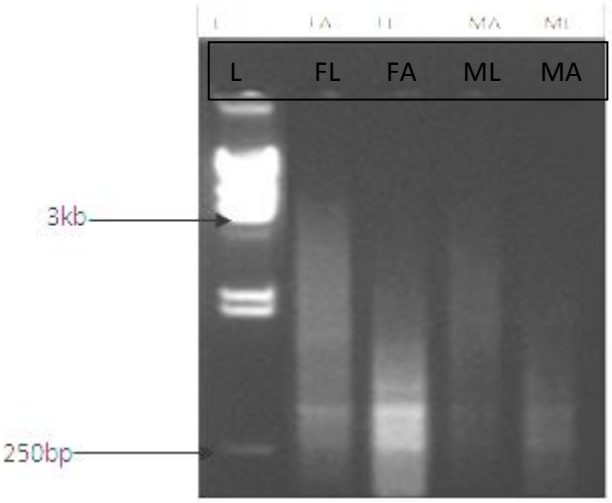

Plate 4.6: 1% Agarose gel of cDNA library constructed from female and male *G. f. fuscipes* antennae and leg. Ladder (L) = 1kb, FA – female antennae, FL – female leg, MA – male antennae, ML – male leg

The internal control GAPDH did not amplify negative control cDNA in both male and female *G. f. fuscipes.* GAPDH with a band size of 377bp amplified male and female *G. f. fuscipes* antennae and legs (Plate 4.7).

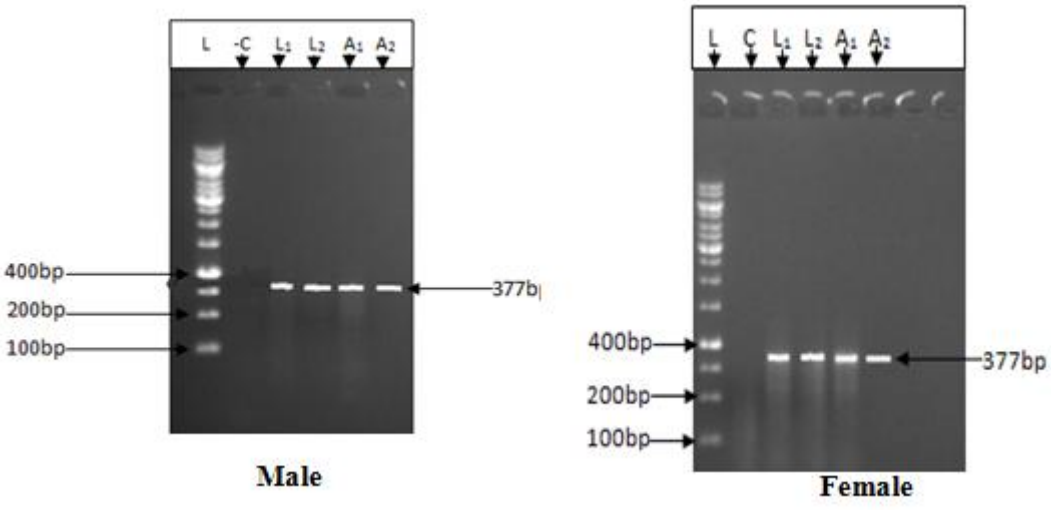

Plate 4.7: 1% agarose gel of GAPDH on first strand cDNA of male and female *Glossina fuscipes fuscipe*s antennae and leg. Ladder (L) 1kb plus, A_1 and A_2= antennal cDNA, L_1 and L_2=Leg cDNA, A-= negative control of antennal cDNA. **A=**male and **B**=female

Out of 33 *G. morsitans morsitans* OBP primers designed for RT-PCR, only 14 primers (GAPDH, OBP1, OBP2a, OBP3, OBP4, OBP5a, OBP6, OBP8a, OBP9, OBP13, OBP19, OBP23 OBP26 and OBP27) amplified female *G. f. fuscipes* genomic DNA with a band size between 410bp and 250bp (Plate 4.8). While 17 primers (GAPDH, OBP1, OBP2a, OBP3a, OBP4, OBP5a, OBP6, OBP9, OBP11, OBP11, OBP17, OBP18, OBP19, OBP20, OBP22, OBP26 and OBP28) amplified male *G. f. fuscipes* DNA (Plates 4.9, 4.10, 4.11).

Primers that did not amplify OBP genes in both males and female genomic DNA included OBP2b, OBP5b, OBP7, OBP8b, OBP10, OBP11, OBP12, OBP14, OBP15, OBP16, OBP17, OBP18, OBP20, OBP21, OBP22, OBP24, OBP25 and OBP28 while in females OBP3, OBP5A, OBP5B, OBP6, OBP8A, OBP8B, OBP12, OBP13 OBP21, OBP23, OBP24, OBP25 and OBP30. However primers OBP28 and OBP29 showed weak amplification in males (Plates 4.8, 4.9, 4.10, 4.11).

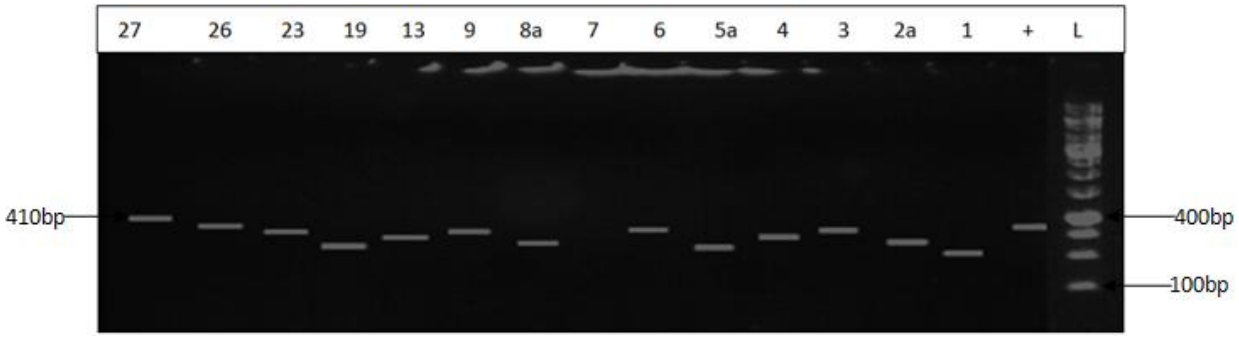

Plate 4.8: 1% agarose gel primer validation for female *G. fuscipes fuscipes* genomic DNA. GAPDH (+), 1 - OBP1, 2a - OBP2a, OBP3, OBP4, OBP5a, OBP6, OBP8a, OBP9, OBP13, OBP19, OBP23 OBP26 and OBP27. While primers OBP2b, OBP5b, OBP7, OBP8b, OBP10, OBP11, OBP12, OBP14, OBP15, OBP16, OBP17, OBP18, OBP20, OBP21, OBP22, OBP24, OBP25 and OBP28 did not amplify.

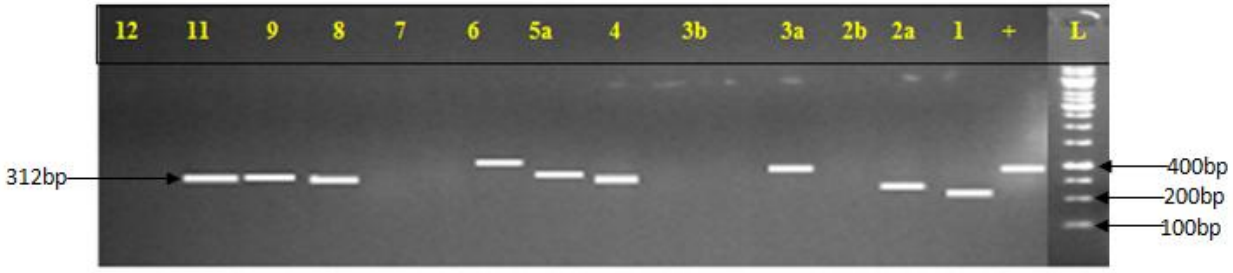

Plate 4.9: 1% agarose gel primer validation in male *Glossina fuscipes fuscipes*, primers for GAPDH (+), OBP1, OB2a, OBP3, OBP4, OBP5a, OBP6, OBP8, OBP9 and OBP11 showed positive amplification.

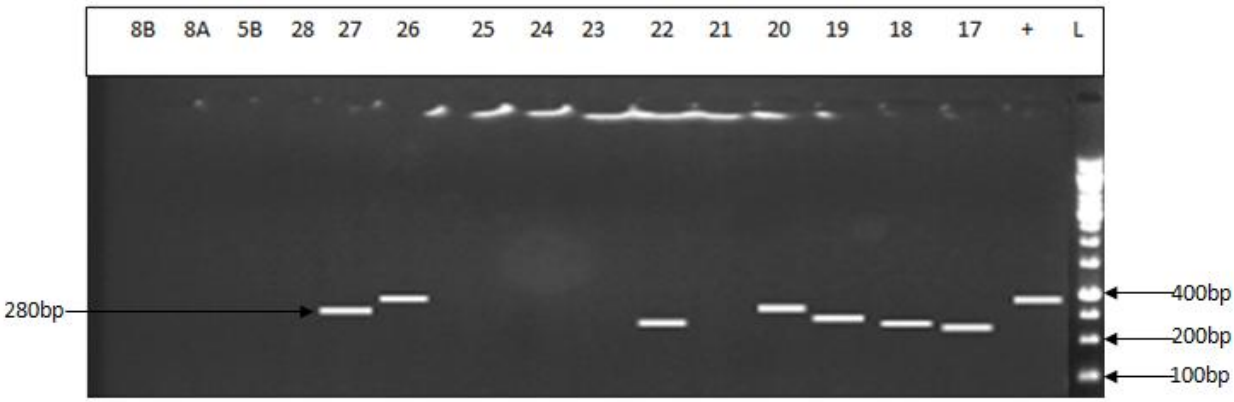

Plate 4.10: 1% agarose denaturing gel-electrophoresis primer validation in male *Glossina fuscipes fuscipes*, primers for GAPDH (+), 17, 18, 19, 20, 22, 26 and 27 showed positive amplification of the respective OBP genes.

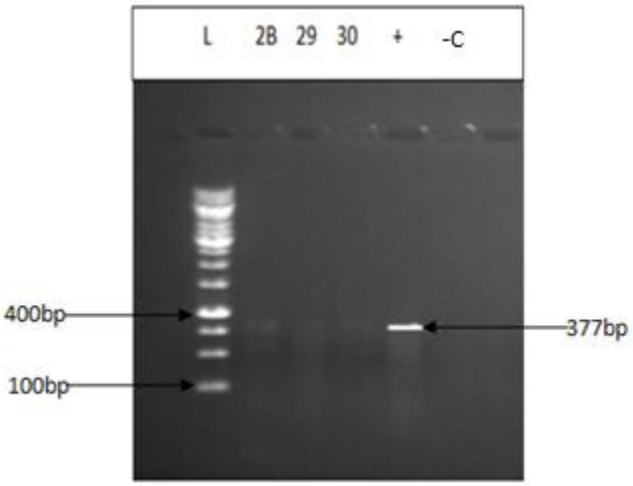

Plate 4.11: 1% agarose denaturing gel-electrophoresis primer validation in male *Glossina fuscipes fuscipes*, only GAPDH (+) showed positive amplification while primers 2B, 29 and 30 showed weak amplification for their respective genes.

4.5 Primer efficiency (E) and Regression Coefficient (R^2)

The efficiency (E) and regression coefficient (R2) was determined for primers that amplified male and female *G. f. fuscipes* DNA (Larionov *et al*., 2005). A standard curve was developed and used to determine efficiency and regression coefficient of the primers (Appendix five; Appendix six; Appendix seven). According to the standard curve for the reference gene GAPDH, the transcript abundance was found to be (3.0±0.07) $x10^5$ and (2.5±0.02) $x10^5$ copies/ng total RNA in the antennae and legs respectively. From the results, 8 primers (GAPDH, OBP 1, OBP 6, OBP 8a, OBP 9, OBP16, OBP22, and OBP 27) showed efficiencies of between 98%-100% (Appendix five; Appendix six; Appendix seven), Three (3) primers (OBP11, OBP14, and OBP 15) had an efficiency of above 100%, 11 primers (OBP2a, OBP4, OBP5a, OBP7, OBP10, OBP13, OBP17, OBP18, OBP20, OBP22 and OBP26) had efficiencies of between 95 and 97 (Appendix five). Only 2 OBP primers (OBP3 and OBP19) had efficiencies of below 95% (Appendix 5)

Among the primers that had efficiencies of between 98% to 100%, 4 amplified Female DNA (OBP6, OBP8a, OBP9 and OBP 27) 2 amplified male DNA (OBP16 and OBP22) and 2 primers amplified both female and male (GAPDH and OBP 1). All OBP primers with efficiencies above 100% amplified exclusively in male DNA tissues.

On the other hand, primers with efficiencies between 95% and 97%, 6 amplified males (OBP7, OBP10, OBP17, OBP18, OBP20 and OBP22), 3 in females (OBP5a, OBP13 and OBP26) and 2 in both males and females (OBP2a and OBP4). Among the two OBP primers with efficiencies below 95%; 1 amplified female (OBP3) and the other amplified both male and female (OBP19). For all the primers the regression coefficients were within the acceptable range of between 0.90 and 0.999 (Table 4.4). The standard curve slope was within the acceptable range of between -3.58 to -3.10 which translates to an efficiency of between 90% to 110% (Appendix seven).

To determine the expression levels of *G. morsitans morsitans* OBP genes in *G. fuscipes fuscipes* antennae and legs, quantitative RT-PCR was carried out with primers that amplified the *G. f. fuscipes* genomic DNA. The *G. f. fuscipes* male leg was used as a control tissue in the relative expression rates of *G. m. morsitans* OBP genes in male *G. f. fuscipes* antennae and female *G. f. fuscipes* antennae and leg.

4.6 Quantitative Polymerase chain reaction (qRT-PCR) data Analysis

A total of 16 OBP genes (GmmOBP1, GmmOBP3, GmmOBP5a, GmmOBP6, GmmOBP7, GmmOBP8a, GmmOBP13, GmmOBP16, GmmOBP17, GmmOBP18, GmmOBP19, GmmOBP20, GmmOBP21, GmmOBP22, GmmOBP26, GmmOBP27 and GmmOBP28) were highly transcribed in female *G. f. fuscipes* antennae than male (Figure 4.14). GmmOBP19 and GmmOBP20 had the highest expression levels while five OBPs (GmmOBP1 and GmmOBP7; GmmOBP13, GmmOBP16 and GmmOBP27) had relatively similar expression levels but different from those of GmmOBP5a and GmmOBP26 (Figure 4.14).

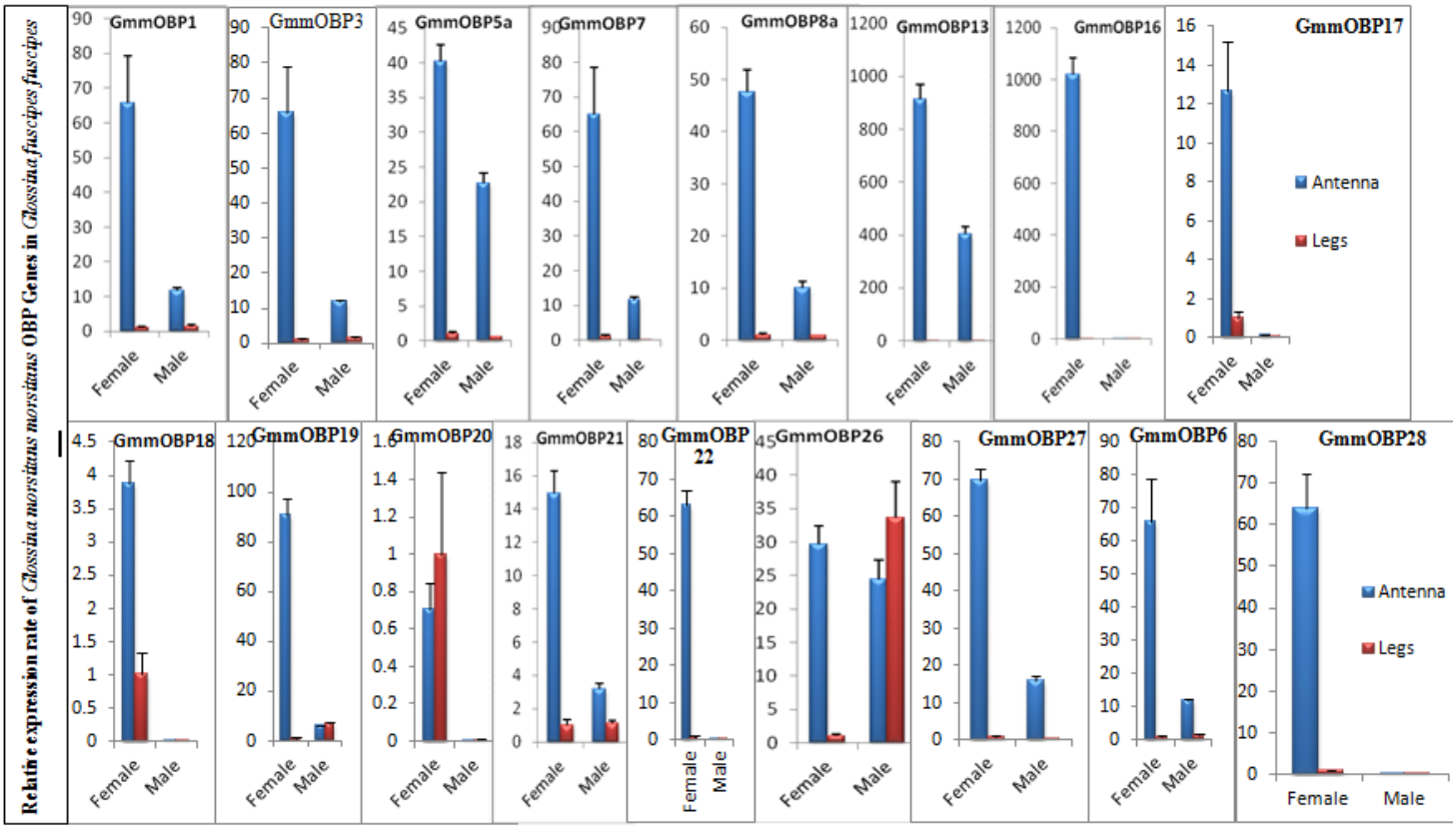

Figure 4.2: OBPs genes with high relative expression rate in *Glossina fuscipes fuscipes* female antennae than male

The OBP genes reported to be highly expressed in the female *G. f. fuscipes* leg than the male leg included GmmOBP2, GmmOBP10, GmmOBP14, GmmOBP15, GmmOBP20 and GmmOBP22 (Figure 4.17). GmmOBP22 had the highest transcription while GmmOBP15 and GmmOBP20 had the same transcription (Figure 4.15).

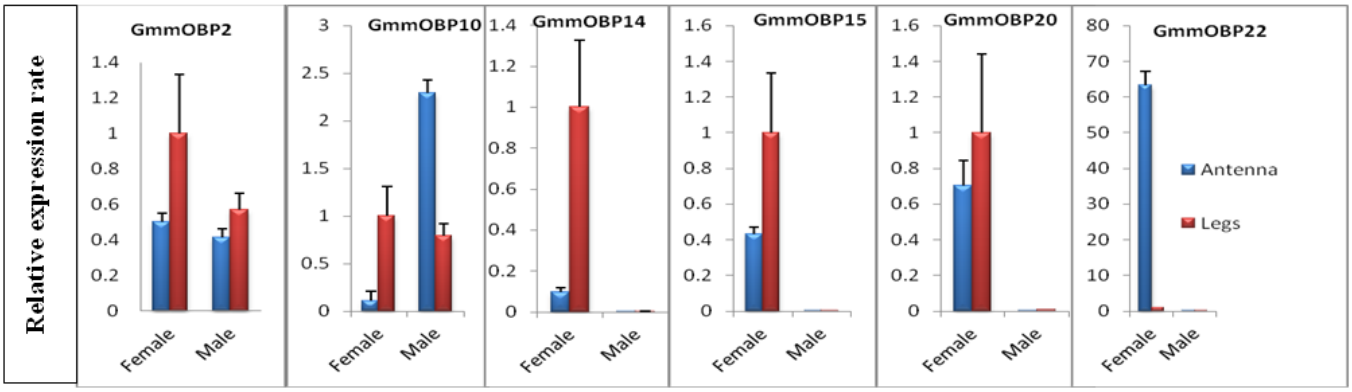

Figure 4.3: qRT-PCR results showing higher *Glossina morsitans morsitans* OBP genes relative expression rate in *Glossina fuscipes fuscipes* female leg than male.

The expressions of five OBPs (GmmOBP4, GmmOBP9, GmmOBP10 and GmmOBP23) were found to be higher in male *G. f. fuscipes* antennae than females with transcription level (Figure4.16).

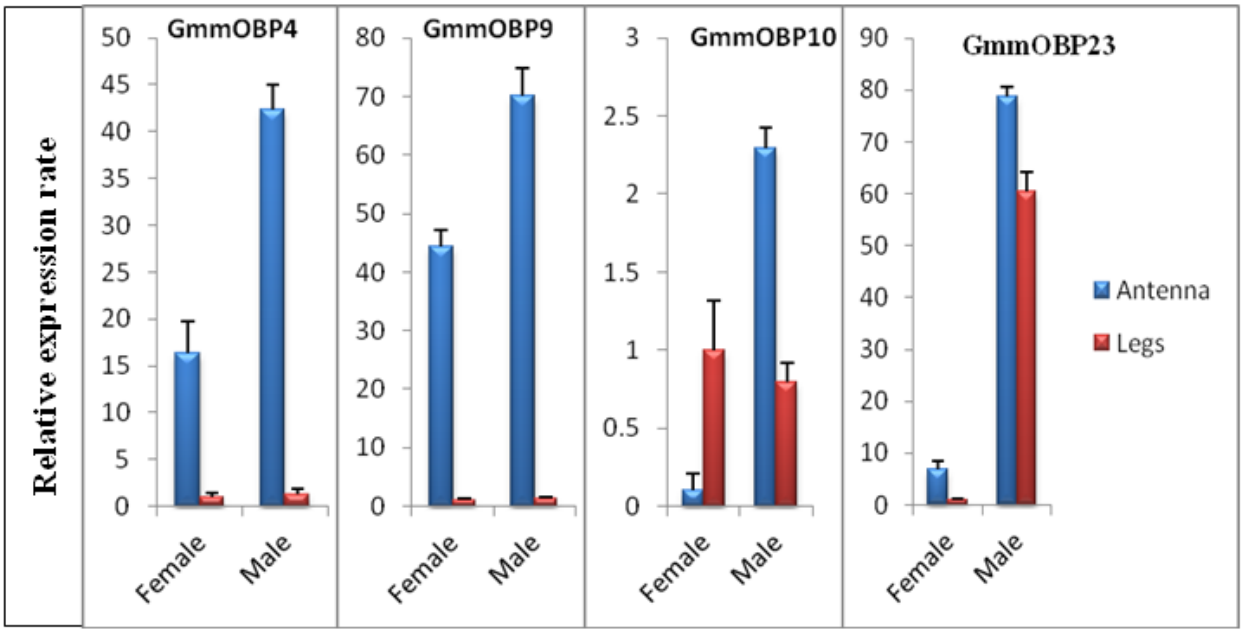

Figure 4.4: qRT-PCR showing high *Glossina morsitans morsitans* OBP genes relative expression rate in *Glossina fuscipes fuscipes* male antennae than female.

Four OBPs (GmmOBP11, GmmOBP21, GmmOBP23 and GmmOBP26) were found to be highly transcribed in the male leg than female leg. GmmOBP23 had the highest transcription level while GmmOBP11, GmmOBP21 and GmmOBP26 reported the least transcription levels (Figure 4.17).

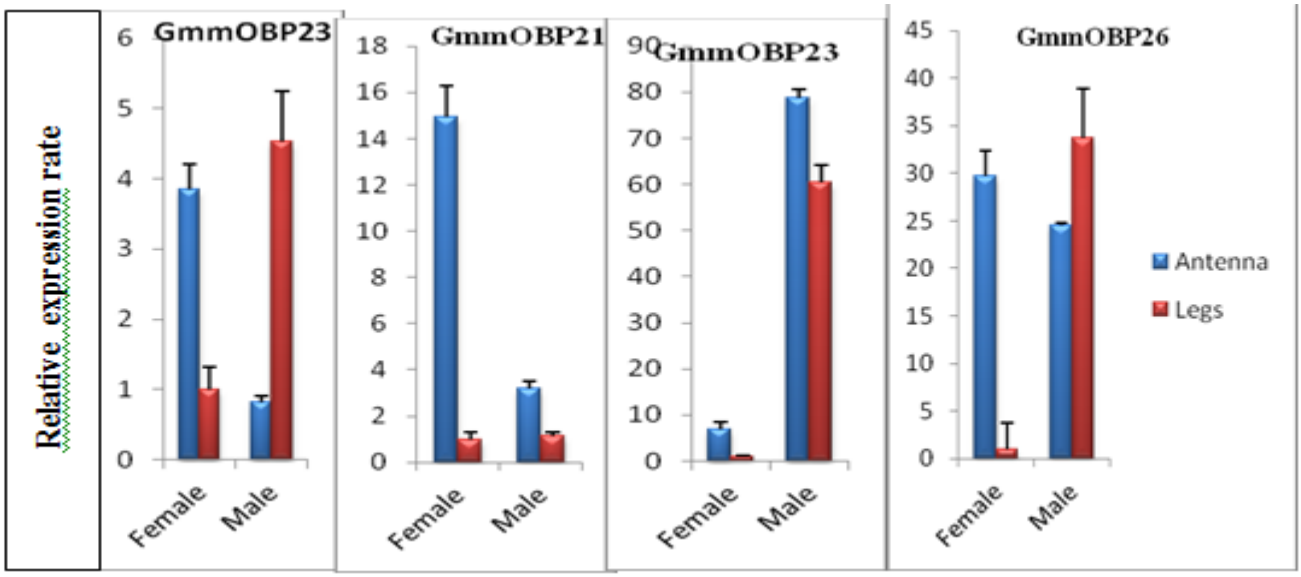

Figure 4.5: qRT-PCR results indicating high *Glossina morsitans morsitans* OBP genes relative expression rate in *Glossina fuscipes fuscipes* male leg than female

Generally, the results show that most *G. m. morsitans* OBP genes were expressed in the antennae than in the legs with majority highly expressed in the female *G. f. fuscipes* antennae and leg tissues than in the male *G. f. fuscipes*.

4.7 Bioinformatics Analysis

The *G. m. morsitans* amino acid sequence homology comparison to those of annotated *D. melanogaster* shows that they belong to three distinct subgroups i.e. Classical OBPs, Minus-C and pheromone binding proteins. The classical OBPs include GmmOBP1, GmmOBP2b, GmmOBP3, GmmOBP4, GmmOBP5a, GmmOBP5b, GmmOBP8a, GmmOBP10, GmmOBP11, GmmOBP13, GmmOBP14, GmmOBP15, GmmOBP16, GmmOBP19, GmmOBP21, GmmOBP22, GmmOBP25, GmmOBP26, GmmOBP27, GmmOBP28 and GmmOBP29. Four OBPs (GmmOBP2, GmmOBP11, GmmOBP18 and GmmOBP23) were classified as pheromone binding proteins while two OBPs (GmmOBP2 and GmmOBP21) were classified as Minus-C OBPs (Table 4.4).

Table 4.4: Bioinformatics analysis of *Glossina morsitan morsitans* Ortholog Odorant Binding Protein gene Sequences in *Drosophila melanogaster*

OBP No.	Accession No	No. of Amino acid	Relative transcription level in female antennae	Relative transcription level in female leg	Relative transcription level in male antennae	Relative transcription level in male leg	*Drosophila* OBP orthologs accession number	*Drosophila* OBP orthologs Percentage identities	*Drosophila* OBP orthologs E-Value	Orthologs function in *Drosophila*
GmmOBP1	GMOY000890	141	**High**	**Very Low**	**low**	**Very low**	CG10840-RF	100%	0.468151	General OBP 99c
GmmOBP2a	GMOY002826	144	**Medium**	**Low**	**medium**	**low**	CG43340-RQ	100%	1.89064	PBP99d
GmmOBP2b	GMOY002825	153	**Very high**	**Low**	**Medium**	**00**	CG5919-RA	100%	1.94052	OBP 44a, Gen. OBP
GmmOBP3	GMOY005549	284					CG43782-RH	100%	0.960398	sensory perception of chemical stimulus 83ef
GmmOBP4	GMOY007757	163	**medium**	**Low**	**high**	**low**	CG10069-RC	100%	2.14908	sensory perception of chemical stimulus 83ef
GmmOBP5a	GMOY006521	184					CG34145-RD	100%	0.155938	19c GOBP
GmmOBP5b	GMOY006522	157					CG1785-RA	100%	2.06747	19b GOBP
GmmOBP6	GMOY009708	145	**Very high**	**Low**	**medium**	**low**	CG31622-RD	95.5%	0.48192	PBP2 phenylalakylamine binding, OBP, PBP
GmmOBP7	GMOY005548	240					CG6224-RB	100%	0.151582	PBP2 phenylalakylamine binding, OBP, PBP
GmmOBP8a	GMOY004317	150	**High**	**Very low**	**low**	**Very low**	CG30015-RE	100%	1.97225	GOBP, GPBP
GmmOBP8b	GMOY004316	261					CG11421-RC	91.1%	1.67E-11	No hit

OBP No.	Accession No	No. of Amino acid	Relative transcription level in female antennae	Relative transcription level in female leg	Relative transcription level in male antennae	Relative transcription level in male leg	*Drosophila* OBP orthologs ID	*Drosophila* OBP orthologs Percentage identities	*Drosophila* OBP orthologs E-Value	Orthologs function in *Drosophila*
GmmOBP9	GMOY005184	150	**Medium**	**Very high**	**low**	**low**		**85.7%**	9.8063E-19	PBP6
GmmOBP10	GMOY004316	261	**Very low**	**low**	**Very low**	**Very low**	GB:AE014134	**100%**	0.0430533	GOBP56e
GmmOBP11	GMOY005550	**140**					GB:AE014297	**87.8%**	0.0118214	OBP 99a GOBP
GmmOBP12	GMOY005184	**150**					GB:AE014297	**85.7%**	1.008e-18	PBP6,
GmmOBP13	GMOY002859	**134**	**Very high**	**low**	**medium**	**Very low**	GB:AE014134	**85.7%**	9.8063E-19	OBP56g IsoA, GOBP56h
GmmOBP14	GMOY006523	**149**	**Very low**	**Very low**	**00**	**00**	GB:AE014296	**100%**	0.196876	GOBP19a
GmmOBP15	**GMOY012229**	109	**Very low**	**Very low**	**00**	**00**	GB:AE014297	**92.9%**	0.0394446	GOBP56eL
GmmOBP16	GMOY005163	**115**	**very high**	**low**	**00**	**00**	GB:AE014134	**95.7%**	0.150391	GOBP57c
GmmOBP17	**GMOY012281**	126					GB:AE014134	**100%**	0.0418666	
GmmOBP18	GMOY003978	**144**	**Medium**	**low**	**00**	**00**	GB:AE014296	**100%**	0.19004	PBP1
GmmOBP19	GMOY006522	157	**Very high**	**Very low**	**low**	**low**	GB:AE014297	**100%**	0.186991	GOBP19b
GmmOBP20	GMOY006417	**261**	**Very low**	**Very low**	**00**	**00**	GB:AE013599	**100%**	0.00566579	

OBP No.	Accession No	No. of Amino acid	Relative transcription level in female *G. f. fuscipes* antennae	Relative transcription level in female *G. f. fuscipes* leg	Relative transcription level in male *G. f. fuscipes* antennae	Relative transcription level in *G. f. fuscipes* male leg	*Drosophila* OBP orthologs accession number	*Drosophila* OBP orthologs Percentage identities	*Drosophila* OBP orthologs E-Value	Orthologs function in *Drosophila*
GmmOBP21	**GMOY006418**	**140**	**Medium**	**low**	**low**	**low**	GB:AE014297	**100%**	0.72931	GOBP99a,
GmmOBP22	**GMOY001476**	143	**Very high**	**Very low**	**00**	**00**	GB:AE014297	**100%**	0.745517	GOBP8a
GmmOBP23	**GMOY000657**	**125**	**low**	**Very low**	**Very high**	**High**	GB:AE014297	**100%**	0.164063	PBP4
GmmOBP24	**GMOY005400**	**144**					GB:AE014134	**100%**	0.00019729	PBP2 & 5
GmmOBP25	**GMOY005876**	**134**					GB:AE014296	**100%**	0.0446346	GOBP56d
GmmOBP26	**GMOY005931**	**114**	**high**	**low**	**Medium**	**high**	GB:AE014134	**100%**	0.149024	GOBP76a, 76c & 6a
GmmOBP27	**GMOY006081**	**121**	**Very high**	**low**	**medium**	**low**	GB:AE014296	**100%**	0.289153	GOBP59a
GmmOBP28	**GMOY006523**		**Very high**	**low**	**Very low**	**Very low**	GB:AE014296	**100%**	0.202456	GOBP19a
GmmOBP29	GMOY007293	227	**00**	**00**	**00**	**00**	GB:AE014298	**100%**	0.186991	GOBP73a
GmmOBP30			**00**	**00**	**00**	**00**				

Bioinformatics analysis of *Glossina morsitans morsitans* Odorant binding proteins indicating the accession number, number of amino acids, their corresponding expression level in *Glossina fuscipes fuscipes* and their orthologs and putative functions in *Drosophila melanogaster*

CHAPTER FIVE

5.0 DISCUSSION, RECOMMENDATIONS AND CONCLUSION

5.1 Discussion

This study showed that *G. m. morsitans* OBP homologous genes are expressed in male and female *G. f. fuscipes* antennal and leg hence probably having conserved functions despite having different hosts range and mates. This is an indication that *G. m. morsitans* (savanna species) and *G. f. fuscipes* (riverine species) could be using the same OBPs while looking for hosts to feed on, mates and larviposition sites. Odorant binding protein genes identified in *G. f. fuscipes* were highly expressed in the antenna. This is consistent with what has been previously reported for OBP genes in *G. m. morsitans* (Liu *et al*., 2010). However, there is no documentation of OBP genes reported in *G. f. fuscipes*. Previous studies have shown that *G. m. morsitans* and *G. f. fuscipes* feed on specific hosts determined by the tsetse fly geographical habitat (Gikonyo *et al*., 2002; 2003; Omolo *et al*., 2009). It is probable that these two species, *G. m. morsitans* and *G. f. fuscipes*, are expressing similar OBP genes which could be involved in modulating their behavior that influences their interaction with their environment. We also found that more *G. m. morsitans* OBP genes were expressed in the female than in male *G. f. fuscipes.* It has been reported that female tsetse fly actively search for hosts to fed on while males mainly swarm around the hosts waiting for the female for mating. This could therefore explain why females are expressing many olfactory OBPs than males (Liu *et al.,* 2010).

Identification of more homologous OBP genes in the head of both male and female *G. f. fuscipes* compared to other body parts is an indication of the richness of olfactory sensillia in the head. The tsetse head is known to have olfactory and gustatory appendages including antennae, proboscis and palps (Bruyne & Baker, 2008) which could explain the abundance of more OBP genes in the head. The *Anopheles gambie*

head play a critical role in olfaction since it houses olfactory organs like labial palp and maxillae (Rutzler & Zwiebel, 2005; Bruyne & Baker, 2008).

The distribution of homologous OBPs in different body parts of *G. f. fuscipes* may suggest that some OBPs are involved in functions other than olfaction. This has been reported in other insects such as Hawk moth *Manduca sexta* (*Lepidoptera*: *Sphingidae*) *Anopheles fenestus* and *Anopheles gambiae* (*Diptera*: *Culicoidae*) **(**Liu *et al.,* 2007e; Vosshall & Stocker, 2007; Xu *et al.,* 2010; Gu *et al.,* 2013. Studies by Pelletier and Leal, (2009) on *Culex pipiens quinquefasciatus,* identified 13OBPs to be involved in semiochemical response. These OBPs were found in non chemosensory tissues. The OBP genes were found localized in the legs of *C. p. quinquefasciatus* (Leak, 1999).

The homologous OBP genes that were exclusively found in either female or male *G. f. fuscipes* tissues could probably represent sex specific genes in each gender for reproductive purposes. Previous studies have shown that such genes could perform sex specific roles as reported in female *Ae. aegypti* (Sengul & Zhijian, 2009) and *An. gambiae* (Biessmann *et al.,* 2005; Li *et al.,* 2005).

The expression profile studies used 33 homologous OBP genes identified from the *G. m. morsitans* genome (IGGI, 2014). It is evident that more OBPs were localized in the *G. m. morsitans* genome contrary to the 22 OBP genes discovered from tissue specific cDNA library and whole genome shotgun technique (Liu *et al*., 2010). Gene profiling revealed that the OBP genes were expressed highly in the female *G. f. fuscipes* antennae than in males which is consistent with other studies that reported the same trend in other insects species (Shanbhag *et al.,* 2000; Zhou *et al*., 2008; Sengul & Tu, 2010; Liu *et al*., 2010; Deng *et al*., 2013; Zhang *et al*., 2013). This confirms that the antennae is an important olfactory tissue not only in tsetse flies but also in other insects (Pelletier & Leal, 2009) and the high abundance of OBP genes in female *G. f. fuscipes* than in males further pinpoint the involvement of female tsetse fly in specific functions including larviposition and host location. The OBP genes that were highly expressed in male *G. f. fuscipes* antennae could be involved in mediating male behaviors like mate detection and

courtship (Leak, 1999). Like other heamotophagous insects, female tsetse fly require blood meal from the hosts than males hence the need to have more OBP genes (Bruce *et al.*, 2005). Such OBP genes are potential targets for control of insect vectors through transgenesis because of the critical role they play in olfaction (Carey & Carlson, 2011).

The *G. m. morsitans* OBP genes not detected in *G. f. fuscipes* could represent species specific OBPs that are important in aiding *G. m. morsitans* interact with its environment. The two tsetse species are known to occupy different geographical habitats and the expression of OBP genes that mediate the interaction of the tsetse with its surrounding are certainly different (Liu *et al*., 2010). This has also been reported in other insect species like *An. gambie* that had some OBP genes lacking in *Ae. aegypti* **(**Bill & Marcus, 2011). The male *An. gambie* are known to feed on nectar (Foster and Takken, 2004) and the female rely on blood meal to provide nutrient for the eggs to mature (Foster & Takken, 2004). *Aedes aegypti* are known to be generalized feeder while female *An. gambie* prefer feeding on human beings. These mosquitoes exhibit diversity in the olfactory proteins and mechanism that aid them to identify their host (Bill and Marcus, 2011).

OBP genes that were detected in the male and female *G. fuscipes fuscipes* legs could be involved in non olfactory functions. Such OBP genes could be specific for detecting safe landing places and detection of non-volatile odors as reported in *Culex quinequesticus* (Pelletier & Leal, 2009).

Classifying *G. m. morsitans* OBPs into three distinct classes namely Classical OBPs, minus C and pheromone binding proteins confirm that they are true OBPs possessing all the olfactory features hence are probably involved in olfaction (Pelosi *et al.,* 2007). Classical OBPs have been reported to have 3 disulfide bonds formed by 6 cysteine residues (Vogt & Riddiford 1981).This could be an indicator that *G. m. morsitans* OBP genes whose orthologs in *D. melanogaster* are GOBP are also classical OBPs. In *D. melanogaster*, OBP99a is classified as minus C (-C) OBP having lost two conserved cysteines, which is an ortholog of GmmOBP11 and GmmOBP21 (Hekmat-Scafe *et al*.,

2002). This could be probable that the two *G. m. morsitans* (GmmOBP11 and GmmOBP21) are having similar functions. *G. m. morsitans* OBP orthologs with 100% identity are likely to be performing the same functions in both insects. Odorant binding protein orthologs that have specific functions of binding pheromons could be involved specifically in mating, courtship and determining larviposition sites (Liu *et al.*, 2010). However GmmOBP2 whose ortholog is a male specific pheromone binding protein could be specifically involved in helping male *G. m. morsitans* and *G. f. fuscipes* in locating female mates as the relative transcription rate in both female and male antennae was considerably high.

5.3 Conclusion

From this study we conclude that:

1. Different tsetse species (*G. m. morsitans* and *G. f. fuscipes*) could be using similar OBP genes even though they occupy different ecological niches.

2. Absence of some *G. m. morsitans* homologs OBPs gene sequences in *G. f. fuscipes* could suggest differences in species specific OBPs or amplification conditions that were not favorable.

3. More OBPs were found to be expressed in female than in male *G. f. fuscipes* which could indicate the importance of olfaction in females compared to males.

4. The head had more OBPs when compared to the abdomen, thorax and legs which could point to the fact that it comprises of important olfactory organ.

5. The antennae were observed to have a high relative expression rate of OBPs compared to the legs which could imply that the antennae play a critical role in olfaction.

5.2 Recommendation

1. The functional roles of highly expressed *G. m. morsitans* OBPs in *G. f. fuscipes* tissues to be determined by gene knockdown and electrophysiological assays.

2. The protein structures of OBPs identified in *G. m. morsitans* and *G. f. fuscipes* to be determined *in silico* and used for modeling and binding assay studies.

3. Synthetic ligands to be synthesized and their binding affinities to identified tsetse OBPs determined.

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APPENDICES

Appendix 1

Number of *Glossina fuscipes fuscipes* collected per trap at the sampling sites in Sori, Homa Bay County.

DAY 1					**DAY 2**				
Trap No.	**Sub-site A**		**Sub-site B**		**Trap No.**	**Sub-site A**		**Sub-site B**	
1	15		17		1	20		73	
2	8		19		2	2		82	
3	32		5		3	7		84	
4	17		2		4	19		49	
5	32		28		5	20		58	
6	11		5		6	2		18	
7	18		4		7	16		26	
8	4		7		8	23		13	
9	3		14		9	11		32	
	Female	**Male**	**Female**	**Male**		**Female**	**Male**	**Female**	**Male**
SUB-TOTAL	76	64	46	55	**SUB-TOTAL**	66	54	231	204
SUB-TOTAL	140		101		**SUB-TOTAL**	120		435	
SUB-TOTAL	241				**SUB-TOTAL**	555			
TOTAL	796								

Number of tsetse collected in Sub-site A and B per trap, sub-total of both males and female flies per sub-site, subtotal of summation of male and female flies collected per day for the separate sites, subtotal of the summation of collection in Sub-site A and B per day and then the total collection for the two days.

Appendix 2

Estimation of DNA concentration and purity for samples extracted from male and female *Glossina fuscipes fuscipes* body parts

	Female tissues of *Glossina fuscipes fuscipes*			
Parameter	**Head**	**Abdomen**	**Thorax**	**Legs**
Quantity (µM/ml)	20	80	74	53
Purity (260A/280A)	1.85	2.02	2.01	1.83
	Male tissues of *Glossina fuscipes fuscipes*			
Quantity (µM/ml)	70	78	86	97
Purity (260A/280A)	1.98	1.87	1.81	1.89

The genomic DNA of different tsetse fly body parts tissues depicting their quantity **(**µM/ml) and purity (260A/280A).

Appendix 3

Total RNA quantification and purity ratio.

Tissue	Concentration (ng/µl)	A260	A280	A260/A280
MA1	1448.5	36.213	20.558	1.76
MA2	2364.6	59.114	29.017	2.04
MA3	1119.3	27.982	18.595	1.8
MA4	1232	30.799	18.095	1.7
ML1	998.7	24.968	16.576	1.81
ML2	829.9	20.749	12.888	1.81
ML3	1877.1	46.928	25.646	1.83
ML4	1290.5	32.263	17.956	1.8
FL1	1224.8	24.496	13.98	1.75
FL2	877.5	17.551	10.923	1.61
FL3	1582.8	31.657	18.561	1.71
FA1	3014.5	60.29	30.946	1.95
FA2	1226.8	24.536	14.452	1.7
FA3	1507.9	30.159	17.799	2.2

Purity estimate of total RNA from male and female *G. f. fuscipes* antennae and legs confirmed that the sample ratios were within the acceptable range of 1.8 to 2.10 except the negative controls and eight samples (F1, FL 2; FA, FA FA1, FA3) (Table 4.6). The cDNA concentrations were above 2000ng/µl except FA3 (1732.6ng/µl) and negative control (MA-C, -423.5 ng/µl; ML-C,427.5 ng/µl; FL-C, -422.5 ng/µl and FA-C, 420.5 ng/µl

Appendix 4

Quantification and purity ratios for first strand cDNA from male and female *G. f. fuscipes* antennae and legs

	Sample ID	cDNA Conc.	Unit	A260	A280	260/280
1	MA -C	-423.5	ng/µl	-8.549	-4.781	1.01
2	MA1	2329	ng/µl	46.579	27.852	1.87
3	M A2	2619.7	ng/µl	52.395	31.608	1.86
4	M L1	2430.2	ng/µl	48.604	29.341	1.86
5	M L2	2267.3	ng/µl	45.347	27.348	1.86
6	ML-C	-427.5	ng/µl	-8.549	-4.781	1.01
7	F L1	2409.2	ng/µl	48.185	27.918	1.83
8	FL2	2916.4	ng/µl	58.327	34.117	1.81
9	FL3	2517.1	ng/µl	50.342	29.35	1.82
10	F L4	2881.6	ng/µl	57.631	33.875	1.7
11	F L5	3187.5	ng/µl	63.749	37.062	1.72
12	F L6	2452.7	ng/µl	49.053	28.821	1.7
13	FL7	2586.3	ng/µl	51.725	30.142	1.82
14	FL8	2710.6	ng/µl	54.212	31.426	1.83
15	F L-C	-422.5	ng/µl	-8.549	-4.781	1.01
16	F A1	2942.7	ng/µl	58.854	34.526	1.7
17	FA2	3228.6	ng/µl	64.572	36.919	1.75
18	F A3	3549.9	ng/µl	70.997	41.822	1.7
19	FA-C	-420.5	ng/µl	-8.549	-4.781	1.01
20	F A4	2904.3	ng/µl	58.086	34.322	1.99
21	F A5	2634.9	ng/µl	52.698	31.234	1.79
22	FA5	1732.6	ng/µl	34.651	21.257	1.93
23	F 6	2829.9	ng/µl	56.597	33.202	1.7

Concentration and purity ratio of first strand cDNA from male and female *G. f. fuscipes* antennae and legs Male antennae negative control (MA-C), male antennae positive (MA), male leg negative control (ML-C), male leg positive (ML), female antennae negative control (FA-C), female antennae positive (FA), female leg negative control (FL-C) and female leg positives (FL).

Appendix 5

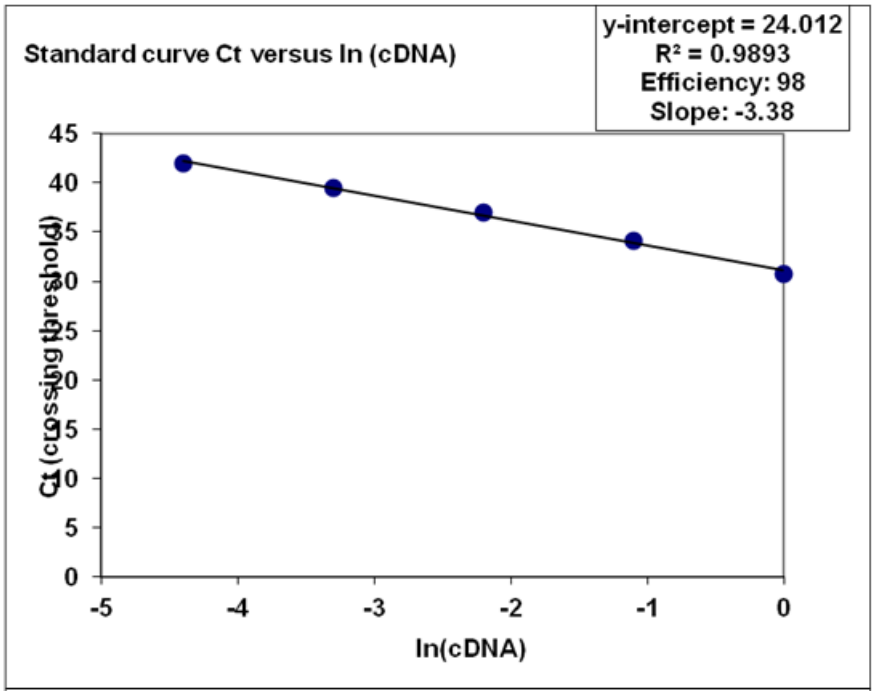
Quantitative RT-PCR primers standard curve values for Y-intercept, slope and coefficient of Regression

***GLOSSINA* PROJECT STANDARD CURVE RESULTS**					
OBP PRIMER	**SLOPE**	**Y-INTERCEPT**	**REGRESSION COEFICIENT (R^2)**	**PERCENTAGE EFFICIENCY**	**COMMENTS**
GAPDH	-3.38	24	0.989	98	Reference gene
OBP 1	-3.38	24	0.988	98	F & M
OBP 2a	-3.39	23	0.978	97	F & M
OBP 3	-3.47	24	0.981	94	F
OBP 4	-3.45	25	0.978	95	F & M
OBP 5a	-3.42	23	0.991	96	F
OBP6	-3.38	24	0.976	98	F
OBP7	-3.39	25	0.987	97	M
OBP 8a	-3.38	24	0.996	98	F
OBP 9	-3.35	24	0.956	99	F
OBP 10	-3.42	23	0.967	96	M
OBP 11	-3.29	23	0.982	101	M
OBP 13	-3.45	24	0.947	95	F
OBP 14	-3.21	26	0.957	105	M
OBP 15	-3.16	23	0.973	107	M
OBP 16	-3.38	23	0.951	98	M
OBP 17	-3.39	24	0.969	97	M
OBP 18	-3.39	24	0.987	97	M
OBP 19	-3.5	25	0.998	93	F & M
OBP 20	-3.42	22	0.965	96	M
OBP 22	-3.45	24	0.974	95	M
OBP 23	-3.38	24	0.981	98	F
OBP 26	-3.39	26	0.997	97	F
OBP27	-3.38	24	0.975	98	F

Standard curve slope, Y-intercept, regression coefficient (R^2), primer efficiency and tissue in which the genes were amplified. F=Female, M=Male F&M=Female and Male

Appendix 6

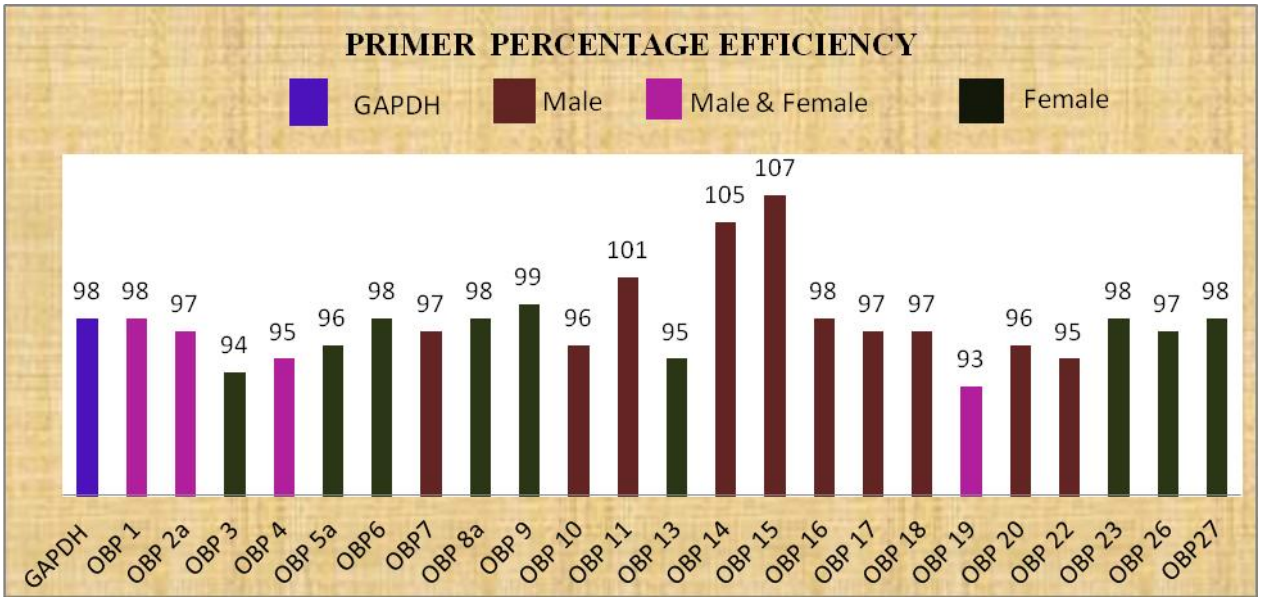
Standard curve dilution curve values



Standard curve for GAPDH indicating efficiency (E), regression coefficient (R^2) and the slope

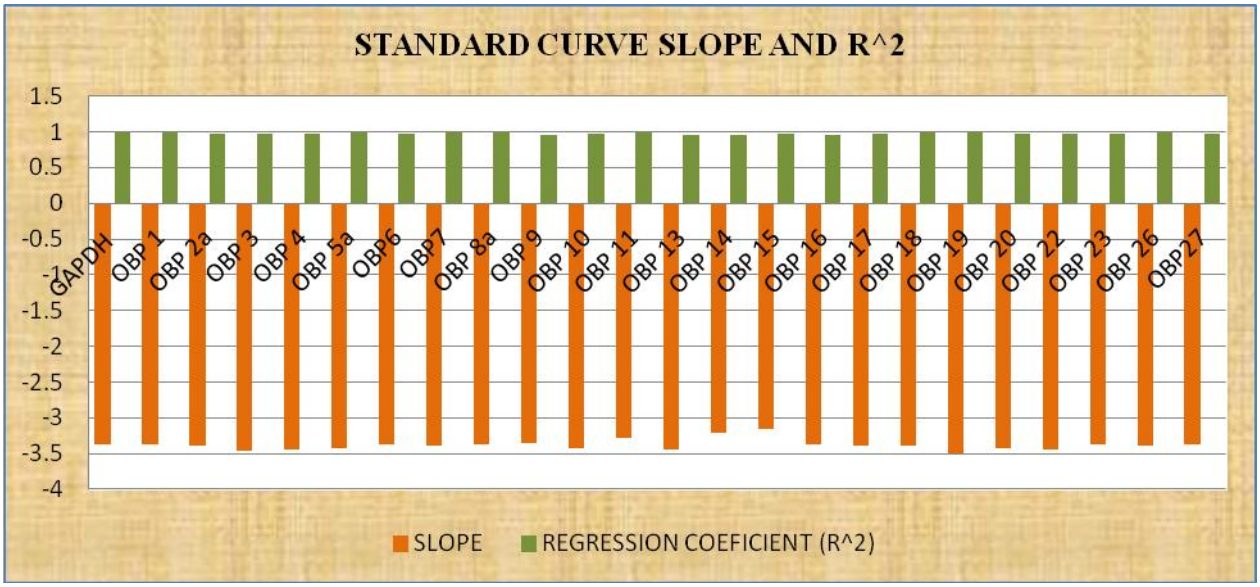
Appendix 7

Primer percentage efficiency and the respective tissues in which the OBP primers amplified.



Appendix 8

The standard curve slope for 24 *G. morsitans morsitans* OBP primers that amplified in *G. fuscipes fuscipes* tissues and the respective regression coefficients



Appendix 9:

Reagent Preparation

Preparation of x10 MOPS electrophoresis buffer

Requirements:

1. 0.2M MOPS (209.39g/L)
2. 0.05M NaAc (82.03g/L)
3. 0.01EDTA (292.02g/l)
4. NaOH pallets

Computations

1M MOPS ⟶ 209.39g/L

0.2M MOPS ⟶ 41.878/L

0.2M MOPS ⟶ 20.989g/0.5L

1M NaAc ⟶ 82.03g/L

0.05M ⟶ 4.10105g/L

0.05M ⟶ 2.05075g/0.5L

1M EDTA ⟶ 292.02g/L

0.01M ⟶ 2.9202g/L

0.1M ⟶ 1.4601g/L

The solution was then titrated with NaOH pallets to pH 7.0

Preparation of T.E buffer

10Mm Tris-HCl pH 8.0, 0.25mM EDTA

X10 T.B.E Electrophoresis buffer

Tris base (108g), boric acid (55g), 0.5M EDTA (20mL). add distilled water to 1 liter to make X10 T.B.E

Preparation of RNase free water

Add 0.1% of DEPC to 1000ml distilled water shake well and incubate at room temperature overnight. Autoclave twice until DEPC scent is no more. Let it cool to room temperature

Preparation of X1 formaldehyde gel running buffer (1 liter)

X10 MOPS gel buffer……100ml

37% Formaldehyde……..20ml

RNase free water……880ml

1000ml

Boil for 3 minutes in a microwave le cool to 60ºC add 900µl of 37% formaldehyde and 3µl of ethidium bromide in a fume hood

Preparation of 1.2% formaldehyde gel

Agarose……..0.6g

X10 MOPS buffer…….5ml

RNase free water……45ml

Preparation of X5 loading dye (10ml)

Saturated aqueous bromophenol blue solution……16µl

500µl EDTA pH 8.0……..80µl

37 % (12.3M) formaldehyde……..……720µl

100% glycerol (High grade)……2ml

X10 formaldehyde gel buffer……..4ml

RNase free water up to 10ml……...2.84ml

(Kept at 4ºC for three months from the date of preparation)

Preparation of x6 DNA loading dye

Saturated aqueous bromophenol blue…….25mg

Double distilled water……..6.7ml

Xylene cyanol……...25mg

Glycerol……...3ml

Store at -20ºC

Appendix 10

Treatment of plastic and glassware for use

Treatment of RNase free plastic ware

Rinse with1% SDS for two hours

Sock in 0.1M NaOH and incubate for two hours at 37ºC

Rinse thoroughly in DEPC treated water then autoclave

Treatment of RNase free glassware

Clean with laboratory detergent thoroughly then soak in 0.1% DEPC treated water.

Incubate at 37ºC for two hours.

Bake overnight and autoclave

Preparation of RNase free Electrophoresis tank

Clean with 1% SDS

Rinse with RNase free water then absolute ethanol

Incubate in 3% hydrogen peroxide for 10 minutes at room temperature

Rinse with DEPC treated water.

Appendix 11

Field photos

(a)

(b)

(c)

(d)

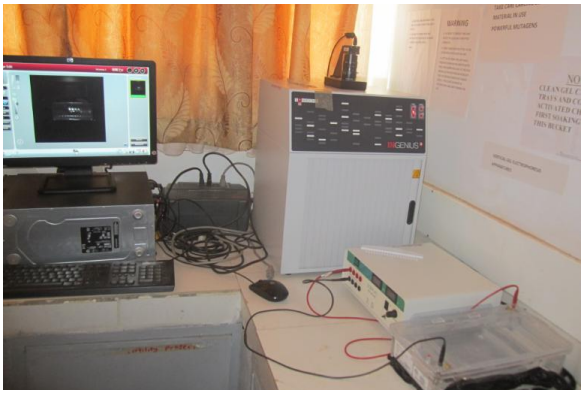
(a) Field technician from ICIPE Mbita point Mr. George Owino setting up one of the traps
(b) Mr. George Owino and Mr. Martin Ogwang' having a field discussion after setting up a trap
(c) Trap showing tsetse fly entry point into the trap
(d) Trapped tsetse flies
(e) Collecting trapped tsetse flies and replacing the trap nets

Appendix 12

Laboratory Equipments used

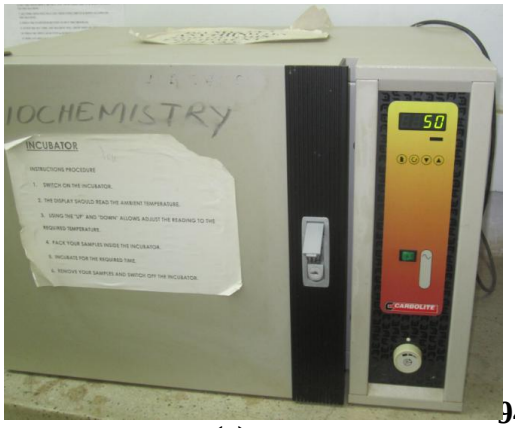
(a)

(b)



(c)

(d)



(e)

(a) Real time PCR machine from Fischer thermo scientific **(b)** ABM PCR machine **(c)** Gel documentation and Gel electrophoresis machines **(d)** Ice maker **(e)** Incubator