GENE EXPRESSION PROFILING OF ODORANT BINDING PROTEINS IN THE TSETSE FLY *Glossina brevipalpis*

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Gene expression profiling of odorant binding proteins in the tsetse fly *Glossina brevipalpis*

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

I dedicate this work to my mother Esther Murithi and father Stephen Murithi for being very understanding and supportive. Thank you for your patience and for encouraging me to believe that I am destined for greater achievements. I appreciate the many sacrifices you have made to ensure that I get quality education and for all your provision. May the Lord almighty bless you abundantly and may He see you through all your endeavours.

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TABLE OF CONTENTS

DECLARATION ii
DEDICATION iii
ACKNOWLEDGEMENTS iv
TABLE OF CONTENTS v
LIST OF TABLES vi
LIST OF FIGURES vii
LIST OF ABBREVIATIONS AND ACRONYMS viii
LIST OF SYMBOLS ix
ABSTRACT x
CHAPTER ONE 1
INTRODUCTION 1
1.1Background information 1
1.2 Statement of the problem 3
1.3 Justification 4
1.4 Objectives 5
1.4.1 General objective 5
1.4.2 Specific objectives 5
1.5 Null hypothesis 5
CHAPTER TWO 6
LITERATURE REVIEW 6
2.1 Tsetse fly distribution 6
2.2 Economic significance of tsetse flies 9
2.3 Trypanosomiasis control techniques 10
2.3.1 Use of trypanocides 10
2.3.2 Use of insecticides 12
2.3.3 Sterile insect technique (SIT) 12
2.3.4 Targets and traps 13

2.4 Olfaction in insects....16
2.4.1 Insect olfactory system....17
2.5 Olfactory proteins....18
2.5.1 Odorant receptors (ORs)....19
2.5.2 Ionotropic receptors (IRs)....19
2.5.3 Sensory neuron membrane proteins (SNMPs)....20
2.5.4 Chemosensory proteins (CSPs)....21
2.5.5 Odorant binding proteins (OBPs)....21
2.6 Sequence comparison analysis tools....24
2.7 Phylogenetic analysis....25
2.8 Quantitative real time polymerase chain reaction (qRT-PCR)....26
CHAPTER THREE....30
METHODOLOGY....30
3.1 Study site....30
3.2 Experimental design....30
3.3 Sample collection....31
3.4 Computational identification and characterization of *G. brevipalpis* OBPs using Bioinformatics tools....33
3.5. Primer design for *G. brevipalpis* OBP sequences....33
3.6. Total RNA extraction....35
3.7. DNAse treatment of total RNA....36
3.8. Determination of RNA yield and quality....36
3.9. Determination of RNA integrity....36
3.10. Complimentary DNA (cDNA) synthesis....37
3.11. Polymerase chain reaction (PCR) amplification of the cDNA....37
3.12. Agarose gel electrophoresis....38
3.13. Recovery of amplified DNA from the gel....38
3.14. Quantitative real time polymerase chain reaction (qRT-PCR)....39

3.14 Quantitative real time polymerase chain reaction (qRT-PCR) data analysis 39
CHAPTER FOUR 40
RESEARCH RESULTS 40
4.1. The putative *G. brevipalpis* OBPs 40
4.2 Spectrophotometry of extracted RNA...46
4.3 Analysis of RNA integrity using gel electrophoresis 47
4.4 Screening of *G. brevipalpis* OBP genes in the differentially starved flies 47
4.5 Screening of *G. brevipalpis* OBP genes in the developmental stages 49
4.6. *Glossina brevipalpis* OBPs extracted in the teneral (control) group 49
4.7. Amplification and dissociation of *G. brevipalpis* OBPs..50
4.8. Expression profiles of *G. brevipalpis* OBP genes in differentially starved flies 52
4.9. Quantitative expression of *G. brevipalpis* OBPs in the developmental stages.......53
CHAPTER FIVE..55
DISCUSSION 55
CHAPTER SIX 60
CONCLUSIONS & RECOMMNDATIONS 60
6.1 Conclusions...60
6.2 Recommendations and Scope for further work...60
REFERENCES..61

LIST OF TABLES

Table 3.1 Primer sequences for amplifying putative *G. brevipalpis* OBPs.......................34

Table 4.1 G. m. morsitans OBPs best hit matches to *G. brevipalpis* and *D. melanogaster* proteome databases...42

Table 4.2 Nanodrop readings of RNA extracted from *G. brevipalpis* samples.................46

LIST OF FIGURES

Figure 2.1. Tsetse fly distribution in Africa (Area shaded in grey indicates countries infested by tsetse flies). 6

Figure 2.2. Distribution of forest group tsetse flies in Africa (Area shaded in red show countries with suitable vegetation for forest group tsetse flies). 8

Figure 2.3. Traps used in the field for tsetse collection..15

Figure 2.4. Tsetse fly olfaction at the periphery..18

Figure 2.5a. Quantitative real time polymerase chain reaction amplification curve 28

Figure 2.5b. Quantitative real time polymerase chain reaction dissociation curve 29

Figure 3.1. The female tsetse fly genitalia 32

Figure 4.1. Multiple sequence alignment (MSA) of putative *G. brevipalpis* OBPs.........43

Figure 4.2. Phylogeny of *G. brevipalpis, G. m. morsitans* and *D. melanogaster* OBPs...45

Figure 4.3a. and 4.3b Formaldehyde agarose gel images of G. brevipalpis RNA. 47

Figure 4.4. 2% Agarose gel electrophoresis of the amplified *G. brevipalpis* OBPs in the tenerals and differentially starved adults...48

Figure 4.5. 2% Agarose gel images of *G. brevipalpis* OBPs that amplified in the developmental stages...49

Figure 4.6. 2% Agarose gel image of *G. brevipalpis* OBPs in the tenerals......................50

Figure 4.7a. Amplification curve of *G. brevipalpis* OBP2 and GAPDH 51

Figure 4.7b. Dissociation curve of *G. brevipalpis* OBP2 and GAPDH 52

Figure 4.8. Relative expression profiles of *G. brevipalpis* OBPs in the differentially starved adults...53

Figure 4.9. Relative expression profiles of *G. brevipalpis* OBPs in the developmental stages. 54

LIST OF ABBREVIATIONS AND ACRONYMS

aa	Amino acid
AAT	Animal African trypanosomiasis
BHC	Beta-Hexachlorocyclohexane
Bp(s)	Base pair (s)
cDNA	Complimentary deoxyribonucleic acid
CSPs	Chemosensory proteins
DNA	Deoxyribonucleic acid
DNA	Deoxynucleotriphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium Bromide
GpSGHV	Glossina pallidipes salivary gland hypertrophy virus
GRs	Gustatory receptors
HAT	Human African trypanosomiasis
HCL	Hydrogen chloride
iGluRs	Ionotropic glutamate receptors
IRs	Ionotropic receptors
KCl	Potassium chloride
$MgCl_2$	Magnesium chloride
MOPs	3-(N-morpholino) propanesulfonic acid
NFW	Nuclease free water
NGU trap	Nguruman trap
OBPs	Odorant binding proteins
ORs	Olfactory receptors
OSNs	Olfactory sensory neurons
pbm	Post blood meal

PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
SIT	Sterile insect technique
SNMPs	Sensory neuron membrane proteins
VSGs	Variant surface glycoproteins
WHO	World Health Organisation

LIST OF SYMBOLS

Romans Symbol	**Quantity**
µl	Micro liter
ng	Nanogram
ºC	Degrees Celsius
mm	Millimetre

ABSTRACT

Glossina brevipalpis is a vector of trypanosomes that cause Animal African Trypanosomiasis. Chemoreception in tsetse flies is critical for identifying larvipositioning sites, hosts and mates. Odorant binding proteins (OBPs) are thought to mediate insect chemoreception by shuttling odours to receptors on olfactory sensory neurons. Little is known about the roles of OBPs in tsetse fly chemoreception. The genome of *G. brevipalpis* has been sequenced and 28 OBPs identified. This study aimed at profiling the expression levels of *G. brevipalpis* OBP genes at different starvation periods and developmental stages. G*lossina morsitans morsitans* OBP genes retrieved from VectorBase were queried against *G. brevipalpis* proteome. Putative *G. brevipalpis* OBP genes were then searched against *Drosophila melanogaster* proteome in FlyBase. Multiple sequence alignment of *G. brevipalpis* OBPs identified six conserved cysteines for most of the OBPs. Phylogenetic analysis of *G. brevipalpis*, *G. m. morsitans* and *D. melanogaster* OBPs showed that *Glossina* OBPs clustered closely. Wild *G. brevipalpis* collected from Shimba Hills National Park in Kwale County were fed on rabbits and starved for 2, 24, 48, 72 and 96 hours. Larvae, pupae and tenerals (newly emerged unfed flies) were also obtained. From the adults and tenerals, a pool of ten pairs of antennae plus one head were used for RNA extraction. Subsequently, one larva and one pupa were also used for RNA extraction. The RNA extracted was used for cDNA synthesis. Conventional PCR was used to screen the 28 putative *G. brevipalpis* OBP genes and the amplified genes were quantified by qRT- PCR. *Glossina brevipalpis* OBP8 was highly expressed in the differentially starved adults while two (GbrOBP2 and GbrOBP7) were found to be significantly expressed in the larval and pupal stages. Expression of OBPs in the adult flies confirmed their involvement in olfaction while expression in larva and pupa may suggest their involvement in non-olfactory processes. This study is the first to show the expression levels of OBPs at the starvation and developmental stages of *G. brevipalpis*. The findings suggest their roles in tsetse chemoreception and give insights on development of specific and environmentally friendly control strategies.

CHAPTER ONE

INTRODUCTION

1.1Background information

Tsetse flies are the sole biological vectors of African trypanosomes, the causative agents of Human African Trypanosomiasis (HAT) or sleeping sickness in humans and Animal African Trypanosomiasis (AAT) or nagana in cattle (Leak, 1998). Some trypanosomes such as *T. congolense* and *T. vivax* can also be transmitted mechanically by tabanids and stomoxys (Desquesnes & Dia, 2003). Trypanosomes are protozoan parasites belonging to the genus Trypanosoma that cause trypanosomiasis to humans and animals (Hoare, 1972).

Trypanosomiasis is a neglected tropical disease that mostly occurs in rural areas in sub-Saharan region where health systems are weak or non existence (Simarro *et al*., 2008) hence slows down development and contributes to food insecurity by low production in agriculture and livestock sectors (Shaw *et al*., 2014). The disease puts at least 70 million people and about 60 million cattle at risk of infection in sub-Saharan Africa (Raffaele, 2009, Simarro *et al*., 2012, WHO, 2013). However, the accurate estimate of the disease prevalence in the region remains unknown as most cases remain undetected (Aksoy, 2003).

Due to organised screening and treatment of people across sub-Saharan Africa in the pre-colonial period by the colonialists, the annual incidence of sleeping sickness between 1931 and 1961 reduced by 90% from >60,000 to <5000 cases/year (Simarro *et al*., 2008). However a rollback was experienced in the post independence period because of the collapse of these control strategies resulting from political and economical instability (Simarro *et al*., 2008a). Moreover, a report by WHO projected an annual occurrence of 300,000 cases towards the end of the 20th century. This steered formation of

collaborations between the WHO with the public, private and international partners in 2001 that were aimed at increasing awareness of HAT epidemiology and setting a platform for developing effective control strategies (Simarro *et al*., 2010). In the same period, the heads of states and governments in the African Union also made eradication of tsetse flies a priority and formed a Pan African Tsetse fly and Trypanosome Eradication Campaign (PATTEC)- a programme funded by African Development Bank (Kabayo, 2002).This contributed positively to a 82.3% reduction in number of annual cases from 37,991 reported in 1998 to 6,743 cases in 2011 in the sub-Saharan region (Simarro *et al*., 2011).

Tsetse flies (order: Diptera) belong to the super family Hippoboscoidea and family Glossinidae. They consist of the genus *Glossina* which is divided into; Fusca (forest), Morsitans (savannah) and Palpalis (riverine) groups (Gooding and Krafsur, 2005). These groups consist of about 23 species and 33 sub-species which have a limited distribution to sub-Saharan Africa (Ducheyne *et al*., 2009, Samdi *et al*., 2011). *Glossina brevipalpis* is a forest fly and vector of AAT or nagana in animals (Kuzoe & Schofield, 2004). The species is widely distributed in central and western parts of Africa but is also distributed sparsely in the southern and eastern regions. In Kenya it is found along the southern east region (Cecchi *et al*., 2008). Nagana results in loss of animal production and drought manpower (Samdi *et al*., 2011).

Tsetse fly control and eradication strategies that have been used in the past include; spraying the tsetse infested areas with insecticides, use of targets impregnated with insecticides, odour baited traps and sterile insect technique (SIT) (Allsopp, 2001). Reduced meat and livestock production from livestock, trypanosomiasis treatment and tsetse fly control results to an annual loss of US$ 1.2 billion while the total annual losses incurred in the efforts to control trypanosomiasis amount to approximately US$ 5 billion (Samdi *et al*., 2011). No vaccine has yet been developed due to the evasive nature of trypanosome from the hosts' immune system by formation of variant surface

glycoproteins (VSGs) (Tetley *et al*.,1987). Hence, long term control of trypanosomiasis will target the tsetse vector and genes related to olfaction are potential targets in developing better and improved methods of tsetse control based on traps, baits and olfactory mediated behaviour (Leak, 1998). Tsetse flies rely mainly on chemoreception to execute critical behaviours such as host location, predator avoidance, mate pursuit and identification of larviposition and resting sites (Masiga *et al.*, 2014). Chemoreception involves a cascade of events by olfactory proteins. The olfactory proteins consists of odorant binding proteins (OBPs), pheromone binding proteins (PBPs), odorant degrading enzymes (ODEs) and chemosensory proteins (CSPs) which belong to soluble proteins and are found in the chemosensilla lymph (Leal, 2013) while receptor proteins consists of (olfactory receptors (ORs), gustatory receptors (GRs), ligand-gated ionotropic receptors (IRs) and sensory neuron membrane proteins (SNMPs)). The OBPs and CSPs bind hydrophobic ligands, transport them through the sensilla lymph and activate the ORs (Leal, 2013). The OBPs were initially thought to be antenna specific from early studies done on the Lepidopterans and other insects but sub sequent studies have revealed their presence in other body parts such as the wings and legs and suggests that they may have other roles in addition to olfaction (Pelosi *et al*., 2005).

This study focussed on the expression of OBPs at different starvation periods and developmental stages of *G. brevipalpis*. The findings could inform on the molecular involvement of individual OBPs in chemoreception and could contribute to the development of better control strategies targeting the tsetse vector.

1.2 Statement of the problem

Management of trypanosomiasis in the past was largely achieved through chemotherapy. However over reliance on the trypanocidal drugs resulted to emergence of resistance in the trypanosomes. The drugs are also costly and toxic. Development of a vaccine towards trypanosomes has not been successful due to existence of variant surface glycoproteins

(VSGs) that enable the trypanosomes to evade the host's immune response (Gadelha *et al*., 2011). Efforts have now been shifted to vector control by use of odour baited traps which are limited by lack of specificity. The precise role of olfactory proteins in tsetse chemoreception is not known yet they play critical role in survival of tsetse flies. Although expression of OBPs has been studied in the savannah tsetse fly *G. m. morsitans* (Liu *et al*., 2010), little is known about the expression of OBPs in the ancient forest species *G. brevipalpis.* Also, control approaches targeting the tsetse fly OBPs to disrupt chemoreception and hence reduce trypanosomes transmission cycle to hosts have not been fully exploited. Therefore this study focused on the expression of OBP genes in the starvation periods and developmental stages of the tsetse fly *G. brevipalpis* in order to contribute to the molecular understanding of chemoreception in tsetse flies.

1.3 Justification

Chemoreception is vital for development and survival of tsetse flies as it facilitates behaviours such as mating, larvipositioning and host finding. *Glossina brevipalpis* transmit *T. brucei brucei*, *T. suis and T. simiae* that cause Animal African trypanosomiasis hence reducing livestock productivity and agricultural output. Use of insecticides including; organochlorines (DDT and dieldrin), Organophosphates (malathion and parathion), carbamates (carbamyl) and pyrethroids (deltamethrin) has not been very effective as they also affect non-target insects. Moreover sterile insect technique is not very effective. Trapping of tsetse flies which applies the principle of olfaction has significantly reduced the tsetse populations. Studying expression of odorant binding proteins is useful as they are vital in olfaction and could enable development of better control strategies. Secondly, this information could enable development of synergetic compounds to augment the existing vector control strategies, thirdly the mechanism of action of the compounds that are used in disrupting olfaction can be clearly outlined and finally, this principle could be applied in developing control approaches for other disease vectors and pest insects.

1.4 Objectives

1.4.1 General objective

To characterize *G. brevipalpis* OBPs and determine their gene expression profiles in differentially starved adults and developmental stages of *G. brevipalpis*

1.4.2 Specific objectives

1) To identify and determine phylogenetic relationship of putative *G. brevipalpis* OBPs with their homologues from selected dipterans.
2) To determine the expression profiles of odorant binding protein genes in tenerals (newly emerged unfed flies) and differentially starved *G. brevipalpis* adults
3) To determine the expression profiles of odorant binding protein genes in the developmental stages of *G. brevipalpis*

1.5 Null hypothesis

Expression of *G. brevipalpis* OBP genes is not dependent on the starvation periods or developmental stages of the fly.

CHAPTER TWO

LITERATURE REVIEW

2.1 Tsetse fly distribution

Tsetse flies are confined within 37 countries of sub-Saharan Africa with an area coverage of about 10 million km^2 (Cecchi *et al*., 2008) (Figure 2.1). There are about 23 species and 33 sub-species that are divided into three groups consisting of Forest (*fusca*), Riverine (*palpalis*) and Savannah (*morsitans*) based on their distribution and morphology (Samdi *et al.*, 2011). Their geographical distribution ensures high densities occurrence in habitats with suitable land cover and hosts (Ducheyne *et al*., 2009).

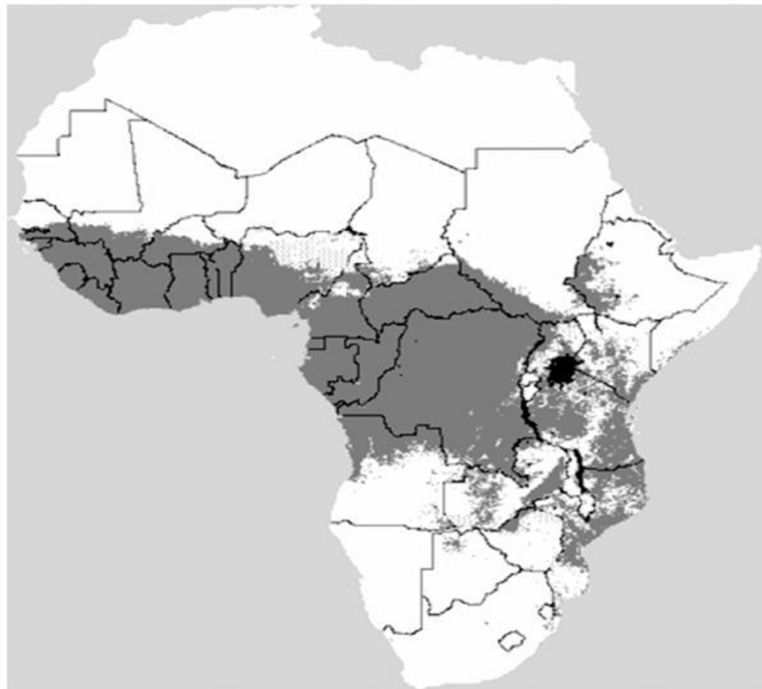

Figure 2.1. Tsetse fly distribution in Africa (Area shaded in grey indicates countries infested by tsetse flies) (Abd-Alla *et al*., 2013)**.**

The distribution of tsetse flies is influenced by the level of their adaptation to the newly created domestic cycle and also on human pressure. For instance, human destruction and fragmenting of tsetse habitats in South Africa caused either disappearance of some tsetse species or their persistence at lower densities in unsuitable habitats (Van den Bossche, de La Rocque, Hendrickx, & Bouyer, 2010).

The forest species are the largest (9.5-14 mm) compared to the savannah and riverine groups. *Glossina fuscipleuris* Austen 1911, *G. fusca fusca* Walker 1849 and *G. frezili* Gouteux 1987 are mostly found in moist forests of west and central Africa. However, *G. brevipalpis* Newstead 1910 occur discontinuously in eastern and southern Africa (Cecchi *et al*., 2014, Krafsur, 2009) (Figure 2.2). A study on *G. brevipalpis* distribution in Matutuine district, Maputo province in Mozambique revealed that they were mostly found in the dense vegetation and in the east wetlands of the Maputo river. The tsetse belt in Matutuine was also found to border closely with that of Kwa-Zulu natal in South Africa which is also widely characterized with *G. brevipalpis* (Sigauque *et al*., 2000). In Kenya, *G. brevipalpis* are found along the south east region in Kwale and Kilifi Counties (Devisser and Messina 2009) and they mainly feed on hippopotamus and bovines such as the cattle, bush pigs, bushbucks and buffaloes (Clausen *et al*., 1998). During feeding the infected *G. brevipalpis* transmits *T. b. brucei, T. suis* or *T. simiae* that causes nagana in livestock. *Trypanosoma suis* and *T. simiae* results to high mortalities in domestic pigs however *T. simiae* has also been found to affect camels, horses and cattle (Aksoy, 2003).

Figure 2.2.Distribution of forest group tsetse flies in Africa (Area shaded in red show countries with suitable vegetation for forest group tsetse flies) (Cecchi *et al*., 2008).

The savannah flies (*G. morsitans morsitans* Westwood 1850, *G. pallidipes* Austen 1903, *G. austeni* Newstead 1912, *G. swynnertoni* Austen 1923) occupy the woody savannah habitats of west, central and east Africa. They are medium sized and mainly transmit both nagana and sleeping sickness. They mainly feed on ungulates and other large animals (Gikonyo *et al*., 2003). Blood meal studies from *G. m. morsitans* and *G. centralis* in (Luangwa valley, Zambia), *G. pallidipes* in (Lambwe valley, Kenya), *G. swynnertoni* in (Serengeti, Tanzania) and *G. pallidipes* (Nguruman and Busia, Kenya and Uganda) showed that tsetse flies get their blood meals from different wild animals (Muturi *et al*., 2011) . However, Gikonyo and colleagues observed that savannah species rarely feed on common game animals such as waterbuck, impala, giraffe, and hartebeest which repel the them (Gikonyo *et al*., 2002).

The riverine flies (*G. fuscipes fuscipes* Newstead 1911, *G. caliginea* Austen 1911, and *G. fuscipes martini* Zumpt 1935) are the smallest in size and inhabit gallery forests and riverine vegetation (Guerrini *et al*., 2009). Thirty percent of their preferred habitation is 'woodland savannah' which is characterized by areas that are less densely vegetated. Exceptionally, *G. tachinoides* are widely distributed in more arid areas compared to other riverine species (Cecchi *et al*., 2008). The *G. tachinoides* still transmit trypanosomes even at low densities (Bouyer *et al*., 2006, Guerrini and Bouyer, 2007). They mainly feed on reptilian hosts and humans to cause nagana and sleeping sickness respectively (Omolo *et al*., 2009).

2.2 Economic significance of tsetse flies

Tsetse flies transmit pathogenic parasites that cause nagana and sleeping sickness in animals and humans respectively (Mattioli *et al*., 2004). The main causative agents of nagana in cattle are *T. Congolense*, *T. vivax* and *T. b. brucei,* (Samdi *et al*., 2011). Others including *T. suis, T. godfreyi* and *T. simiae* affect mostly domestic pigs while *T. evansi* is primarily a camel parasite. *Trypanosoma brucei* is further sub divided into *T. b. gambiense* and *T. b. Rhodesians* causing chronic Gambian sleeping sickness (west and central Africa) and acute Rhodesian sleeping sickness (east and southern Africa) respectively in humans (Simarro *et al*., 2012). However, there have been sporadic reports on sleeping sickness cases resulting from non-human pathogenic species of trypanosomes such as *T. b. brucei, T. congolense* and *T. evansi* (Brun *et al*., 2010).Normally, it is difficult for humans to be infected by animal species because of the presence of a trypanolytic factor in human serum. However some species for instance *T. congolense* and *T. evansi* have showed some degree of resistance to human plasma (Joshi *et al*., 2005).

Sleeping sickness occurs in two stages; first is the early stage in which parasites are found in the hemolymph (hemolymphatic) followed by the late stage which occurs at the central nervous system (meningoencephalitic). A chancre is usually the first to appear at

the site of the tsetse bite. For *T. b. rhodesiense* infections, the patient experiences intermittent fevers associated with parasitemic waves within weeks while for *T. b. gambiense* infections, the patient experiences frequent lymph adenopathy, oedema and anaemia. Some common late stage symptoms for both types include epileptic attacks, maniacal behaviour, somnolence and coma. In both cases, the rate of survival reduces drastically once the trypanosomes cross from the blood to the central nervous system (Simarro *et al*., 2008).

Animals infected with nagana appear very thin, lose appetite, have low milk production, have premature births and males have damaged testicles (Stich *et al*., 2002). Epidemic cases of nagana are rare. Instead, tsetse infested livestock areas experience endemic trypanosomiasis. Endemicity occurs when susceptible livestock are fed on by tsetse flies or due to continued use of trypanocidal drugs to eradicate the virulent strains. However, untreated animals affected by virulent strains eventually die (Ducheyne *et al*., 2009).

2.3 Trypanosomiasis control techniques

Various strategies have been developed to control trypanosomiasis with some targeting the tsetse fly vector and others the trypanosome pathogens. Those targeting trypanosomes include; application of trypanocides while those targeting tsetse flies include; use of insecticides, sterile insect techniques (SIT), targets, traps e.t.c.

2.3.1 Use of trypanocides

Trypanocides have been mainly used to control establishment of trypanosomes in the hosts. The early stage of Eastern and Gambian HAT can be treated using suramin and pentamidine respectively while the late stage for both are treated using melarsoprol. Suramin inhibits several of the trypanosome enzymes and rarely causes serious side effects or mortalities (Bouteille *et al*., 2003). Pentamidine have side effects including; reversible renal toxicity, hypotension, cardiac or pancreatic toxicity and painful intramus-

cular injections (Cattand, 2001). Melarsoprol on the other hand is arsenic, penetrates the blood-brain barrier and puts the patient at a 2-12% incidence of developing side effects or death (Blum, Nkunku, & Burri, 2001). Eflornithine, a recent drug is effective against both stages of Gambian infection. However its availability, administration and affordability is difficult (Bouteille *et al*., 2003).

Nagana in cattle is treated treated using diminazene actuate (bedevil). Although it has similar side effects to those of pentamidine, the injection is less painful, it is cheaper, has a shorter treatment period and it is highly produced (Bouteille *et al*., 2003). Selective administration of diminazene actuate to cattle in the year 1996-2003 by farmers in Zambia reduced the number of drugs administered and even mortalities. However, it caused an increase in drug resistance (Masumu *et al*., 2012). Isometamidium chloride (samorin/ trypan) is excellent prophylaxis for AAT but resistant cases have been reported against it in east and west Africa (Diarra *et al*., 1998).

Combined therapy of eflornithine-nifurtimox has been tested for improved efficacy, simplified administration and reduced drug resistance but it has also not been embraced as it is faced by limitations such as, expensive intravenous administration which requires skilled personnel and can only be used for second stage treatment of Gambian HAT (Simarro *et al*., 2008a).

Other trypanocides (quinapyramine sulphate and suramin sodium) can be used as prophylactic while Homidium salts (homidium chloride and homidium bromide) can be used for either therapeutic or prophylactic purposes. Although heavy reliance on these synthetic drugs has resulted to resistance mechanisms by the trypanosomes, their administration is very useful in epidemic cases where a swift action is needed as was the case in Mozambique in the period after the civil war when trypanosomiasis constrained restocking of cattle (Sigauque *et al*., 2000). Less effort is being put to research on developing new drug targets as this would require lots of resources and commitment for it to be actualized. Moreover, no vaccination strategy has been developed due to presence

of over 1000 variant surface glycoproteins (VSGs) which enables the trypanosome to evolve and evade the host immune response (Marcoux *et al*., 2010).

2.3.2 Use of insecticides

The technique involves ground or aerial spraying of persistence insecticides such as dithiothreitol (DTT) or dieldrin or aerosol spraying of non persistent insecticide droplets in the resting points of tsetse flies (Vreysen *et al*., 2013). Ground spraying of tsetse with insecticides (DTT and Beta-Hexachlorocyclohexane (BHC)) was first done in 1945 but was later discontinued as it was found to have negative effects on the non target organisms (Kuzoe & Schofield, 2004). Annual ground spraying with DDT and aerial spraying of deltamethrin in low doses was used for control of *G. m. centralis* in Okavango Delta in Botswana for 20 years before switching to odour baited targets. This method ceased to be used because it resulted to: insecticide resistance in endemic areas, reinvasion pressure and in addition, it was labour intensive and costly (Kuzoe & Schofield, 2004). During a pilot study at Mangapwani in Zanzibar, a pyrethroid pour on technique reduced the fly catches to zero in 37 days (Vreysen *et al*., 2014).

Aerosal spraying with either helicopters or aircrafts has been used in Rwanda, Kenya, Tanzania and Zambia. This technique was successfully used in Zambia to eliminate *G. m. morsitans* in an area of 15,000 square kilometers (Vreysen *et al*., 2013). Although an effective control method, aerosol spraying with helicopters is laborious, requires close supervision and careful planning (Vreysen *et al*., 2013).

2.3.3 Sterile insect technique (SIT)

Sterile insect technique is a non-insecticidal control approach in which male flies are sterilized with gamma radiations and released to mate with the wild female virgins. This hinders embryogenesis and hence no viable offsprings are brought forth (Vreysen, 2001). Repeated release of sterile males continually reduces occurrence of the target insects and

may eventually render them extinct (Abd-Alla *et al*., 2013). The technique is specific to target organisms and is effective at low tsetse densities (Kuzoe and Schofield, 2004). In Zanzibar, SIT was effectively used alongside the pour-on technique to reduce *G. austeni* populations in the Unguja Island from mid-1994 to mid-1996. Additionary, integration of SIT with insecticide impregnated targets and traps was used in controlling populations of *G. palpalis gambiensis* and *G. tachinoides* in the Sideradougou area in Burkina Faso and of *G. palpalis palpalis* in the Lafia area of Nigeria while in Tanzania, SIT integrated with aerial spraying of insecticides was successfully used to control *G. m. morsitans* (Vreysen *et al*., 2011).

The success of SIT technique in eradicating tsetse flies is however faced by various challenges. Vulnerability of tsetse colonies to infection by Salivary Gland Hypertrophy virus (GpSGHV) which causes a SGH syndrome has led to their collapse (Abd-Alla *et al*., 2008). For instance, a SIT colony initiated in 2007, to eradicate *G. pallidipes* in the southern rift valley of Ethiopia and another colony at the Insect Pest Control Laboratories (IPCL) Seibersdorf Laboratories, Austria collapsed due to infection by GpSGHV (Abd-Alla *et al*., 2012). Other challenges of SIT include mass rearing of the male flies of which sterile males should constitute 80% of the male population and requirement of extensive planning failure to which there is a low probability of success (Vreysen *et al*., 2014).

2.3.4 Targets and traps

Targets and traps are control methods that are based on use of bait technology (Vreysen *et al*., 2013). Stationary insecticide targets which consists of a screen of blue and black cloth, treated with biodegradable pyrethroid such as deltamethrin is a simple and cheap tsetse control method. In both targets and traps, tsetse flies use their visual cues to detect the blue colour which they find attractive but on landing they move towards the black colour and succumbs to the insecticides-in the case of a target or enters the trapping net-in the case of a trap (Kuzoe & Schofield, 2004). In both cases, appropriate odour baits

have been tried for their effectiveness in tsetse attraction. Using baits is advantageous because it has minimal, localized and temporary effects on non-target species. Various odours have been widely tested for their effectiveness in suppressing tsetse populations. For instance, effective odour baits were developed for control of *G. f. fuscipes* species (Omolo *et al*., 2009) and black rhinoceros odour in trapping tsetse flies and biting flies (Mihok *et al*., 1996).

Following successful eradication of tsetse fly at Mangwapani in Zanzibar in 1987, the government attempted to do an island-wide eradication of tsetse flies in the Unguja Island in 1988-1993 using the pour on technique in areas with abundant livestock and insecticide impregnated screens (IIS) in those with none. However no much impact was observed and the use of IIS proved to be a challenge (Vreysen *et al*., 2014).

The first tsetse trap developed by Harris in 1931 was effective in trapping *G. pallidipes* in South Africa (Kuzoe & Schofield, 2004). Since then, different traps that are effective for capturing live tsetse species in various parts of Africa have been developed. For example, pyramidal trap in Congo (Gouteux & Sinda, 1990), vavoua trap in Côte d'Ivoire (Laveissière & Grébaut, 1990), biconical (Challier & Laveissiere, 1973), epsilon, Nzi and Nguruman (NGU) traps (Figure 2.3). *Glossina brevipalpis* and *G. austeni* species in Kwa-Zulu Natal (South Africa) were initially trapped using the vavoua and siamese traps but were ineffective as only 21-41% of the flies trapped flew vertically to be collected in the cones. This was resolved by development of the hatch trap (H-trap) that was designed with side cones enabling the flies to be trapped as they flew horizontally (Kappmeier, 2000). Baited Nzi traps were found to be more effective for trapping biting flies than tsetse flies (Mihok *et al*., 2007). The biconical trap was first developed in 1973 to control spread of palpalis and fusca groups in west Africa but is now widely being used to control a wide range of species mostly in the west and central Africa (Kuzoe & Schofield, 2004). Odour-baited biconical traps were effectively used to attract *G. longipalpis* in Jopa-Cobiana Forest in Guinea-Bissau (Jaenson *et al*., 1991). Although cost effective,

traps are difficult to maintain, they require continous replenishment of odours, require use of specific cloth with the right reflectivity pattern and use of suitable trap to target specific species and in different geographical distribution (Vreysen *et al*., 2013).

Figure 2.3. Traps used in the field for tsetse collection.

The figure above shows some of the traps that have been used to trap tsetse flies. **A:** Biconical **B:** Hatch (H) and **C:** Nguruman (NGU) traps. The traps are normally baited with synthetic odours which are placed near them, the blue colour attracts the tsetse flies from afar but when they get close they detect the odour that is made to mimic that of the hosts, they move towards the black cloth and enter the trap. While inside the trap the tsetse fly vertically (biconical and NGU) or horizontal (H-trap) and are trapped in the nets

from which they cannot escape. (Photos were taken by Mary Murithi during field collections in Shimba Hills National Park, 2014).

More recently, push-pull technology that integrates use of traps and/or targets and "tsetse collars" has been developed (Saini and Hassanali, 2007). These techniques apply the olfactory principal whereby the collar repellants push tsetse flies away while the attractants pull them into the traps. Some of the attractants that have been effectively used in trapping tsetse include acetone, 4-methyl-phenol, 1-octen-3-ol, carbon dioxide. Other extracts from the hosts' such breath, urine and sweat have been used as well. Repellants that have been synthesized and used include, guaiacol, methyl-ketones, d-octalactone and 2- methoxy-4-methylphenol (Gikonyo *et al*., 2003).

2.4 Olfaction in insects

Insects including tsetse flies, rely on olfaction to execute critical functions such as host finding, identifying mates, detection of suitable larviposition or oviposition sites and resting positions among others (Carey & Carlson, 2011).

The antenna is the primary olfactory organ in insects but maxilliary and/or labial palps may also function in odour detection. The antenna houses sensilla hairs which are characterized by various shapes and structures but they all principally function to house and protect the dendrites on the olfactory sensory neurons (OSNs) (Hansson and Stensmyr, 2011). The sensilla are also distributed in other parts of the insect's body and are often used for purposes unrelated to feeding including mechano-, hygro-, and thermo reception (Bruyne, Foster, Carlson, & Haven, 2001). The long sensilla with thick walls are trichoid 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) (Couto *et al*., 2005, Vosshall *et al*., 2000). The advantage of having multiple olfactory organs in insects is not clearly understood. In some insect species such as *Anopheles*

gambiae and *Manduca sexta*, maxillary and labial palps house OSNs that detect CO_2 enabling them to locate food resources while in others such as *D. melanogaster* this function is executed by antennal OSNs and their maxiliary palps function in taste enhancement (Shiraiwa, 2008). As to whether the sensillar architecture influences the detection of specific chemicals is not known but it has been reported that trichoid sensilla house OSNs tuned to pheromones while coeloconic sensilla house OSNs that detect water soluble amines and acids. Furthermore, sexual dimorphism of the sensilla observed in some insects may be linked to functions. For instance, female mosquito *An. gambiae* have thrice the number of sensilla compared to the males as they feed on both blood and nectar (Carey & Carlson, 2011).

2.4.1 Insect olfactory system

The morphological diversity of the insects' peripheral olfactory system is an indication of existence of selection pressure but the principle of olfaction is unchanged. During olfaction, the volatile hydrophobic odours from the environment penetrate sensilla through the cuticle pores to the sensillar lymph. Their hydrophobic characteristic enables them to bind to specific OBPs or CSPs. The resulting complex then diffuses through the sensilla lymph and releases the odorant to the dendrites which are on the surface of the OSNs. The odour signals are transduced into higher brain centers for further processing and determines the behaviour elicited by the fly (Figure 2.4) (Leal, 2013) (Masiga *et al*., 2014).

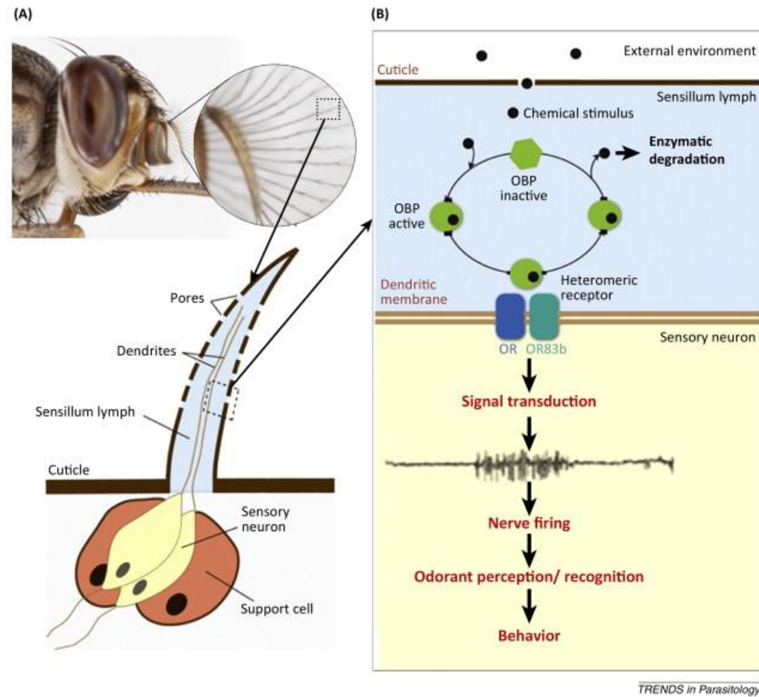

Figure 2.4.Tsetse fly olfaction at the periphery (Masiga *et al*., 2014)

The olfactory system in the antennae. The antenna consists of many sensillar hairs. During olfaction, the hydrophobic molecules get into the sensilla through the cuticle pores. The molecules bind the OBP to form a complex that diffuses through the sensilla lymph. The OBP deposits the molecule on the olfactory receptor which is on the surface of the dendritic membrane. The molecule is transduced further to the higher brain centers and this elicits the behaviour observed in the fly.

2.5 Olfactory proteins

Chemoreception in insects is dependent on a cascade of events by multigene families of proteins consisting of receptors proteins (ORS, iGluRs, IRs, GRs and SNMPs) and soluble proteins (CSPs, ODEs & OBPs) (Leal, 2013).

2.5.1 Odorant receptors (ORs)

Insect ORs are a multigene family of about 370 – 400 amino acids that are characterized by seven-transmembrane domains with an inverted topology of extracellular (cytoplasmic) N terminal and an intracellular C terminal in the plasma membrane and are expressed in OSNs (Benton *et al*., 2006). They are heterodimers resulting from a co-expression of olfactory receptor protein (ORx) with a highly conserved, seven transmembrane odorant co-receptor (OR83b/Orco). This co-expression improves odorant responsiveness (Larsson *et al*., 2004). The insects OR/Or83b complex also differs from the vertebrate G-protein coupled receptors which rely on second messengers to activate the ion channels (Benton *et al*., 2009). Insect ORs are classified as either generalists or specialists depending on their specificity to food (Kaupp, 2010). The number of OR genes reported varies with *Drosophila* having 62 ORs (Clyne *et al*., 1999) while the *Anopheles* mosquito has 80 ORs identified (Fox, Pitts, Robertson, Carlson, & Zwiebel, 2001). In *G. m. morsitans* 46 ORs have been identified through genomic annotation (Obiero *et al*., 2014). Generally, insects ORs are very divergent with little sequence conservation within and across insect orders and species (Clyne *et al*., 1999). In the *Drosophila*, OSNs expressing similar ORs project their axons to the same glomeruli in the antennal lobe. Most *Drosophila* ORs are expressed in the basiconic and trichonic OSNs with an exception of OR35 which is expressed in the coeloconic OSN (Couto *et al*., 2005).

2.5.2 Ionotropic receptors (IRs)

The IRs are the most recently identified odorant receptors in insects that diverged from ionotropic glutamate receptors (iGluRs) (Benton *et al*., 2009). They are highly expressed in the coeloconic sensilla in *Drosophila* (Yao *et al*., 2005). There are two types of IRs, antennal IRs and divergent IRs. The antennal IRs are conserved across insects while the divergent IRs are species specific and are expressed at the peripheral nervous system and

internally in the gustatory neurons (Croset *et al*., 2010). This suggests their involvement in sensing tastes and food. They detect various acids including; aldehydes, amines and ammonia. Although IRs are similar to iGluRs in the aspect that they have S1 and S2 lobes separated by an ion channel domain, they lack the conserved residues found in iGluRs that interact with glutamate (Benton *et al*., 2009). Unlike the ORs which only allow expression of similar receptors per neuron, the IRs exhibits the ability to be co-expressed in a neuron. *Drosophila* consists of 66 IR genes that have been identified with a high divergence but their ion channel pore is highly conserved to that of iGluRs hence they might be implicated in ion channelling (Benton *et al*., 2009). Insect genome studies have revealed IRs present in other species. Olivier and colleagues, reported presence of 12 putative IR encoding genes from an EST library of the male moth *Spodoptera littoralis*. This expression was observed in adult antennae, pupae and larvae and they were characteristically similar to DmelIRs (Olivier *et al*., 2011). Sequencing and annotation of *G. m. morsitans* genome identified 18 IRs (Macharia *et al*., 2016).

2.5.3 Sensory neuron membrane proteins (SNMPs)

Sensory neuron membrane proteins are related to CD 36 in vertebrates that binds lipoprotein and uptakes cholesterol and lipids. Screening for selective expression of 39 uncharacterized genes in *Drosophila* by RT-PCR revealed an antenna enriched gene encoding SNMP which was homologous to *Anopheles* and *Antiheroes polyp emus* antennal specific SNMP. The SNMPs are grouped into two namely; SNMP1 and SNMP2. Further studies in Drosophila show that SNMPs are expressed in OSNs involved in pheromone detection hence they are necessary for eliciting responses of OSNs expressing OR67d to cis-vaccenyl acetate (cVA) that is elicited by the female Drosophilas (Kurtovic *et al*., 2007). They also activates HR13 (a pheromone receptor in moths) by (Z)-11-hexadecanal pheromone ligand (Große-Wilde *et al*., 2007).

2.5.4 Chemosensory proteins (CSPs)

Chemosensory proteins are soluble proteins consisting of a four conserved cysteine signature and two disulphide bridges linking cys1 to cys2, and cys3 to cys4 to result in formation of two small loops (Picone *et al*., 2001). This makes the CSPs more flexible than the OBPs that have a rigid structure and only have a flexible C- and N-terminal ends. The CSPs have a tunnel that accommodates long carbon chain molecules leaving the two ends to interact with the solvent molecules (Jansen, 2005). They also consist of a shorter sequence of about 110 amino acids and weigh 12-13 kilo Daltons. The CSPs were first identified in *D. melanogaster* and were later reported in other insects. The CSPs were initially reported to be expressed in the chemosensilla lymph but have subsequently been found in non-chemosensory organs such as the legs and wings (Pelosi *et al*., 2005). For instance, CSPMbraA6 that is expressed in the trichodea sensilla of the moth *Mamenstra brassicae* not only binds to Z11-16:Ac component in the pheromone blend of *M. brassicae* but also binds fatty acids (Lartigue *et al*., 2002). CSP-sg4 from the locust *Shistocera gregaria* although closely related to the pheromone detecting moth group 2 CSP , does not show affinity to pheromones but instead binds plant volatiles and aromatic compounds. No CSPs in *S. gregaria* have been implicated to have pheromone binding properties. The CSP ASP3c in *A. mellifera* has been suggested to be a general lipid carrier protein(Jansen, 2005).

2.5.5 Odorant binding proteins (OBPs)

Odorant binding proteins are small (120-150 amino acids), soluble, globular, extracellular proteins of about 14-16 kilo Daltons (Swarup *et al*., 2011). The OBPs are synthesized by the support cells and secreted into sensillium lymph where they bind volatile odors and transport them to ORs located within OSNs (Pelosi *et al*., 2005). They consist of a short signal sequence at the N terminal that enables them to be secreted. They also have a conserved six cysteine signature that determine their 3 dimensional-

protein structures bound by covalently linked disulphide bridges that pair cys1 to cys3, cys2 to cys5 and cys4 to cys6 for maintaining their stability. The helices are rigid while the N-terminal and C-terminal are flexible and can open or close the cavity. The α-helical structure of insect OBPs differs from that of mammalian OBPs which have a β-barrel fold, a carboxy terminal α-helix and belong to a lipocalin superfamily (Campanacci *et al*., 2001). Odorant binding proteins have mainly been classified into four large sub-families namely: classic (six cysteine signature), plus-C (more than six cysteines), minus-C (lack the second and fifth cysteines) and dimers (two classic OBPs) (Fan *et al*., 2011).

Genome annotations have revealed significant number of OBP genes in various insects; 32 in *G. m. morsitans* (IGGI, 2014), 28 in *G. brevipalpis* (unpublished), 51 in *D.melanogaster* (Hekmat-scafe *et al*., 2002), 69 in *An. gambiae,* 111 in *Ae. Aegypti*, 109 in *Cu. quinquefasciantus* (Manoharan *et al*., 2013), 44 in *Bombyx mori* (Gong *et al*., 2009), 46 in *Tribolium castenium* and 21 in *Apis mellifera* (Forêt, 2006). The lower number of OBPs in tsetse flies relative to those in other insects can been attributed to their hematophagous nature and also their host specificity (IGGI, 2014).

Dipteran OBPs have shorter amino acid sequences (around 125 amino acid residues) compared to those of Lepidopterans such as moths (around 140 amino acid residues) as they lack the extended C terminus to take over the binding pocket at low pH. Some OBPs passively solubilize and shuttle hydrophobic odorants across the chemosensillar lymph to the dendrites while others are actively involved in the recognition of these odorants. This can explain the large number of OBPs within various insect species. OBPs were first discovered in a giant male Silk moth *Anthrea polyphemus* antenna which was bound to a component of the female sex pheromone (*E, Z*)-6,11-hexadecadienyl acetate. Expression of Pheromone binding proteins (PBPs) mainly on the male antennas to detect pheromones from females and that of general OBPs (GOBPs) to detect general volatiles in the atmosphere were later unveiled in many other insects (Fan *et al*., 2011).

Differential expression of numerous OBPs in the different antennal sensilla (trichonic, basiconic and coeloconic) could mean different odorant binding specificities. Also, the presence of OBPs in non-olfactory organs such as wings, legs, abdomen and thorax suggests that they may be involved in non-olfactory processes; for instance a study on the paper wasp *Polistes dominalus* showed that some OBPs were also expressed in legs and wings in addition to the antennas (Calvello *et al*., 2003). In another study on the fire ants, an OBP protein was observed in the thorax and was associated with the insects' social behaviour (Krieger & Ross, 2002).

The term "OBP" was first used in *Drosophila* and has since been adopted in other insects to indicate that the gene belongs to the OBP-like family of genes. The number assigned to the OBP was based on the chromosomal location (cytogenic location) of the gene and the letter preceding the number indicates the position of an OBP relative to other OBPs on the same cytogenic location (Galindo and Smith 2001) . The OBP gene clusters in *Drosophila* appear either in tandem arrays or in opposite orientations implying that they evolved by tandem duplication or complex duplication coupled with rearrangement events respectively. OBPs that are closely linked may however exhibit significant variations in their expression patterns. Also, most *Drosophila* OBP genes have 0-3 introns located within conserved cysteines and have a signal peptide at the N terminal (Hekmat-scafe *et al*., 2002).

Studies carried out on mutant *Drosophila's* lacking "LUSH" OBP showed defective response towards; ethanol, benzaldehyde and the courtship pheromone- 11-cis vaccenyl acetate (Hekmat-scafe *et al*., 2002) while a mutation in the *D. sechellia* OBP57e gene caused by a 4 base pair insertion results in the species attraction to octanoic and hexanoic acid and hence its specialization to the host plant *Morinda citrifolia* (Swarup *et al*., 2011). The antennal specific AmeASP1 in drone and worker *Apis mellifera* (honeybees) detects two queen pheromones; 9-keto-2E decenoic acid and 9-hydroxy-2E decenoic acid. LmaPBP found only in female cockroaches *Leucophae maderae* detect the con specif-

ic male sexual pheromone blend which consists majorly of butane-2,3 diol, 3-hydroxy-butan-2-one, (E2)-octenoic acid and little amounts of cenecioic acid (Jansen, 2005). Molecular characterization studies of the Asian mosquito Anopheles stephensi OBP1 (AsteOBP1) and OBP7 (AsteOBP7) revealed their high expression in female antennas and were hence suggested to be involved in female olfactory response and also in blood feeding (Sengul, 2008)(Meryem Senay Sengul and Zhijian Tu, 2010).

2.6 Sequence comparison analysis tools

DNA or protein sequence homology between organisms is a quick way to infer functions of newly synthesised genes. Numerous tools used for searching sequence databases use parameters that enable measurement of similarity between sequences and thus determine functions (Altschul, 1990). Sequences may be compared either globally or locally. Global comparison entails comparison of complete sequences including the regions with low similarity. This is however not common. In contrast, local similarity compares regions that show some level of conservation and are hence more relevant (Zhang, 2003). Basic local alignment search tool (BLAST) is the most widely used local alignment tool by most researchers to align sequences. The BLAST algorithm is fast and is able to detect weak but biologically significant sequence similarity (Mount, 2007). Various scoring protein matrices bearing different similarity scores exist, the choice of which one to use depends on the output required and the nature of work being done e.g. PAM30, PAM70, BLOSUM45, BLOSUM62 and BLOSUM80. These scoring matrices assign scores to the alignment based on the presence of a match, mismatch or a gap. The BLAST expect value (e-value) is the threshold of statistical significance between sequences. Smaller e-values (closer to zero) are preferred as they indicate that the alignment hit between sequences did not occur by chance (Altschul *et al*., 1997).

Various BLAST programs exist each defined on the type of query and search database being run; BLASTn (a nucleotide sequence is compared with a nucleotide sequence da-

tabase), BLASTp (an amino acid sequence is compared with a protein sequence database), BLASTx (a translated nucleotide sequence is searched against a protein sequence database), tBLASTn (a protein sequence is compared against a translated nucleotide database) and tBLASTx (a translated nucleotide sequence is searched against a nucleotide sequence database) (Zhang, 2003). On retrieving the homologs, the sequences can be compared using multiple sequence alignment (MSA) and phylogenetic analysis. MSA can be done through algorithms such as; MUSCLE, T-COFFEE, MAFFT and CLUSTALW. MUSCLE (Multiple sequence comparison by log expectation) algorithm is widely preferred as it is characterised by speed and biological accuracy. The resulting alignments can be visualized and edited using; Jalview (Waterhouse *et al*., 2009), Seaview (Gouy *et al*., 2010), MEGA (Molecular Evolutionary Genetics Analysis) (Tamura *et al*., 2013) e.t.c.

2.7 Phylogenetic analysis

Phylogeny is the estimation of evolutionary relationship between sequences (homology). Homologous genes in different species that perform similar functions are known as orthologous genes while paralogues are homologues that result from gene duplication in multigene families within the same species. Phylogenetic trees consist of branches and leaves (internal and external) (Baldauf, 2003). Branching pattern of the phylogeny may be grouped as either; clade (monophyletic), paraphyletic or polyphyletic. Branch lengths indicate the divergence between species in the same leaf. Cladograms however do not show any evolution between species. The root of a phylogenic tree is the oldest point in the tree and determines the branching pattern of the tree. Rooting of a tree is achieved by putting an outgroup (a reference group that does not belong to the group of interest). A phylogenetic tree without an outgroup may have its root at the middle or may be presented without a root (Harrison and Langdale, 2006).

Phylogenetic trees may be constructed using either distance-matrix methods (Unweighted pair group method with arithmetic mean (UPGMA), neighbour-joining, Fitch-Margoliash) or discrete data methods (parsimony, maximum likelihood, Bayesian Methods). Although distance method is simple, straight forward and fast, discrete method is preferred as it examines each alignment column independently and is much more informative (Holder and Lewis, 2003). Various programs that may be used to construct phylogenies include PHYLIP, MEGA, PAUP*. Phylogenetic accuracy is tested by assigning bootstrap values. This tests the percentage of data that supports the tree. Values above 70% indicate that the alignment is reliable.

2.8 Quantitative real time polymerase chain reaction (qRT-PCR)

Quantitative RT-PCR is a sensitive and specific procedure that amplifies minimal DNA in a sample measuring the amplified product as the reaction progresses (after each cycle) (Arya *et al*., 2005). Amplicons may be detected via different chemistries which include; dsDNA-binding dyes (SYBR Green), fluorescent/ hydrolysis probes (taqman probes), molecular beacons or scorpions (Bustin and Mueller, 2005). The template required for qRT-PCR can either be genomic DNA or cDNA which is obtained by reverse transcribing RNA with the enzyme reverse transcriptase. The PCR step requires target specific primers that should not show primer dimers. Evaluation of primers is best achieved using SYBR Green 1 chemistry and melting curve analysis (Bustin *et al*., 2005). Real time PCR results can only be compared when similar priming strategy and reaction conditions are used (Ståhlberg *et al*., 2004).

Principally, quantification of target gene is equivalent to its abundance and this is implicated in the amplification or threshold cycle (Ct) number. The Ct is the cycle at which the fluorescence resulting from amplification is detected by the instrument. In the initial cycles, the fluorescence is below detection threshold and it defines a baseline. qRT-PCR amplification curves are characterised by a plot of fluorescence signal versus cycle number (Figure 2.5a) (Bustin and Mueller, 2005).

Quantification of target gene can be achieved by using absolute quantification or relative quantification. In absolute quantification, a standard curve (log of copy numbers versus Ct values) is generated by amplifying known standards (dilutions) with known copy numbers to obtain the Ct values. The curve obtained is then used to determine the copy numbers of the target gene in the unknown samples (Raeymaekers, 2005). This method is mostly used when measuring small gene numbers (Dumur *et al*., 2004) and in viral load quantification (Borg *et al*., 2003). On the other hand, relative or comparative quantification entails the use of a reference gene also known as a normalize, reference or endogeneous control gene. The reference gene is a house keeping gene whose expression in an organism is not dependent on the treatment, the type of tissue, starting materials or other factors. Theoretically, expression of a reference gene should not vary although experimentally some genes have been observed to vary. Studies evaluating the stability of relative genes have recommended the use of more than one reference genes so as to minimize errors and avoid making biased conclusions. There are numerous genes that can be considered as possible reference (house-keeping) genes such as, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Beta-tubulin (β-Tubulin), Alpha-actin (α-actin), Beta-2-microglobulin (B2M), Ubiquitin C (UBC) etc. The commonly used reference gene is GAPDH which functions in oxidoreduction in glycolysis and gluconeogenesis (Vandesompele *et al*., 2002). Suggestions have been made to use several reference genes in a single experiment and their resulting mean expression used for normalization However, the choice of the appropriate normalizer gene to be used by a researcher is dependent on the nature of the experiment being done (Vandesompele *et al*., 2002).

In qRT-PCR analysis, the raw Ct values of the target gene and the reference gene in the control sample are first obtained. The difference between the two values is known as the delta Ct (dCt). Similarly, the dCt value in the test or treated sample is also calculated. The difference between the two dCt values in the treated and in the control sample gives the deltadelta Ct (ddCt) value and this can be used to determine the fold expression of the target gene relative to the normalizer gene (2^{-ddCt}) (Livak and Schmittgen 2001). Alterna-

tively, reference genes can also be tested for their efficiency and the values obtained used to normalize the gene of interest using the Pfaffl method (Bustin *et al*., 2005).

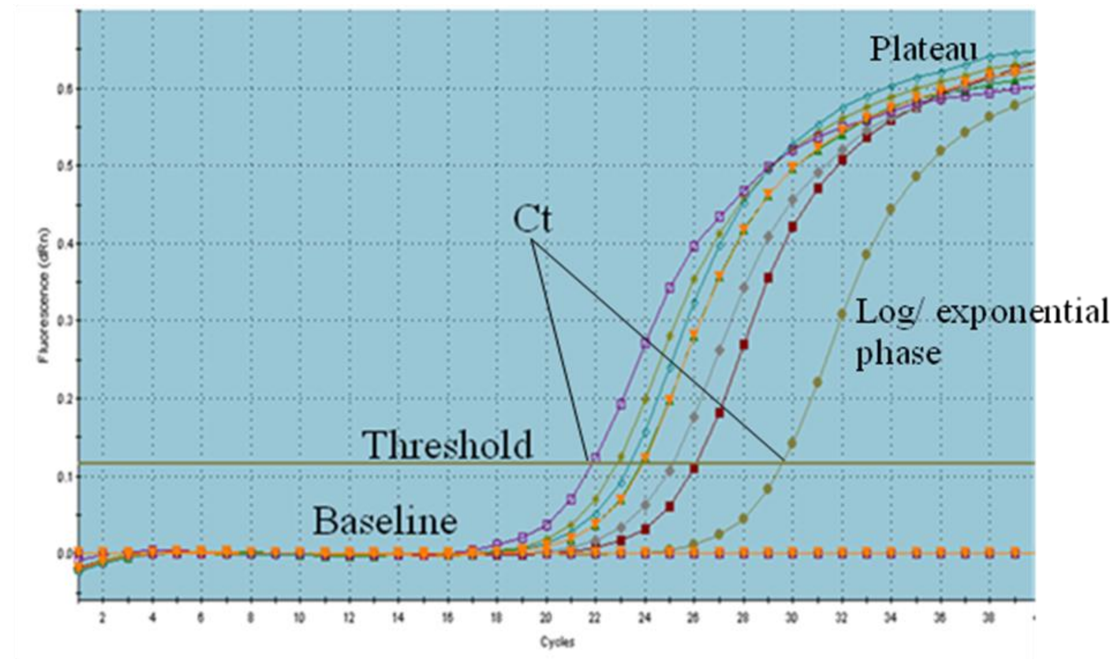

Figure 2.5a. Quantitative real time polymerase chain reaction amplification curve (Bustin & Mueller, 2005).

The figure above shows a qRT-PCR amplification curve. The baseline is the period at which fluorescence is not being detected. The threshold is a predefined level within the exponential phase of the amplification curve at which the fluorescence is readily detectable and is recorded as the threshold cycle (Ct). If the sample contains a high concentration of nucleic acid the resulting Ct will be low and if the concentration is low the Ct will be high. At the plateau the reagents in the reaction mixture have been depleted.

To confirm that the qRT-PCR amplification is specific, a melt/dissociation curve analysis is performed post PCR. During the analysis, the amplicons are melted at 95 ºC, equilibrated at 60 ºC and slowly reheated (dissociated) back to 95 ºC. The melting temperature (Tm) is observed at the center of the peak with primer dimers forming a

peak at lower temperatures. Primer dimers are mostly prevalent in negative samples (Figure 2.4b) (Bustin and Mueller, 2005).

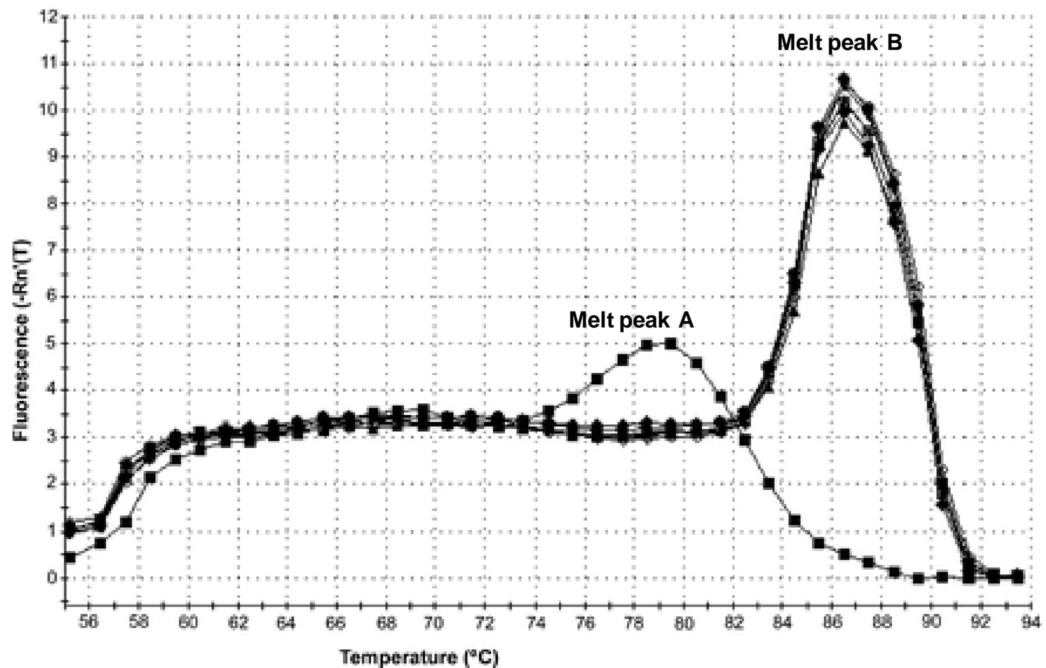

Figure 2.5b. Quantitative real time polymerase chain reaction dissociation curve (Bustin & Mueller, 2005)

The melting temperature (Tm)-the temperature at which the double strand DNA is denatured halfway is observed as the peak of the curve. Amplification due to the gene of interest is observed as a smooth peak at high temperatures (melt peak B =87 ºC) but if amplification is as a result of primer dimers or unspecific amplification then the peak at will be observed at low temperatures (melt peak A =79 ºC)

CHAPTER THREE

METHODOLOGY

3.1 Study site

Tsetse flies were collected from Shimba Hills National Park, in Kwale County. The area is about 250 km^2 and has an altitude of 120-450 m. The area is characterized by rainforest, woodland and grassland cover. The temperature in the area ranges from 24ºC to 36ºC and rainfall average of 900-1200 mm per annum. The short rains occur in October - December while the long rains occur from March-June. The park hosts various animals such as elephants, buffaloes, water bucks, giraffes, sable antelopes, duikers, bush pigs e.t.c. The tsetse fly species found in the area include *G. brevipalpis*, *G. pallidipes* and *G. austeni*. The experiments were carried out at the international centre of insect physiology and ecology in the molecular biology and bioinformatics unit laboratory.

3.2 Experimental design

For this study, random deployment of traps was done. Collection of flies was done in the mid morning and late afternoon. *Glossina brevipalpis* were separated from other non target insects. The population of the female *G. brevipalpis* after the 10 days collection was notably higher than the males (a total of 500 *G. brevipalpis* females and 60 males were collected). Larva and pupa were obtained from gravid females. The adult flies were grouped into five. Each group was fed on live rabbit and antennas extracted after appropriate starvation period (2 hours, 24 hours, 48 hours, 72 hours and 96 hours) post blood meals. The tenerals (newly emerged unfed flies) were used as the control group for both the developmental stages and the differentially starved adults. Pilot RNA extractions were carried out before field collections to determine the appropriate amounts of starting material. One larva, one pupa, and a pool of ten antennal pairs enriched with one head for the tenerals and differentially starved adults were observed to give sufficient RNA for

downstream processes. The 28 putative *G. brevipalpis* OBPs were first screened in all the experimental groups using conventional PCR and those that amplified successfully were further analysed quantitatively using qRT-PCR.

3.3 Sample collection

Wild *G. brevipalpis* female flies were obtained from Shimba Hills National Park, in November 2014. Biconical and NGU (Nguruman) traps baited with acetone, 1-octen-3-ol and 4-methylphenol were randomly placed at least 100m apart and allowed to stand for 24 hours before the first collection. Subsequent ccollections were done twice a day (at midday and in the late afternoon) for ten days. *G. brevipalpis* were identified and isolated from other tsetse fly species and non targeted insects (stomoxys and biting flies) using morphological characteristics as follows; *G. brevipalpis* (fusca) were distinguished from the savannah and riverine species as they are characterized by large hairs beside the thorax, below the wing insertion point and have a dark spot on their wing. The male *G. brevipalpis* differ from the females as they have a hypopygium (a structure located at the posterior tip folded beneath the last two abdomen segments) on the external genitalia which lacks in the females. Additionally, the front of the hypopygium consists of hairy plates called hectors (Pollock, 2015). *G. brevipalpis* were maintained exclusively on rabbit blood until the period of analysis. The flies were placed in cages and reared in the colony whose temperature was set at 25ºC – 30ºC and 80 humidity. Ten antennae plus one head of *G. brevipalpis* females were extracted from flies starved at 2 hours, 24 hours, 48 hours, 72 hours and 96 hours post blood meal periods and the samples stored in liquid nitrogen. Larvae, pupae and tenerals (newly emerged unfed flies) were also obtained.

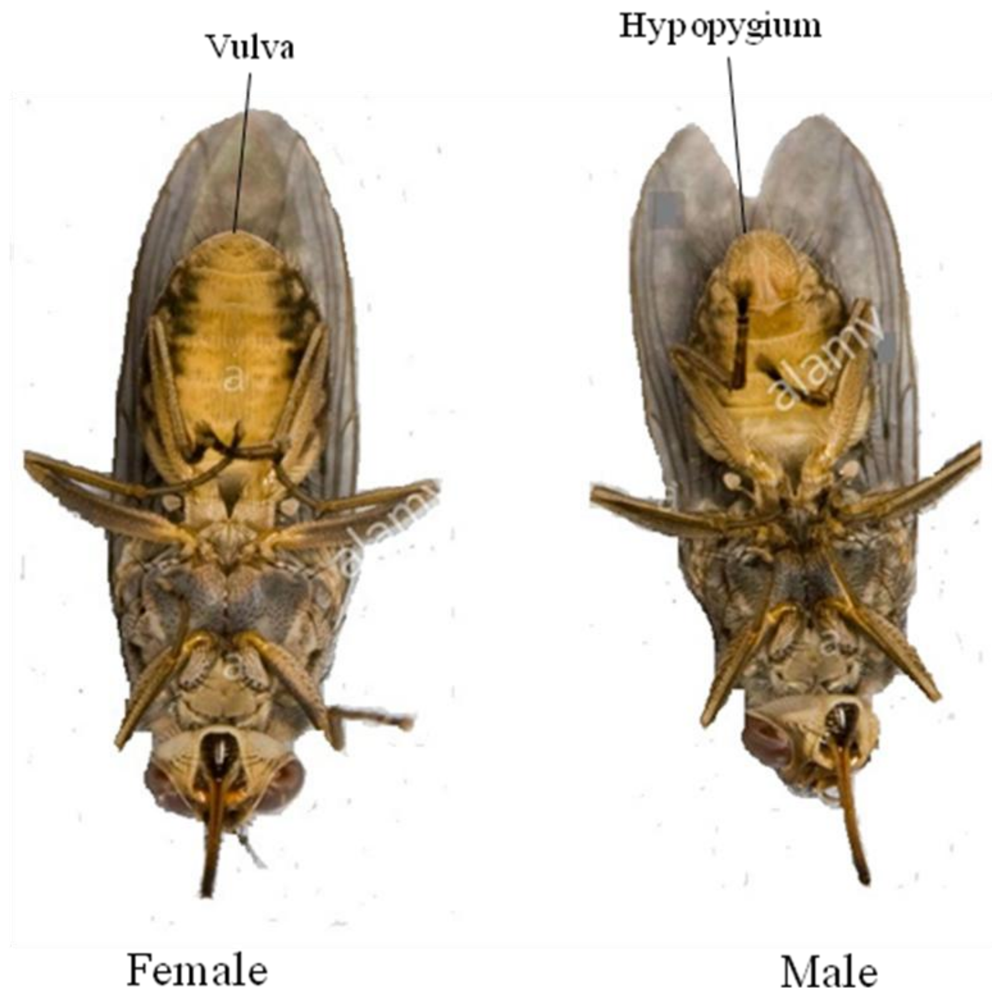

Figure 3.1. The female tsetse fly genitalia

The female tsetse flies have a smooth round abdomen known as vulva while that of the males has a distended hump on the lower abdomen called a hypopygium (John Pollock, 1982)

3.4 Computational identification and characterization of *G. brevipalpis* OBPs using Bioinformatics tools

Odorant binding protein sequences of *G. m. morsitans* were retrieved from VectorBase (VB-2015-06) by doing a protein name search (Giraldo-Calderon *et al*., 2014). The obtained sequences were queried against *G. brevipalpis* proteome in VectorBase to get the putative OBP sequences (Giraldo-Calderon *et al*., 2014). The putative *G. brevipalpis* OBPs were then queried against *D. melanogaster* proteome in FlyBase to obtain the orthologous sequences (Marygold *et al*., 2013). The BLASTP algorithm cut off e-value <10e-20 and substitution matrix BLOSUM62 were used. The N-terminal signal peptides of the *G. brevipalpis* putative OBPs were confirmed using a SignalP 4.1 Server (Petersen *et al*., 2011) and multiple sequence alignment performed using multiple sequence comparison logarithmic expectation (MUSCLE) tool (Edgar, 2004). The aligned sequences were visualised using Jalview (Waterhouse *et al*., 2009). The amino acid sequences of *G. brevipalpis* putative OBPs and their orthologs in *G. m. morsitans* and *D. melanogaster* OBPs were used to construct a phylogenetic tree using Molecular Evolutionary Genetics Analysis Version 6.0 (MEGA 6) using Maximum Likelihood method based on the Jones-Taylor-Thornton (JTT) model with NNI topology search (Tamura *et al*., 2013). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 5.2264)). All positions with less than 95% site coverage were eliminated (Jones *et al*., 1992). An initial tree was generated with 1000 bootstrap alterations (Tamura *et al*., 2013).

3.5. Primer design for *G. brevipalpis* OBP sequences

Primers of the putative *G. brevipalpis* OBP genes were designed using the web based Primer3Plus tool (Untergasser *et al*., 2012). The primer sizes were between 18 and 20 base pairs with melting temperature (Tm) values ranging from 55 ℃ to 60 ℃ and the product size range of 150 - 200 base pairs (Table 3.1).

Table 3.1 Primer sequences for amplifying putative G. brevipalpis OBPs

GENE ID	PRIMER SEQUENCES (5' TO 3')	
	FORWARD	**REVERSE**
GbrGAPDH*	GTCGAGTCTACGGGTGTGTTTAC	GTATAACTTTTCCCACGGCTTTAG
β- tubulin*	TTGATGCGACTGGTGCTTAC	CAAAATTATCAGGGCGGAAA
GbrOBP1	TCACCAGGTGCTACATCGAG	GTCGCTCTTCTCGGTGTTCT
GbrOBP2	ATGGCAGTATCCCGAAGATG	CGCACTTTGCAATCTTTTCA
GbrOBP3	CAGGCCGAAGTGAACGTTAT	GGGATTTGTCAGCAAAGCAT
GbrOBP4	CCTTCTTCAGCGAGAACAGG	GTACAGGCAGTCCAGGAAGC
GbrOBP5	CGAAGATCAGCCTACCATGA	GCCGCAGCAATTGATATAAG
GbrOBP6	CTGGCAAGGGAATGCACTAT	CTTTTTGCATGTTCCACAGC
GbrOBP7	TGGACCAGTGTTTAGCACCA	TTATCAAAGCCCTGCGTCTC
GbrOBP8	AAGACCGGTACCACAGAGGA	CAATGTGTTGAACGGTTTCC
GbrOBP9	CGAGGCGTGATGATGAAGTA	TAATGCACACGTCTCACAGG
GbrOBP10	AAATGGGTTTGGTTGACGAC	GATGGAACCACCAAGCCTTA
GbrOBP11	CACCAACTGCTACGTGAAATG	CGATTAGCCCAAGTGCAAGT
GbrOBP12	TGAGTTGGGAGCAGTTGATG	TGCCTCTGGATCAGCTTTTT
GbrOBP13	ACTTAACTCATCGCCCAACG	CGTGAACTGCTGCAAACATT
GbrOBP14	CCGACAGCTACAAGGACGA	GGTTGCTCACCAGGAAGAAG
GbrOBP15	GAGCACATCAGCATCAAGGA	AGGTACATGATGGCCACCTC
GbrOBP16	CAAGCTGCAGTACCTGAACG	CCAGATGCTGAAGCCGTACT
GbrOBP17	GCGTGAAATTTATGCGGCTA	TTTTCGTCATCCCAGTTGTG
GbrOBP18	CTTCTTCCAGTACGCCATCC	TCAGGAAGTGCATGAAGGTG
GbrOBP19	ACAAGGACAAGGTGGTGGAC	TCCTTCAGCTGGTTCAGGAT
GbrOBP20	GATCCAGGAGCTGAAGTTCG	GCAGTCCTCGATGATCTCCT
GbrOBP21	ATCGCCAAGCAGTTCAAGAT	CCTCCTGCCTCTTCTTGATG
GbrOBP22	CAGATCTGGGACAACAACCA	ACGTCCTCCTTGAACCACTC
GbrOBP23	CTTCCAGACCCCCATCTACA	CTGAAGCTGCTGTTGCACTC
GbrOBP24	AACGAGTGCAAGGACGAGAC	CTTCTCCTTCACCAGCTTGC
GbrOBP25	CCCGAGCTGAGGAAGAAGTA	CAGGATGCTGCTGCTGTAGT
GbrOBP26	GCCTGGTGATGATGAGGAAC	TGATGAAGAACAGCCTGTCG
GbrOBP27	GACAGCGTGATGAAGAACGA	TAGATGCCGTTGCTGATGTC
GbrOBP28	ACAAGGACAAGGTGGTGGAC	TTGAAGCCCTTCTCCTTCAG

* normalizer (house keeping) genes

The table above shows primer sequences for *G.brevipalpis* GAPDH, β-tubulin and the 28 putative OBPs which were designed using Primer3plus software. The primer lengths were in the range of 18-20 amino acids long although those of GAPDH were longer.

3.6. Total RNA extraction

Total RNA was extracted from the antennas and head samples at (2 hours, 24 hours, 48 hours, 72 hours and 96 hours post blood meal, tenerals) as well as from larva and pupa using the TRIzol method (Chomczynski and Sacchi, 1987). The samples were thoroughly homogenized in 350μl of 1× PBS pH 7.4 (PBS Buffer; 1.37M sodium chloride, 27M potassium chloride, 100mM disodium hydrogen phosphate, 18mM potassium dihydrogen phosphate and distilled water) and vortexed for 30 seconds. The homogenate was centrifuged at 12000 rpm for 10 minutes at 4ºC (Eppendorf AG 5417R centrifuge, Hamburg, Germany) and 250μl of the supernatant transferred to a micro centrifuge tube containing 750μl of TRIzol® reagent (Invitrogen, Carlsbad CA, USA). This was mixed thoroughly and left to stand at room temperature for 10 minutes. Then 0.2 v/v Chloroform was added, vortexed vigorously for 15 seconds and incubated at room temperature for 10 minutes. The mixture was centrifuged at 12000 rpm for 10 minutes at 4ºC. Thereafter, 500μl of the upper aqueous phase containing the RNA was aliquoted into a clean 1.5 ml microcentrifuge tube into which 1μl glycogen and 500μl of 100% isopropanol were added. The mixture was precipitated for 2 hours at -80ºC and centrifuged at 12000 rpm for 10 minutes at 4ºC to obtain the RNA pellet. The supernatant was carefully discarded in a container to avoid losing the pellet. The pellet obtained was washed by adding 50μl of 75% ethanol and inverting the tube gently. The sample was centrifuged again at 12000 rpm for 10 minutes at 4ºC and the liquid discarded. Further centrifugation was carried out for 30 seconds and excess liquid removed with a fine tip pipette. The pellet was dissolved in 15μl nuclease free water and left at room temperature for 5 minutes.

3.7. DNAse treatment of total RNA

RNase-free-DNAse 1 (Fermentas, Thermoscientific, UK) was used to digest any contaminating genomic DNA from the extracted RNA. The RNA sample was put in a tube containing final concentrations of 0.5× DNase 1 reaction buffer and 0.125U/all DNAse1. The mixture was incubated at 37ºC for 30 minutes. The treatment was terminated by adding 2.7mM EDTA and incubated at 65ºC for 10 minutes.

3.8. Determination of RNA yield and quality

The yield and quality of total RNA was determined by measuring the absorbance of 2µl of RNA using spectrophotometry (Nanodrop 2000 Spectrophotometer, Thermoscientific, USA). The ratio of optical density at wavelengths of 260nm and 280nm was used to assess the purity of RNA. A ratio between1.8-2.0 denotes that the absorption in the ultraviolet range is due to nucleic acids, one lower than 1.8 indicates the presence of proteins and/or other ultraviolet absorbers while that higher than 2.0 indicates that the samples may be contaminated with chloroform or phenol. RNA yield was measured at a concentration factor of 40ng/µl and the final RNA concentration in mg/ml calculated as follows:

$$\text{RNA concentration (mg/ml)} = \frac{\text{Absorbance at 260*Dilution factor *40}}{1000}$$

3.9. Determination of RNA integrity

Integrity of the RNA extracted was validated using denaturing 1.2% formaldehyde agarose gel electrophoresis (0.6g agarose, 5ml 10× MOPS buffer, 45ml distilled water, 900µl 37% formaldehyde and 3µl ethidium bromide). 3µl RNA was mixed with 5× RNA loading dye, incubated at 70ºC for 5 minutes and chilled on ice for 3 minutes, 5 all of the mixture was then loaded on the gel alongside a DNA ladder and run at 60 volts for 1 hour.

3.10. Complimentary DNA (cDNA) synthesis

The RevertAid First Strand cDNA synthesis kit (Fermentas, Thermoscientific, UK) was used for synthesis of normalised cDNA to final concentration of 20ng/µl. The total PCR volume was 20µl. The first step of the reaction consisting of final concentrations of 20ng/µl RNA, 0.25 µM Oligo$(dT)_{18}$ primer were put in a clean micro centrifuge tube, topped up with nuclease free water to 12 µl, mixed gently, spun down, incubated at 65ºC for 5 minutes (Proflex PCR system, Applied Biosystems, USA) and immediately cooled on ice for 1 minute. Other components constituting of the following final concentrations were added; 1× reaction buffer (250mM Tris-HCl pH 8.3, 250mM KCl, 20mM $MgCl_2$, 50mM Dithiothreitol), 1U/µl Ribolock RNAse inhibitor, 1mM dNTP Mix and 10U/ µl RevertAid M-MULV Reverse Transcriptase. The reaction mixture was spun briefly and incubated at 42ºC for 60 minutes, terminated at 70ºC for 5 minutes and finally chilled on ice. The negative control consisted of all the components minus the enzyme reverse transcriptase.

3.11. Polymerase chain reaction (PCR) amplification of the cDNA

All the 28 Putative *G. brevipalpis* OBP gene primers were screened on the cDNA synthesised. A 10µl reaction containing the final concentrations of the following components was prepared; 1× phusion HF buffer (20mM Tris-HCl, pH 7.8 at 25ºC, 100mM KCl, 7.5mM $MgCl_2$, 1mM Dithiothreitol, stabilizers, 200µg/ml bovine serum albumin, 50% glycerol), 0.2mM dNTPs, 0.25mM of each forward and reverse primers, 0.04U/all phusion polymerase enzyme (Fermentas, Thermoscientific, UK) and 20ng/all cDNA. The mixture was topped up with 5.8µl nuclease free-water. The thermocycling conditions used were as follows; Initial denaturation at 98ºC for 30 seconds, 35 cycles of subsequent denaturation at 98ºC for 15 seconds, annealing at 55ºC for 30 seconds, extension at 72ºC for 30 seconds and a final extension at 72ºC for 5 minutes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Beta-tubulin (β-tubulin)

were used as positive (internal) controls. The reaction was done using a Proflex PCR system (Applied Biosystems).

3.12. Agarose gel electrophoresis

The amplicons were run in a 2% agarose gel stained with ethidium bromide in a 1× TAE buffer (Tris Acetate ethylene diamine tetra acetate). Each well contained 5µl of the amplicon mixed with 0.5 µl of 5× DNA loading dye (Bromophenol blue, xylene cyanol and orange G). The samples were run for 1 hour 20 minutes at 70 volts in a Bio-rad power pack (model 200/2.0). The gel was viewed in a Kodak Gel-Logic 200 transilluminator and photo images taken.

3.13. Recovery of amplified DNA from the gel

DNA bands of interest were cut out using sterile surgical blades and placed in sterile 1.5 micro centrifuge tubes. The DNA was purified using the Quick clean DNA gel Extraction Kit (GenScript Corporation, Piscataway, NJ) using the following procedure; Weight of the cut out gels were obtained, three volumes of binding solution II added into the tubes containing the gels (100 mg=100 µl) and incubated in a water bath at 50ºC for 10 minutes, with occasional vortexing until the solution turned yellow (an indication of complete dissolution of the gels). One volume of isopropanol was added to the solution and vortexed. The mixture was transferred to a quick clean column and centrifuged at 12000 rpm for 30 seconds. The flow through into the collection tube was discarded into a waste container, followed by double wash of the column using 50 µl of washing solution and centrifuged at 12000 rpm for 1 minute. The column with the bound DNA was transferred into a sterile microcentrifuge tube, 20 µl of the elution buffer added carefully onto the bottom of the column and incubated at room temperature for 1 minute. Elution was carried out by centrifuging the tube content at 12000 rpm for 1 minute at 4ºC. Five microliters of the eluted DNA was run on 1% agarose gel stained with ethidium bromide for 1 hour 30 minutes to confirm the recovery of DNA.

3.14. Quantitative real time polymerase chain reaction (qRT-PCR)

The OBP genes that were successfully amplified during screening were further quantified using qRT-PCR. 2× Maxima SYBR Green/ ROX master mix (Fermentas, Thermoscientific, UK) was used for quantification following the manufacturer's instructions with a Stratagene Mx3000P qPCR system (Agilent Technologies Ltd, Cheshire, UK). Each reaction amounted to 10µl volume and consisted of the following final concentrations; 1× Maxima SYBR Green/ROX master mix, 2ng/ µl of cDNA template and 0.3µM of forward and reverse primers. The reaction mixture was topped up using nuclease free water, mixed gently without creating bubbles, centrifuged briefly and placed in the thermo cycler. The following thermocycling conditions were used; one cycle of initial denaturation at 95ºC for 10 minutes, 40 cycles of; 95ºC for 15 seconds, 60 ºC for 30 seconds and 72ºC for 30 seconds. Data acquisition was performed during the extension step. Dissociation curve analysis was done at the end of the PCR to confirm specific amplification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for initial normalization of the template cDNA. Tests were replicated three times.

3.14 Quantitative real time polymerase chain reaction (qRT-PCR) data analysis

The fold expression of the five *G. brevipalpis* relative to the normalizer (reference) gene was calculated by ddCt (Livak's) method. Discrepancy between the Ct for the OBPs (dCt) and GAPDH (dCt) were first calculated to normalize the variation in the amount of cDNA in each reaction (Livak and Schmittgen, 2001). Descriptive statistics (means and plots of averages) were done in R package. Prior to Analysis of variance (ANOVA), data were log transformed using the formula log10 (ddct+0.001) and multiple comparison was performed using Tukey's test where $p<0.05$ was considered significant.

CHAPTER FOUR

RESEARCH RESULTS

4.1. The putative *G. brevipalpis* OBPs

An online query of 32 *G. m. morsitans* OBPs searched against the *G. brevipalpis* proteome in VectorBase resulted to 28 *G. brevipalpis* OBPs. The OBPs were assigned names similar to those of *G. m. morsitans* by attaching a prefix Gbr (for *G. brevipalpis*) to the numerical numbers such as GbrOBP1. *G. brevipalpis* OBPs varied in size with GbrOBP6 having the lowest number of amino acids (100 aa) while GbrOBP20 was the longest (263 aa) (Table 4.1). Twenty two *G. brevipalpis* OBPs had signal peptide at the N-terminal end while six (GbrOBP5, GbrOBP15, GbrOBP16, GbrOBP22, GbrOBP26 and OBP27) did not have the signal peptide sequence. GbrOBP2, GbrOBP4 and GbrOBP11 had the smallest signal peptide (17 aa) while GbrOBP10 had the longest signal peptide (30 aa) (Table 4.1). GmmOBP1 had the highest similarity to GbrOBP1 (98.6% identity, e-value = 1e-97) while GmmOBP17 had the lowest similarity to GbrOBP17 (45% identity, e-value = 3.00e-41). Twenty six *G. brevipalpis* OBPs showed orthologous hits to *D. melanogaster* while (GbrOBP18 and GbrOBP25) did not have any hit. GbrOBP9 had the highest similarity to DmelOBP83a (73.3% identity, e-value= 5.4e-55) while GbrOBP16 had the lowest similarity to DmelOBP57 (25.3% identity, e-value=0.310438).

Multiple sequence alignment of the 28 putative *G. brevipalpis* OBPs using MUSCLE revealed the presence of six conserved cysteines in 19 OBPs (GbrOBP1, GbrOBP2, GbrOBP4, GbrOBP5, GbrOBP11, GbrOBP14, GbrOBP17, GbrOBP22, GbrOBP23, GbrOBP25, GbrOBP28, GbrOBP8, GbrOBP12, GbrOBP13, GbrOBP15, GbrOBP19, GbrOBP21, GbrOBP24, GbrOBP10), 12 cysteine residues in two OBPs (GbrOBP3 and GbrOBP7), less than 6 cysteine residues in four OBPs (GbrOBP6, GbrOBP16,

GbrOBP18 and GbrOBP26) and more than 6 cysteines in two OBPs (GbrOBP9 and GbrOBP20) (Figure 4.1).

G. m. morsitans		G. breviplalpis					D. melanogaster			
OBP	Accession No.	OBP accession and ID	Length (aa)	% identity	e-value	SignalP	OBP	Accession No.	% identity	e-value
GmmOBP1	GMOY000890	GBRI026688(GbrOBP1)	142	98.6	1.00E-97	1-18	DmelOBP44a	NP_001286186	62.2	3.80E-47
GmmOBP2	GMOY002825	GBRI012898(GbrOBP2)	149	77.80	1.00E-79	1-17	DmelOBP99b	NP_001263078	52.4	3.80E-39
GmmOBP3	GMOY005549	GBRI031705(GbrOBP3)	254	91.8	1.00E-174	1-20	DmelOBP83ef	NP_731042	41.2	1.28E-51
GmmOBP4	GMOY007757	GBRI016436(GbrOBP4)	152	62.9	4.00E-67	1-17	DmelOBP56a	NP_611442	34	8.86E-10
GmmOBP5	GMOY006521	GBRI035549(GbrOBP5)	129	82.9	3.00E-73	No	DmelOBP19c	NP_728340	32	3.44E-19
GmmOBP6	GMOY009708	GBRI045128(GbrOBP6)	100	69	2.00E-34	1-28	DmelOBP28a	NP_523505	41	9.33E-10
GmmOBP7	GMOY005548	GBRI031703(GbrOBP7)	239	84.2	1.00E-152	1-19	DmelOBP83cd	NP_649612	41.4	2.49E-05
GmmOBP8	GMOY004317	GBRI031755(GbrOBP8)	158	81.1	2.00E-91	1-23	DmelOBP83a	NP_001287190	49	1.53E-34
GmmOBP9	GMOY005184	GBRI031753(GbrOBP9)	151	93.1	3.00E-99	1-26	DmelOBP83b	NP_524242	73.3	5.48E-55
GmmOBP10	GMOY004316	GBRI031754(GbrOBP10)	154	74.6	1.00E-73	1-30	DmelOBP83a	NP_001287190	60.8	2.16E-50
GmmOBP11	GMOY005550	GBRI031704(GbrOBP11)	140	82.1	2.00E-89	1-17	DmelOBP83g	NP_731043	60.3	2.07E-50
GmmOBP12	GMOY004316	GBRI031756(GbrOBP12)	144	88.7	2.00E-90	1-23	DmelOBP83a	NP_001287190	61.8	6.27E-46
GmmOBP13	GMOY002859	GBRI040269(GbrOBP13)	134	79.1	9.00E-75	1-20	DmelOBP56h	NP_001188979	37.9	2.52E-21
GmmOBP14	GMOY006523	GBRI035551(GbrOBP14)	149	83.9	4.00E-91	1-25	DmelOBP19a	NP_728338	63.2	2.08E-43
GmmOBP15	GMOY012229	GBRI016471(GbrOBP15)	175	51.4	5.00E-37	No	DmelOBP56d	NP_001286620	28.3	7.17E-08
GmmOBP16	GMOY005163	GBRI041963(GbrOBP16)	111	65.6	8.00E-24	No	DmelOBP57c	NP_611481	25.3	0.310438
GmmOBP17	GMOY007314	GBRI036202(GbrOBP17)	181	45	3.00E-41	1-19	DmelOBP56i	NP_725929	26.1	0.0069539
GmmOBP18	GMOY003978	GBRI019127(GbrOBP18)	109	48.2	7.00E-28	1-20	-	-	-	-
GmmOBP20	GMOY006417	GBRI012886(GbrOBP20)	263	73	8.00E-140	1-22	DmelOBP99a	NP_001287586	42.3	1.26731
GmmOBP21	GMOY006418	GBRI012882(GbrOBP21)	164	87.1	5.00E-91	1-28	DmelOBP99c	NP_651711	56.8	1.27E-46
GmmOBP22	GMOY001476	GBRI009351(GbrOBP22)	167	94.9	4.00E-96	No	DmelOBP8a	NP_727322	32.3	1.09E-12
GmmOBP23	GMOY000657	GBRI023685(GbrOBP23)	176	80.2	1.00E-69	1-19	DmelOBP84a	NP_476990	46.8	8.80E-37
GmmOBP24	GMOY005400	GBRI010734(GbrOBP24)	148	91.7	4.00E-93	1-23	DmelOBP19d	NP_788940	45.5	9.62E-24
GmmOBP25	GMOY005876	GBRI010929(GbrOBP25)	253	70.9	4.00E-68	1-21	-	-	-	-
GmmOBP26	GMOY005931	GBRI030526(GbrOBP26)	129	58.8	9.00E-43	No	Dmellush	NP_001163468	37.6	1.46E-21
GmmOBP27	GMOY007293	GBRI008934(GbrOBP27)	108	81.4	4.00E-43	No	DmelOBP73a	NP_001097628	53.8	9.44E-10
GmmOBP28	GMOY006522	GBRI035552(GbrOBP28)	156	89.1	2.00E-101	1-23	DmelOBP19b	NP_608391	40.4	1.38E-31

Table 4.1 G. m. morsitans OBPs best hit matches to G. brevipalpis and D. melanogaster proteome databases.

The complete lengths of *G. brevipalpis* OBPs together with their signal peptides in amino acids (aa) is shown. Percentage identities (% identity) and expect values (e-value) of the orthologous genes of *G. m. morsitans* OBPs to *G. brevipalpis* and *G. brevipalpis D. melanogaster* to indicated. **GmmOBP***, **GbrOBPx** and **DmelOBPx** indicate the gene names used for *G. m. morsitans*, *G. brevipalpis* and *D. melanogaster* odorant binding proteins respectively, the x is the gene identifier number. **aa**; amino acids.

Figure 4.1. Multiple sequence alignment (MSA) of putative *G. brevipalpis* OBPs

Multiple sequence alignment of *G. brevipalpis* OBPs was done in MEGA using (MUSCLE) and visualized in Jalview. GbrOBPX indicate the number allocated to an OBP. The cysteines are highlighted in black. The length of the sequences in amino acids is shown at the top. The conserved regions and the quality of the alignments is represented by the bars below the sequences.

Phylogenetic construction involving a total of 77 OBP amino acid sequences (27 from *G. brevipalpis*, 27 from *G. m. morsitans* and 23 from *D. melanogaster*) were analysed. Two *G. brevipalpis* OBPs (GbrOBP20 and GbrOBP22) had the highest bootstrap value of 99. while one (GbrOBP2) had the least bootstrap value of 67 to that of *G. m. morsitans*.

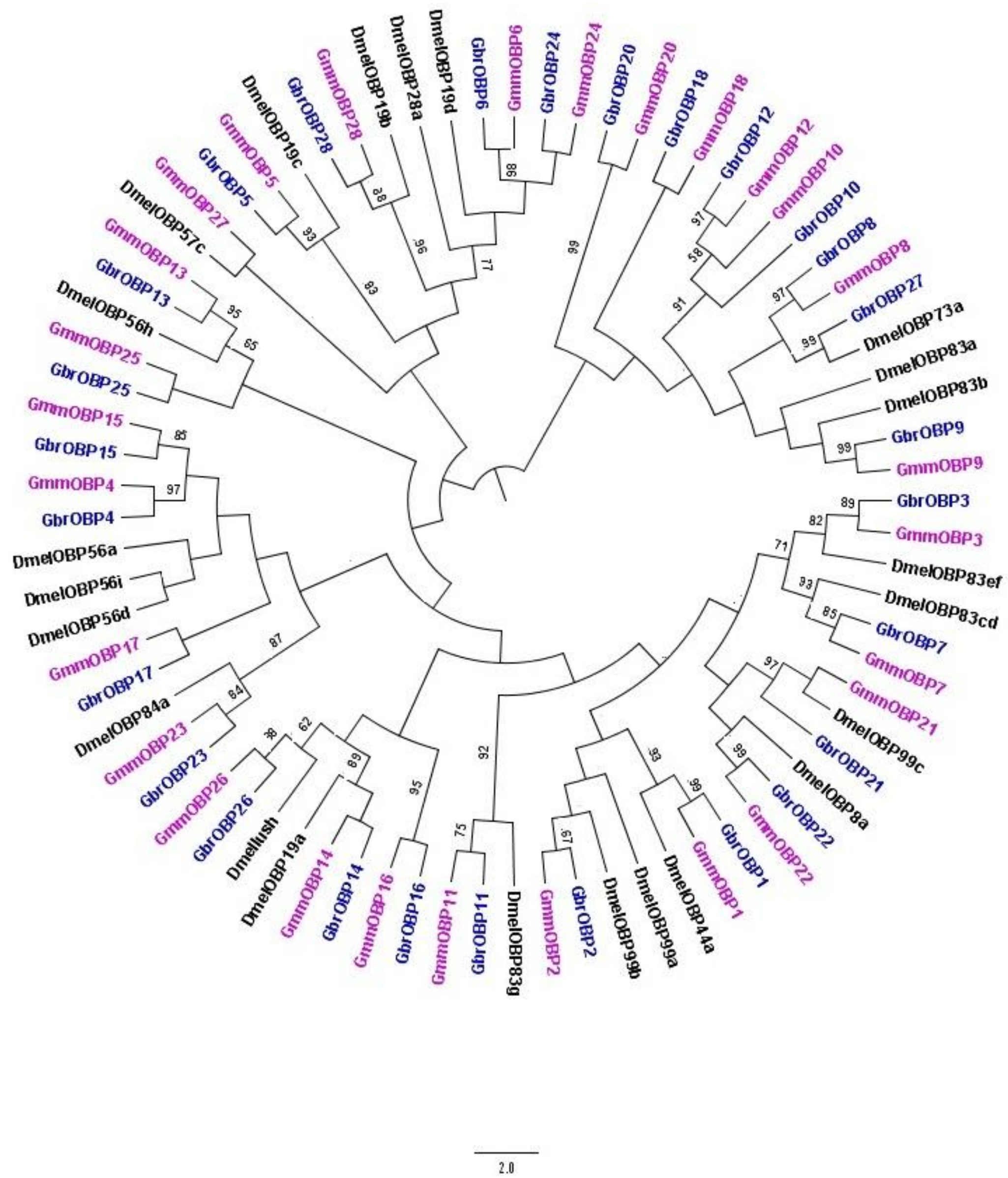

Figure 4.2. Phylogeny of *G. brevipalpis, G. m. morsitans* and *D. melanogaster* OBPs.

The phylogenetic tree above shows the relationship between *G. brevipalpis* (Blue), *G. m. morsitans* (Purple) and *D. melanogaster* (Black) OBPs. The tree was constructed in MEGA6 with 1000 iterations (bootstraps)

4.2 Spectrophotometry of extracted RNA

RNA extracted from two biological replicates of each sample group (larva, pupa and antennas enriched with heads from; tenerals, 2h pbm, 24h pbm, 48h pbm, 72h pbm and 96h pbm) were quantified to obtain their concentrations (nag/all) and quality (Absorbance (A) at 260 nm/280 nm) (Table 4.2). It was observed that the amounts of RNA obtained from larva and pupa were higher than those of the tenerals and differentially starved adult flies. The highest RNA concentration was from the larva (11535 nag/all) while the lowest was from 48h pbm group (185ng/all). The A260/A280 ratio across the sample groups was within the range of 1.7-2.08 (Table 4.2).

Table 4.2 Nanodrop readings of RNA extracted from G. brevipalpis samples

Sample name	Concentration	Absorbance
Larva	11535	2.08
Larva	3547	2.06
Pupa	2549	2.01
Pupa	4231	1.98
Tenerals	378	2.0
Tenerals	617	1.65
Adults (2h pbm)	510.6	1.7
Adults (2h pbm)	328	1.79
Adults (24h pbm)	346.5	1.69
Adults (24h pbm)	205	1.73
Adults (48h pbm)	402.8	1.91
Adults (48h pbm)	185	1.99
Adults (72h pbm)	213.3	1.92
Adults (72h pbm)	193.1	1.95
Adults (96h pbm)	220	1.84
Adults (96h pbm)	278.2	1.95

RNA concentrations (nag/all) and quality (absorbance at wavelength 260nm/280nm) in the two biological replicates of all the sample groups (larva, pupa, tenerals and adults starved at 2h pbm, 24h pbm, 48h pbm, 72h pbm and 96h pbm). **h:** hour, **pbm:** post blood meal, the starvation period is indicated before the hour.

4.3 Analysis of RNA integrity using gel electrophoresis

The integrity of RNA was tested by running the samples on 1.2% denaturing formaldehyde agarose gel electrophoresis. In all the sample groups (larva, pupa, tenerals, 2h pbm, 24h pbm, 48h pbm, 72h pbm and 96h pbm), single bands of about 1.8 kb in size were observed (Figures 4.3a and Figure 4.3b). This indicates that the RNA obtained was of good quality and could be used for downstream processes.

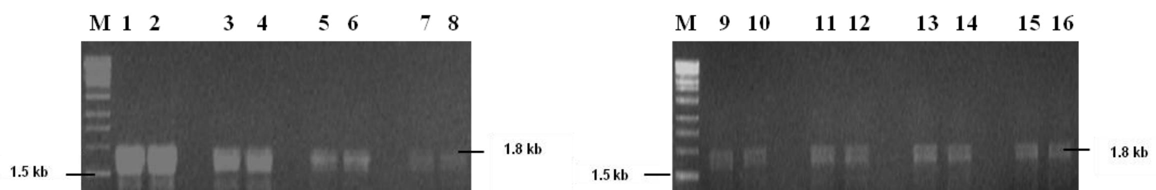

Figure 4.3a Figure 4.3b

Figure 4.3a. and 4.3b Formaldehyde agarose gel images of G. brevipalpis RNA.

Gel images of 18s rRNA from the different sample groups. The band size in all the cases was about 1.8kb. **M**: DNA marker 1kb, **Lane 1 and 2**: Larva, **Lane 3 and 4:** Pupa, **Lane 5 and 6:** 72h pbm, **Lane 7 and 8:**96h pbm, **Lane 9 and 10:** Tenerals, **Lane 11 and 12:** 2h pbm, **Lane 13 and 14:**24h pbm, **Lane 15 and 16:**48h pbm

4.4 Screening of *G. brevipalpis* OBP genes in the differentially starved flies

The 28 putative *G. brevipalpis* OBP genes were screened by amplification in six sample groups (tenerals and differentially starved adults at (2h pbm, 24h pbm, 48h pbm, 72h pbm and 96h pbm)) using conventional PCR. The positive controls GAPDH and β-tubulin were amplified in all the test samples while the negative control (without the reverse transcriptase minus enzyme) did not show any amplification. Six OBPs (GbrOBP2, GbrOBP6, GbrOBP8, GbrOBP10, GbrOBP7 and GbrOBP13) were detected in the tenerals and 48h pbm, five OBPs (GbrOBP2, GbrOBP6, GbrOBP8, GbrOBP7 and GbrOBP13) were amplified in 2h pbm and 24h pbm while four OBPs (GbrOBP2, GbrOBP6, GbrOBP8 and GbrOBP13) were present in 72h and 96h pbm. The band sizes

of GbrOBP2, GbrOBP6, GbrOBP8, GbrOBP10, GbrOBP7 and OBP13 observed in the 2% agarose gel electrophoresis were 159bps, 195bps, 200bps, 200bps, 200bps and 200bps respectively (Figure 4.4)

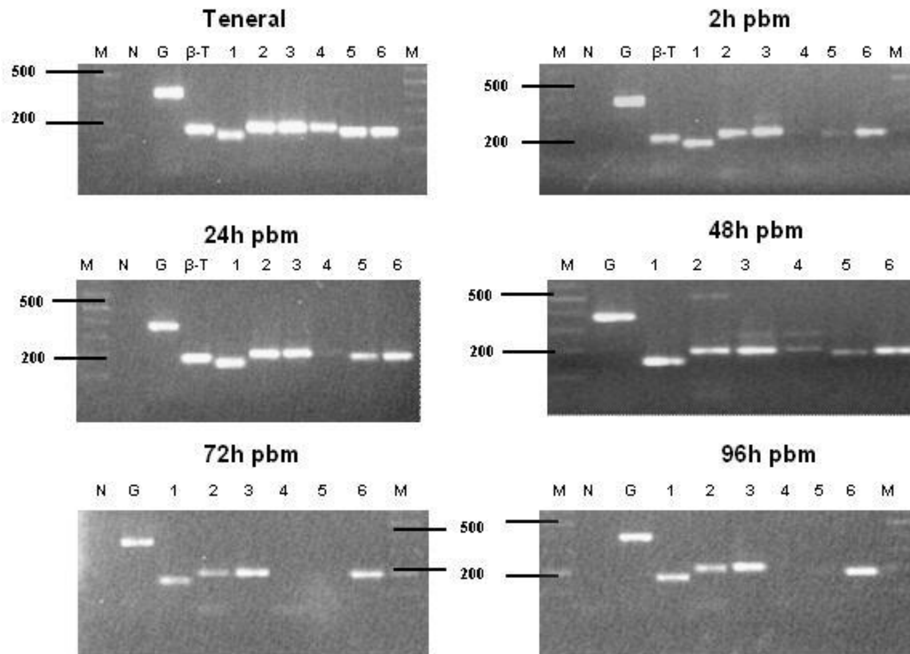

Figure 4.4. 2% Agarose gel electrophoresis of the amplified *G. brevipalpis* OBPs in the tenerals and differentially starved adults

Gel images of *G. brevipalpis* OBP genes that were amplified using conventional PCR in the tenerals and the differentially starved flies (2h pbm, 24h pbm, 48h pbm, 72h pbm and 96h pbm). **M:** 100bp molecular marker (New England Biolabs), **N:** Negative control, **G:** GAPDH, **β-T:** β-Tubulin, **Lane 1:** GbrOBP2, **Lane 2:** GbrOBP6, **Lane3:** GbrOBP8, **Lane4:** OBPGbr10, **Lane 5:** GbrOBP7, **Lane 6:** GbrOBP13**.** GAPDH(positive) amplified in all the groups. All the six OBPs (GbrOBP2, GbrOBP6, GbrOBP8, GbrOBP10, GbrOBP7 and GbrOBP13) amplified in tenerals and 48h pbm samples, five OBPs (GbrOBP2, GbrOBP6, GbrOBP8, GbrOBP7 and GbrOBP13) amplified in the 2h and 24h pbm samples while four OBPs (GbrOBP2, GbrOBP6, GbrOBP8 and GbrOBP13) amplified in the 72h and 96h pbm groups.

4.5 Screening of *G. brevipalpis* OBP genes in the developmental stages

All the 28 putative *G. brevipalpis* OBPs were screened in the developmental groups (Larva, Pupa and tenerals). The positive control genes (GAPDH and β-tubulin) were amplified in all the samples whereas GAPDH did not amplify in the negative control sample (reverse transcriptase minus). Four OBPs (GbrOBP2, GbrOBP7, GbrOBP10 and GbrOBP13) were amplified in larva and pupa while six OBPs (GbrOBP2, GbrOBP6, GbrOBP8, GbrOBP10, GbrOBP7 and GbrOBP13) were detected in the tenerals. The band sizes of GbrOBP2, GbrOBP6, GbrOBP8, GbrOBP10, GbrOBP7 and OBP13 observed in the 2% agarose gel electrophoresis were 159 bps, 195 bps, 200 bps, 200 bps, 200 bps and 200 bps respectively (Figure 4.5)

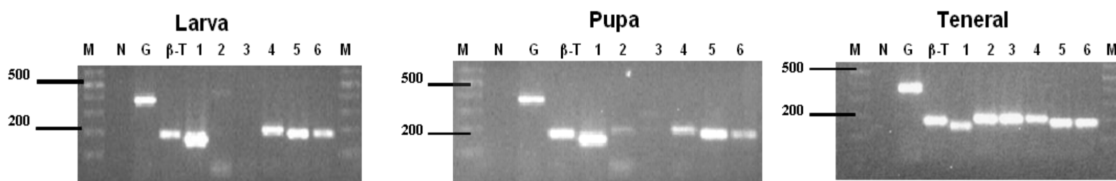

Figure 4.5. 2% Agarose gel images of *G. brevipalpis* OBPs that amplified in the developmental stages

The figure above shows band fragments of six *G. brevipalpis* OBP genes that amplified in the conventional PCR across the different developmental stages (larva, pupa and tenerals). **M:** 100bp molecular marker (New England Biolabs), **N:** Negative control, **G:** GAPDH, **β-T:** β-Tubulin, **Lane 1:** GbrOBP2, **Lane 2:** GbrOBP6, **Lane3:** GbrOBP8, **Lane4:** GbrOBP10, **Lane 5:** GbrOBP7, **Lane 6:** GbrOBP13. The positive controls (GAPDH and β-tubulin) were amplified in all the three groups. Four GbrOBPs (GbrOBP2, GbrOBP7, GbrOBP10 and GbrOBP13) were amplified in larva and pupa while six GbrOBPs (GbrOBP2, GbrOBP6, GbrOBP8, GbrOBP10, GbrOBP7 and GbrOBP13) were detected in the tenerals.

4.6. *Glossina brevipalpis* OBPs extracted in the teneral (control) group

The six *G. brevipalpis* OBPs that amplified in the tenerals were extracted and re-run on 2% agarose gel electrophoresis to verify their presence before sequencing.

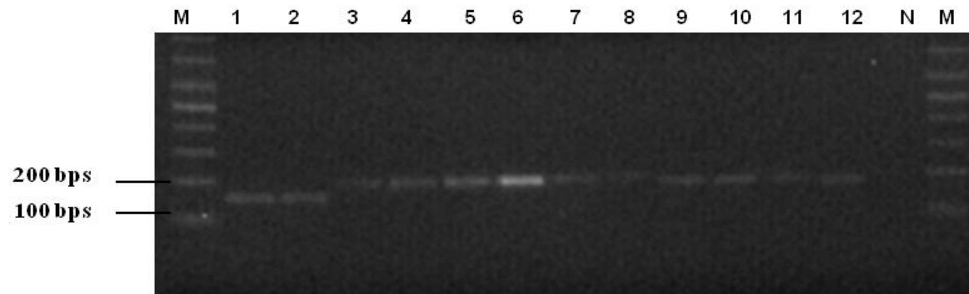

Figure 4.6. 2% Agarose gel image of *G. brevipalpis* OBPs in the tenerals

The figure above shows 2% agarose gel electrophoresis of the *G. brevipalpis* OBPs after their purification using the Genescript Quick clean DNA gel Extraction Kit. **M:** 100bp molecular marker (New England Biolabs), **Lane 1 and 2:** GbrOBP2, **Lane 3 and 4:** GbrOBP6, **Lane 5 and 6:** GbrOBP8, **Lane 7 and 8:** GbrOBP10, **Lane 9 and 10:** GbrOBP7, **Lane 11 and 12:**GbrOBP13, **N:** Negative control

4.7. Amplification and dissociation of *G. brevipalpis* OBPs

The five *G. brevipalpis* OBPs that reliably amplified in conventional PCR were quantified in qRT-PCR. The Ct values from the amplification and the melting temperatures from the dissociation curves for each gene were obtained. For instance, amplification and dissociation profiles of GbrOBP2 gene across all the samples (larvae, pupae, tenerals, 2h pbm, 24h pbm, 48h pbm, 72h pbm and 96h pbm) are shown in Figures 4.7a and 4.7b respectively.

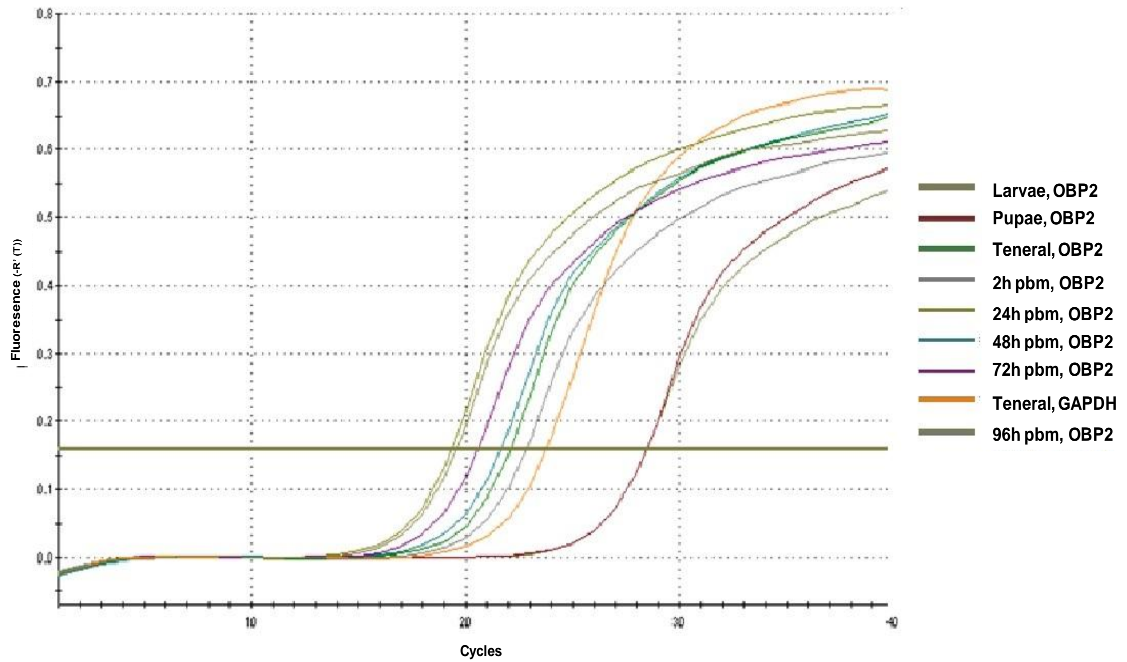

Figure 4.7a. Amplification curve of *G. brevipalpis* OBP2 and GAPDH

The figure above shows amplification curves of GbrOBP2 and GAPDH in all the sample groups. The different coloured plots indicate the different groups (Larva, pupa, tenerals, 2h pbm, 24h pbm, 48h pbm, 72h pbm and 86h pbm). The threshold line cuts through the lag phase of the curve and determines the Ct value at which the dye fluoresces.

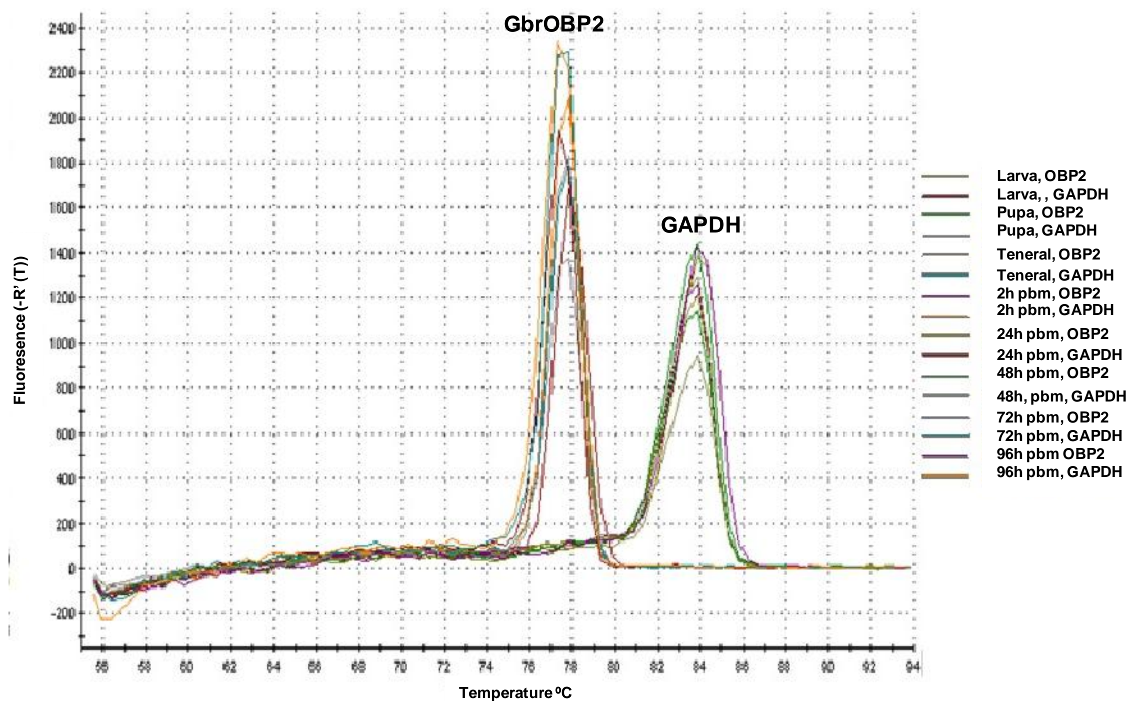

Figure 4.7b. Dissociation curve of *G. brevipalpis* OBP2 and GAPDH

The figure above shows a dissociation curve resulting from change in fluorescence against temperature. The single peaks observed for GbrOBP2 and GAPDH at 77 ºC and 84 ºC respectively are an indication of specific amplification.

4.8. Expression profiles of *G. brevipalpis* OBP genes in differentially starved flies

GbrOBP8 was the most highly expressed than the other four OBPs. Expression patterns of the five putative *G. brevipalpis* OBP genes in the differentially starved flies were varied. The expression of GbrOBP8 was up regulated at 24h pbm, down regulated at 48h pbm and rose gradually up to the 96h pbm. Two OBPs, GbrOBP6 and GbrOBP13 showed some expression while another two, GbrOBP2 and GbrOBP7 had minimal expression (Figure 4.8).

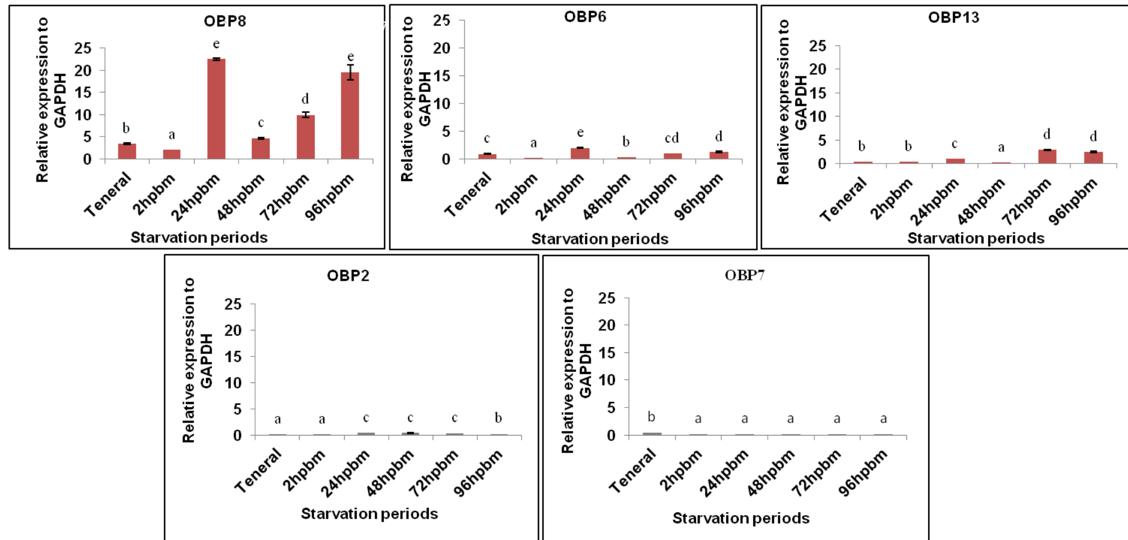

Figure 4.8. Relative expression profiles of *G. brevipalpis* OBPs in the differentially starved adults

Expression of different *G. brevipalpis* OBP genes in tenerals and differentially starved adult flies at 2h, 24h, 48h, 72h and 96h pbm. **h:** hour, **pbm:** post blood meal

4.9. Quantitative expression of *G. brevipalpis* OBPs in the developmental stages

Analysis of the five putative *G. brevipalpis* OBP genes across the developmental stages using qRT-PCR revealed high expression of GbrOBP2 in larva and slight expression in the pupa. GbrOBP7 was significantly expressed in the pupa followed by that in the tenerals and least expressed in the larva. Three other OBPs (GbrOBP6, GbrOBP8 and GbrOBP13) had minimal expression in the larval and pupal stages and were less than those of the teneral stage (Figure 4.9).

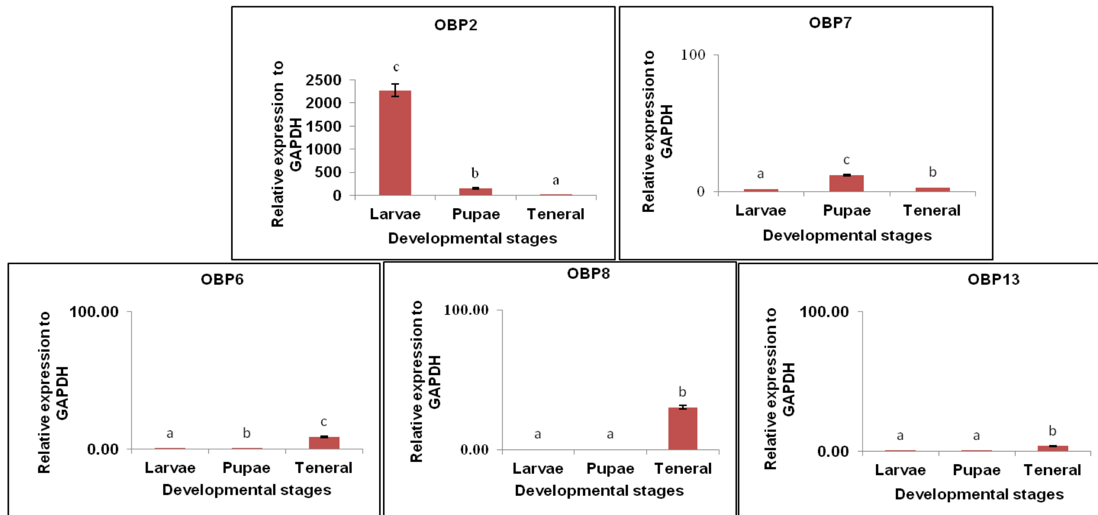

Figure 4.9. Relative expression profiles of *G. brevipalpis* OBPs in the developmental stages.

Expression of five *G. brevipalpis* OBP genes (OBP2, OBP7, OBP6, OBP8 and OBP13) in the developmental stages of the tsetse flies (Larva, Pupa and Tenerals)

CHAPTER FIVE

DISCUSSION

Most of the putative *G. brevipalpis* OBPs had size range between 120 and150 bps which agrees with the size of classical OBPs that have been observed in other insects. However other putative *G. brevipalpis* OBPs were out of range of the classical OBPs and may belong to other classes of OBPs namely minus-C, plus-C or dimers (Fan *et al*., 2011). Presence of signal peptide observed at the N-terminal of OBPs confirms that the OBPs are secretory, a characteristic associated with insect's OBPs (Koganezawa and Shimada, 2002). The *G. brevipalpis* OBPs that lacked signal peptide may be non-secretory and their role needs to be investigated further.

The 28 putative *G. brevipalpis* OBPs were found to be homologous to *G. m. morsitans* OBPs with sequence similarity ranging from 45% to 98.6% while that between the *G. brevipalpis* and *D. melanogaster* was 25.3% to 73.3%. This percentage range varies with the sequence similarities previously reported for OBPs in other insect species. For instance, the percentage sequence similarity between the fruit fly (*Bactrocera dorsalis*) and vinegar fly (*Drosphila melanogaster)* OBPs was found to range from 33% to 63% (Zheng *et al*., 2013a) while that between *G. m. morsitans* and *D. melanogaster* OBPs was between 28% to 82% (Liu *et al*., 2010). The different ranges in amino acid percentage identities between insect OBPs confirms the OBPs divergence among same and different species (Pelosi *et al*., 2005). The two *G. brevipalpis* OBPs (GbrOBP18 and GbrOBP25) that lacked a hit in *Drosophila* may imply that the two genes are specific to the *Glossina* genus.

Multiple sequence alignment revealed that some *G. brevipalpis* OBPs had the conserved six cysteines. This is an important characteristic for OBPs as it enables formation of disulphide bridges during the protein structure formation (Pelosi *et al*., 2005). The

presence of different numbers of conserved cysteines pinpoint the fact that just as in other insects, putative *G. brevipalpis* OBPs belong to different OBP subfamilies (Pelosi *et al*., 2005).

In the phylogenetic analysis, the *G. brevipalpis* OBPs clustered closely with the *G. m. morsitans* OBPs while the *D. melanogaster* OBPs clustered on their own. This may have resulted from their evolutionary history 260 million years ago. First, the Dipterans diverged into Brachycera (Glossina and Drosophila) and Nematocera (Mosquitoes) and later the Calyptratae further branched into Acalyptratae (Glossina) and the Acalyptratae (Drosophila) (Liu *et al*., 2010). The branching points of *D. melanogaster* also were observed to be deeper than those of the Glossina and this may suggest that the Drosophila OBPs evolved earlier.

The total RNA spectrophotometry optical density values ranging between 1.69- 2.08 in the test sample groups was an indication of high purity and confirmed that the RNA was not contaminated with either protein residues or phenol-chloroform. When run on a denaturing 1.2% formaldehyde agarose gel electrophoresis the tsetse RNA forms a single band as opposed to two bands as seen in most eukaryotic RNA. The single band results from an endogeneous "hidden break" in the 28S rRNA during heat denaturation. When heated, the 28S rRNA is split into two fragments that run along the 18S rRNA (1800 bps). Single RNA bands have also been observed in insects such as the honey bee *Apis mellifera* (Winnebeck, Millar, & Warman, 2009) and onion thrips *Thrips tabaci* (Macharia *et al*., 2015). The observed single 18S rRNA band in all the samples confirms the integrity of the mRNA and its suitability to be used in the downstream expression studies (Winnebeck *et al*., 2009).

Expression profiling of five *G. brevipalpis* OBPs (OBP2, OBP6, OBP7, OBP8 and OBP13) showed that they all had varied expression in the antennas of the newly emerged flies (tenerals) and the differentially starved flies. This suggests that the *G. brevipalpis* OBPs may play an important role in tsetse olfaction. Functional studies of various OBPs

have confirmed that OBPs are indeed important in olfaction. For instance, the *Drosophila* LUSH mutants were observed to be insensitive to the pheromone 11-cis-vaccenyl acetate (cVA) and defective for avoidance of concentrated alcohols (Xu *et al*., 2005) while the male silkworm *Bombyx mori* OBP1 has been reported to be sensitive to the pheromone bombykol produced by the females (Grosse-Wilde *et al*., 2011). Altered expression of DmelOBP83a gene in *Drosophila* females showed interference in response to citral (Swarup *et al*., 2011) and detection of volatile pheromones (Siciliano *et al*., 2014).

Notably, high expression of GbrOBP8 among the different treatments in *G. brevipalpis* could suggest that the OBP is critically involved in tsetse olfaction. This finding is consistent with that in *G. m. morsitans* where the orthologous gene GmmOBP8 was also observed to be highly expressed (Liu *et al*., 2010). However, high expression of GbrOBP8 at 24h pbm in *G. brevipalpis* contrasts the findings in *G. m. morsitans* whereby GmmOBP8 was down-regulated at 24h pbm.

High expression of GbrOBP6 at 24h pbm was in agreement with the findings of the orthologous gene (GmmOBP6) in *G. m. morsitans* (Liu *et al*., 2010). The *Drosophila* orthologue DmelOBP28a was however observed to be higher in the virgin female flies than in the mated females suggesting a probable role in reproduction (Zhou *et al*., 2009). Functional studies in *Drosophila* suggested the involvement of DmelOBP28a in regulating feeding and transporting bitter tastants to odorant receptors (ORs). Disruption of OBP28a gene resulted to altered behavioral responses towards 2-ethyl pyrazine and citral and led to increased consumption of bitter tastants such as quinine (Swarup *et al*., 2011).

Low expression of GbrOBP2 agreed with the findings of Liu and colleagues who reported that GmmOBP2 and GmmOBP10 were lowly expressed. GbrOBP2 orthologous gene in Drosophila, DmelOBP99b was observed to have increased transcriptomic profile post mating (Zhou *et al*., 2009). GbrOBP7 was also among the least expressed and its expression in the differentially starved flies did not vary. These observations may suggest

that it may be involved in other non-olfactory processes. GbrOBP13 was highly expressed in the 72h and 96h pbm periods. Functional studies of its orthologous gene in the Drosophila (DmelOBP56h) suggested its involvement in clearing bitter tastants and hence increases consumption of bitter substances by the flies (Swarup *et al*., 2014). The tsetse unlike the Drosophila feeds exclusively on blood therefore it might be difficult to relate this finding in tsetse to that of the Drosophila.

Chemical sensing influences many processes in both adults and immature stages of insects. Olfaction in adult insects is enabled by well structured antennas containing numerous sensilla, which are relatively less well developed in larvae (Jin *et al*., 2015). Notably expression analysis of insect OBPs such as in the Lepidopterans (Moths; *Helicoverpa amigera* and *H. assulta* (Zhang *et al*., 2015)) and Dipterans (Fruit fly; *Batrocera dorsalis* (Zheng *et al*., 2013b) and adult mosquito; *Culex pipiens cuinquefasciantus* (Peiletier & Leal, 2009)) have focused on the adults. However, understanding chemical sensing in juvenile stages of insect development (larvae and pupae) is under-represented. Tsetse flies unlike most other insects are unique as they have adenotropic viviparity (larvae develops *in utero*) whereby the larva obtains its food from the mother's milk glands. On the other hand, the pupal stage, which develops within minutes of larvipositing, is mainly dormant and is nourished by the fat body (International Glossina Genome Initiative, 2014). Therefore, expression studies on OBPs in the tsetse fly larvae and pupae are important as they provide information on chemical sensing during initial development stages.

The significant expression of GbrOBP2 and GbrOBP7 genes in larval and pupal stages may suggest their involvement in development. My results are consistent with the differential OBP gene expression reported in the oriental latrine fly, *Chrysomya megacephala.* In *C. megacephala,* seven OBPs were expressed in larvae among them Cmeg33593_c0, an orthologue of DmelOBP99b and GbrOBP2 was the most abundant (Wang *et al*., 2015). In other studies on the wheat blossom midge *Sitodiplossis*

mosellana, high expression of SmosOBP1and SmosOBP10 in larvae suggested their involvement in larval specific chemical sensing (Gong *et al*., 2014).

In a recent study with *G. m. morsitans,* GmmOBP7 (an orthologue of GbrOBP7) was observed to be highly expressed in the larvae. In this study however, I observed GbrOBP7 to be highly expressed in the pupae. Both observations are consistent with the suggestion of Liu *et al*.,2010 that OBP7 in tsetse is likely to be involved in the development of the fly (Liu *et al*., 2010)Higher expression of OBP1 in *Anopheles stephensi* (AsteOBP1) at the pupal stage was attributed to the physiological development of the chemosensory tissue (Sengul and Tu, 2010). In yet another study with the honey bee *Apis mellifera*, two OBPs, OBP13 and OBP10 were expressed throughout the pupal stages and were suggested to be involved in development as the fly prepares to emerge (Sylvain and Maleszka, 2006).

CHAPTER SIX

CONCLUSIONS & RECOMMNDATIONS

6.1 Conclusions

From this study I concluded that:

1) Expression of OBP genes in the *G. brevipalpis* antennae and head could be associated with olfaction which facilitates host searcing, mating and larvipositioning site identification
2) The two *G. brevipalpis* OBPs (OBP2 and OBP7) that were significantly expressed in the juvenile stages of tsetse may probably be involved in non-olfactory functions and could play a role in metamorphosis and development of tsetse flies

6.2 Recommendations and Scope for further work

1) Expression profiles of *G. brevipalpis* OBPs in both males and females and in the different tissues be studied
2) Expression analysis of *G. brevipalpis* OBPs be studied using flies reared under controlled conditions to eliminate the environmental factors such as age and physiological status that might have influenced the outcome of the experiments
3) The structure and function of the GbrOBP8 that was highly expressed in the tenerals and differentially starved flies as well as GbrOBP2 and GbrOBP7 that were highly expressed in the juveniles should be investigated

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