# TRANSGENIC EXPRESSION OF THE TSETSE FLY *GLOSSINA FUSCIPES FUSCIPES* OLFACTORY RECEPTOR, Or67d, IN *DROSOPHILA MELANOGASTER*

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**DECLARATION**

I declare that this research thesis is entirely my own work and has not been submitted for a degree in any other University.

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## DEDICATION

To my wonderful mother, Agathe Nyembwa and my cheerful siblings (Laetitia, Samira, Cynthia, David and Daniel).

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**ABSTRACT**

Tsetse flies are the sole vectors of Human African trypanosomiasis (sleeping sickness) and Animal African trypanosomiasis (Nagana). The insect is attracted to its suitable hosts through external signals which are perceived by olfactory receptors (ORs); thus representing the basis of transmission of the disease to thousands of people and millions of livestock. A developing approach to efficiently identify the key chemical ligands of odorant receptors entails expressing single ORs in different cell systems for consequent screening analysis. This study aimed to establish the expression of an expanded olfactory receptor family, Or67d of *Glossina fuscipes fuscipes*, a vector of both animal and human trypanosomiasis, in a *Drosophila* system. The receptor homologue is known to mediate responses to *Drosophila melanogaster* male-specific pheromone 11-cis-vaccenyl acetate (cVA) regulating mating behavior of males and females. In *G. f. fuscipes*, five copies of the same gene were found to be homologous to Or67d of *Drosophila melanogaster*. Out of the five copies, four were typically complete and only three of them contained the conserved seven-transmembrane-helix 6 (7tm_6) odorant receptor domain. Phylogenetic reconstruction of the four gene copies suggested a closest relationship between GffOr67d4 and *Drosophila homolog*, DmelOr67d. This gene copy was synthesized in pUC57 vector, amplified by polymerase chain reaction, cloned in pENTR™/D-TOPO® vector then sub-cloned into the destination vector pTW. Sequencing analysis using Bioedit v.7.2.5.0 revealed that the gene was successfully cloned between attB sites, downstream of the upstream activating sequence (UAS). Afterwards, the recombinant plasmid was injected in *Drosophila* embryos by fly genetic services. Transgenic flies presenting red eyes were subjected to RNA extraction, cDNA synthesis and RT-qPCR analysis. All RT-qPCR performed on the *Drosophila* transgenic flies both males and females showed that our gene of interest GffOr67d4 was expressed in *Drosophila* relative to the internal control, alpha-tubulin. Our study revealed that the *Drosophila* system can actually be used as a heterologous cell system for the identification of behavioral and ecologically relevant chemical signals of ORs in tsetse fly species and for the design of olfactory-based strategies to control trypanosomiasis.

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| | |
|---|---|
| AAT | Animal African Trypanosomiasis |
| BLASTp | Basic Local Alignment Search Tool that searches protein database using a protein query |
| cDNA | Complementarty DeoxyriboNucleic Acid |
| CI | Cytoplasmic Incompatibility |
| $CO_2$ | Carbon Dioxyde |
| CSP | ChemoSensory Proteins |
| cVA | 11-cis-Vaccenyl Acetate |
| DALY | Disability Adjusted Life Years |
| DDT | DichloroDiphenylTrichloroethane |
| DNA | DeoxyriboNucleic Acid |
| EBI | European Bioinformatics Institute |
| EDTA | Ethylene DiamineTetraacetic Acid |
| *G. f. fuscipes* | *Glossina fuscipes fuscipes* |
| GRs | Gustatory Receptors |
| HAT | Human African Trypanosomiasis |
| ICIPE | International Centre of Insect Physiology and Ecology |
| IRs | Ionotropic Receptors |
| LB | Luria-Bertani |
| MUSCLE | Multiple Sequence Comparison by Log- Expectation |
| NCBI | National Center for Biotechnology Information |
| NTDs | Neglected Tropical Diseases |
| OBPs | Odorant Binding Proteins |
| ORs | Olfactory Receptors |
| ORNs | Olfactory Receptor Neurons |

| | |
|---|---|
| OSNs | Olfactory Sensory Neurons |
| PCR | Polymerase Chain reaction |
| pENTR™/D-TOPO® | Entry Plasmid for Directional Topoisomerase based cloning |
| PTRE | Post-Treatment Reactive Encephalitis |
| pTW | Plant Tissue Watersoaking |
| pUC | Plasmid from the University of California |
| RNA | RiboNucleic Acid |
| RT-qPCR | Quantitative Reverse Transcription PCR |
| SAT | Sequential Aerosol Technique |
| SIT | Sterile Insect Technique |
| SNMPs | Sensory Neuron Membrane Proteins |
| S.O.C. | Super Optimal Broth (SOB) medium with Catabolite repression |
| TE | Tris-EDTA (Ethylene DiamineTetraacetic Acid) |
| TAE | Tris-Acetate EDTA (Ethylene DiamineTetraacetic Acid) |
| TOPO Cloning | Topoisomerase based cloning |
| UAS | Upstream Activating Sequence |
| US | Unites States |
| WHO | World Health Organization |

# CHAPTER ONE

# INTRODUCTION AND LITERATURE REVIEW

## 1.0 Introduction

Tsetse flies of the genus *Glossina* are the cyclical vectors of African trypanosomes, eukaryotic flagellated parasites that cause African Trypanosomiasis (Baral,2010). The disease affects both humans (Human African Trypanosomiasis, HAT) and animals (Animal African Trypanosomiasis, AAT) in sub-Saharan Africa. It has a tremendous impact on both human and animal well-being, in addition to the region's economic growth (Fenn & Matthews, 2014). The vector is present in 37 African countries invading an entire area of approximately 10 million $km^2$ of sub-Saharan Africa where the disease is endemic. The infested area represents approximately one-third of the whole of Africa (Eshetu & Begejo, 2015). According to the World Health Organization (WHO), about 70 million people and 48 million cattle occupying the tsetse-fly infected zones are at risk of infection (Klug *et al.*, 2014; Desquesnes *et al*., 2013). AAT causes approximately 3 million cattle deaths annually and reduces meat and milk productivity, draught animal power and therefore, less manure is spread on the cultivating fields (Eshetu & Begejo, 2015). Hence, it prevents the incorporation of crop farming and livestock care which contributes to agricultural losses of $4.75 billion per annum limiting development of sustainable agricultural systems (Eshetu & Begejo, 2015). Although the number of HAT cases reported by WHO dropped by 73% from 25,865 in 2000 to 7,106 in 2012 due to the high level of disease control in the affected regions (Simarro *et al.*, 2014), HAT is still ranked as the third most important contributor to the global burden of parasitic diseases after malaria and schistosomiasis, with disability adjusted life years (DALY) of 1.78 million lost across Africa (Fèvre *et al.*, 2008).

Despite such huge impact, current tools for control of trypanosomiasis are still limited as trypanocidal drugs used for chemotherapy are often ancient, difficult to administer and have undesirable side effects, and there are also reports of drug resistance (Barrett *et al*., 2007). No new drug is in the pipeline because it is an unattractive market for pharmaceuticals. In addition to that, the African trypanosomes are poor targets for vaccine development due to the periodic and systematic change of their variant surface glycoproteins, a phenomenon called antigenic variation (Bezie *et al.*, 2014). With the limited control and diagnostic tools, African trypanosomiasis remains one of the most neglected tropical diseases (NTDs).

Hence, the alternative effective measure to control the disease is to target the tsetse vector density, as tsetse flies are required to transmit the parasites to vertebrate hosts (Hyseni *et al.*, 2012). There is an urgent need to improve current and/or develop novel control and diagnostic strategies.

The basis of transmission of the disease to thousands of people resides in the ability of the vector to select its suitable mammalian hosts through visual and odorant signals and cues (Obiero *et al.*, 2014). Odorant responses of tsetse fly to natural cues have been exploited in the design of artificial bait technologies which have been successfully applied in tsetse control (Mangwiro *et al.*, 1999), also in the design of tsetse repellents (Saini & Hassanali, 2007). Although these can potentially be applied in tsetse control, research on tsetse responses to odours is extremely limited and responses are poorly understood (Obiero *et al.*, 2014). Understanding the molecular factors that underpin these responses could enhance development of better tsetse control strategic interventions to control the disease.

The process by which the fly perceives and responds to these odours is mediated by different classes of proteins including odorant binding proteins, chemosensory proteins, odorant and gustatory receptors (Klug *et al.*, 2014). Transduction of odorant molecules inside the cell is initiated upon binding with olfactory receptors, which are critical to this process (Masiga *et al.*, 2014).

The function of a great number of odorant receptors in *Drosophila* and *Anopheles* has been elucidated using an *in vivo* expression system, the *Drosophila melanogaster* empty neuron system (Hallem *et al.*, 2004; Carey *et al.*, 2010). However, the role played by ORs has not yet been illustrated in tsetse fly vector whose OR repertoire genes has been recently published (Obiero *et al.*, 2014). The genome content of ORs in tsetse flies revealed that an expanded cluster of ORs in *Glossina* was homologous to the *Drosophila melanogaster* Or67d (DmelOr67d), which also had multiple copies in several other insects' species. This receptor mediates responses to a male-specific pheromone of *D. melanogaster*, 11-cis-vaccenyl acetate (cVA), that regulates the mating behaviour of both males and females (Kurtovic *et al.*, 2007). This study was directed towards evaluating the effectiveness of the TOPO/Gateway cloning system as an efficient method for the delivery of an OR transgene in the *Drosophila* system. The study also aimed to assess the efficiency of the *Drosophila* system as a reliable expression system for tsetse ORs genes. Understanding the molecular basis of insect olfaction could enhance the development of strategic interventions to control the disease.

## 1.1 Literature review

### 1.1.1 African Trypanosomiasis

African trypanosomes of the genus *Trypanosoma* are extracellular flagellated protozoa that cause sleeping sickness (HAT) in humans and Nagana (AAT) in cattle (Wheeler, 2010; Morrison *et al.*, 2009). The parasites are mostly transmitted cyclically to several mammalian hosts through the bite of the tsetse fly in which the procyclic forms of the parasite undergo a series of transformations and multiplications giving rise to infective metacyclic forms which may be inoculated by the fly with its saliva into a new host (Bezie *et al.*, 2014). The fly thrives in approximately 37 sub-Saharan African countries where the disease is typically endemic (Baral, 2010).

#### A. Human African Trypanosomiasis

Sleeping sickness or Human African Trypanosomiasis is caused by two subspecies of *Trypanosoma brucei*, *T. b. rhodesiense* and *T. b. gambiense* which are responsible for the acute and chronic forms of the disease, respectively (Stich *et al.*, 2002; Franco *et al.*, 2014). The chronic form or gambiense HAT is endemic in West and Central Africa (Figure 1.1), accounting for approximately 98% of the current cases reported, while the acute form or rhodesiense HAT is present in the eastern and southern Africa (Figure 1.1) (Paliwal *et al.*, 2011).

Approximately 70 million people living in tsetse belt are at risk of developing the disease in Africa, where the annual cases were estimated to be around 300,000 people by the end of the $20^{th}$ century (WHO, 1998). Recent reports have shown a significant decline from the peak of 37,991 cases in 1998 to 25,865 cases in 2000 and 7,106 cases in 2012 (Simarro *et al.*, 2012; Simarro *et al.*, 2014). This decline could be an underestimation due to lack of adequate diagnostic tools, technical and infrastructural deficiencies, poor accessibility and political instability in trypanosomiasis endemic areas (Berrang, 2007).

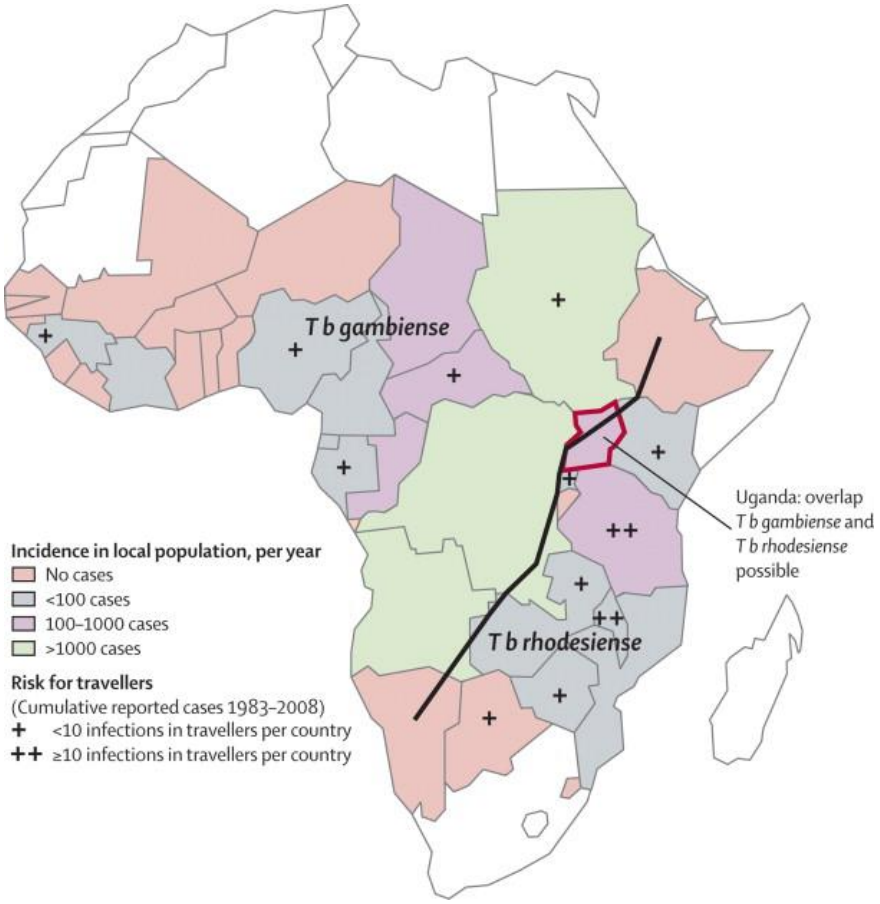


**Figure 1.1. Geographic distribution of Human African Trypanosomiasis.** The black line divides the regions in which *T.b. gambiense* predominates and those in which *T.b. rhodesiense* prevails. The colours show incidents of local population and the signs (+, ++) depict the risk for travellers (Brun *et al.*, 2010).

In both HAT forms, the disease usually develops in two stages, the early stage known as the haemolymphatic stage and the late stage (meningo-encephalitic stage). In the haemolymphatic stage, the parasites are restricted to the lymphatic and blood systems only (Checchi *et al.*, 2008). This stage is characterised by successive headache, fever, joint pain, pruritus and adenopathy (Stuart *et al.*, 2008). At this stage, although the prompt parasite growth is countered by the host immune responses, the parasite's antigenic variation enables immune evasion, resulting in waves of parasitemia. The parasites thereafter cross the blood–brain barrier into the cerebrospinal fluid where they invade the central nervous system causing the second stage of the disease, the meningo–encephalitic stage (Franco *et al.*, 2014). Here, the disease causes progressive neurological damage such as tremors, motor weakness, walking difficulties, sensory disorders, visual impairments, headache, sleep disturbances that

subsequently culminate in coma and ultimately death in the absence of treatment (Fèvre *et al.*, 2008).

The two stages of the disease require different treatments all associated with severe side effects. In addition to the severe adverse effects, these treatment regimens are associated with drug resistance (Baker *et al.*, 2013), thus increasing the need for improvement and development of novel control strategies of the disease. Reduction of vector populations remains the preferred alternative measure to control the disease (Aksoy, 2003).

**B. Animal African Trypanosomiasis**

Animal African Trypanosomiasis (Nagana) is an animal parasitic disease caused by *T. congolense, T. b. brucei* and *T. vivax* (Wheeler, 2010). These parasites are mostly transmitted cyclically through the tsetse fly bite but sometimes mechanically by different biting haematophagous insects (Hoare, 1972; Gruvel, 1980; Desquesnes *et al.*, 2009).

*Trypanosoma vivax* (subgenus *Duttonella*) is mechanically transmitted by tabanids and stomoxes (Mihok *et al.*, 1995; Desquesnes & Dia, 2003b). It affects mainly bovines, sheep and goats and to a lesser extent horses (Mare, 2009; Desquesnes *et al.*, 2013). However, it is less pathogenic to cattle than *Trypanosoma congolense*, which is considered as the single most important cause of AAT, seriously affecting sheep, goats, horses and pigs (Mare, 2009).

*Trypanosoma congolense* (subgenus *Nannomonas*) has been shown to be transmitted mechanically by a common African tabanid, *Atylotusagrestis* (Desquesnes & Dia, 2003a). *T. brucei* subs *brucei* (*subgenus Trypanozoon*) is known to be mechanically transmitted through biting insects, tabanids and stomoxes (Gingrich *et al.*, 1983; Mihok *et al.*, 1995). Horses, dogs, cats, camels and pigs are very susceptible to *T. brucei* infection. The infection varies from mild to chronic or subclinical in cattle, sheep, goats and pigs (Mare, 2009).

The disease is also caused by other species of *Trypanosoma* such as *T. simiae*, which is highly pathogenic to the domestic pigs but non-infective to rodents (Gashumba *et al.*, 1986) and *T. godfreyi*, a newly identified species which infects pigs (Hamill *et al.*, 2013). *T. evansi* that belongs to the Salivaria section of *T. brucei* group (Subgenus *Trypanozoon*) cause a disease called surra (an arthropod-borne disease) mostly in camels, horses and dogs, and to some extent in cattle and domestic buffaloes (Desquesnes *et al.*, 2013). The parasite trypomastigotes are transferred directly from host to host through mechanical transmission by

biting insects such as tabanids and stomoxes; explaining the spread of the parasite outside the tsetse belt in Africa (Sumba *et al.*, 1998; Desquesnes *et al.*, 2009).

Nagana is endemic in tsetse fly infested regions covering 37 sub-Saharan African countries where approximately 48 million cattle are at risk of infection with 3 million annual cattle deaths (Eshetu & Begejo, 2015). The widespread infection of wild animals and domestic livestock by *Trypanosoma* species makes it impossible to maintain a healthy and stable economy in the endemic regions; inflicting serious economic losses in these countries (Chanie *et al.*, 2013; Aksoy *et al.*, 2014). The infection can result in subacute, acute or chronic disease in animals mainly characterized by fever, anemia, weakness, diarrhea and nervous abnormalities; symptoms which are responsible for losses in meat and milk production, draught power and manure, calving rates and growth rates (Shaw, 2009; Mare, 2009; Desquesnes *et al.*, 2013). Preventing crop farming integration and livestock keeping essential to the improvement of sustainable agricultural systems, Nagana can still be considered as one of the major causes of food insecurity, hunger, and poverty in sub-Saharan Africa (Vreysen *et al.*, 2012). It leads to annual livestock and crop production losses of about 4.75 billion US dollars (Eshetu & Begejo, 2015).

### 1.1.2 Control of the disease

A great number of methods have been used for the management of trypanosomiasis, including curative treatment of infected humans as well as prophylactic and curative treatment of animals. Alternative methods consist of reduction of tsetse populations (Vreysen *et al.*, 2012).

#### A. Chemotherapy

In comparison with other tropical diseases, the attention given to the development of chemotherapy for sleeping sickness has lagged behind, the current drugs in use have more or less remained unchanged for more than 50 years (Bacchi, 2009).

The treatment of HAT depends on the stage of the disease and the specific species involved. However, in both cases, drugs are associated with severe adverse effects. The drugs used for early stage *T. b. rhodesiense* and *T. b. gambiense* are Suramin and Pentamidine; exclusively effective in the haemolymphatic stage (Kennedy, 2006). Suramin may cause neuropathy, fatigue, neurotoxicity, hyperglycemia, rash, anemia, hypocalcaemia, renal insufficiency, neutropaenia, and transaminitis (Kaur *et al.*, 2002). Toxic effects of pentamidine involve

nephrotoxicity, liver enzyme failure, leucopenia as well as pains at the injection site (Barrett *et al.*, 2007). Melarsopol is used for treatment of late stage HAT, due to its ability to cross the blood brain barrier. This drug is active against *T. b. rhodesiense* and *T. b. gambiense* (Barrett *et al.*, 2007). However, it is associated with severe post-treatment reactive encephalitis (PTRE) in approximately 10% of treated patients of which 50% die because of toxicity (Kennedy, 2006). Severe side effects of melarsopol include neurological sequelae and convulsions that might lead to death, pyrexia, headache, pruritus and thrombocytopenia (Barrett *et al.*, 2007). Besides its high cost, melarsopol is also estimated to kill approximately 5% of the treated patients due to its toxicity alone (Aksoy, 2003). The recently introduced compound, eflornithine is also used for late stage treatment of only gambiense HAT. It is also associated with different side effects such as headache, fever, macular rash, peripheral neuropathy, tremor, hypertension, and gastrointestinal complications including diarrhea (Chappuis *et al.*, 2005).

## B. Vector control

Vector control remains the preferred method of managing trypanosomiasis due to the limitations of chemotherapy (high cost, drug resistance and toxicity) and the impossibility of vaccine development due to parasite antigenic variation (Aksoy, 2003). The adult fly is the only accessible target stage since eggs and larva are absent in nature and are hidden in the female reproductive organs and the pupa entirely develops in the soil (Vreysen *et al.*, 2012).

Ancient methods of tsetse fly control involving elimination of tsetse flies preferred vegetation and destruction of host game animals were very efficient but were dropped due to their negative effects on the environment (Brightwell *et al.*, 1991).

Current control approaches include the use of insecticides, spraying of land and livestock, sterile insect technique, paratransgenesis, traps and targets as well as the ‘push-pull’ method (Brun *et al.*, 2010).

### 1. Insecticides

This approach involves spraying residual insecticides such as dichlorodiphenyltrichloroethane (DDT), dieldrin and endosulfan in tsetse-infested areas during the dry seasons either from the ground or by the aid of helicopters targeting mainly the day and night resting flies, respectively. In spite of being labor intensive and requiring specific logistics, the method has been effective in controlling trypanosomiasis and has led to

eradication of *G. p. palpalis, G. m. submoristans,* and *G. tachinoides* from a savannah area of 200,000 $km^2$ in northern Nigeria**.**

However, the persistence of the insecticides over an extended period in the environment after spraying leads to the development of vector resistance to the insecticides, killing of non-target insects, including beneficial insects, that may result in the outbreak of several other pests due to the elimination of their natural predators. In addition, this method leads to environmental pollution because insecticides accumulate in food chains and are a health risk to the spraying workers and to the population (Vreysen *et al.*, 2012).

Therefore, in 1980s, the abundant use of insecticides was substituted by the sequential aerosol technique (SAT) which basically consists of spraying non-residual insecticides with the aid of aircrafts or helicopters. These non-residual insecticides target adult tsetse flies and are more environmentally friendly as a result of their short half-lives**.** Depending on the temperature, the spraying cycles are separated by 16-18 days and because the insecticide dropsize is sufficiently small to suspend long in the air but heavy enough to prevent upward drift, they also exterminate emerging flies in the subsequent cycles before they start reproducing (Kgori *et al.*, 2006).

**2. Sterile Insect Technique (SIT)**

The sterile insect technique is a genetic suppression control method that consists of systematic release of irradiated sterile male tsetse flies into a target wild population (Aksoy, 2003). Once the sterile male mates with virgin female flies, they are unable to sire offsprings, reducing tsetse fly populations (Dyck *et al.*, 2005). The sterile males that are released continually in huge numbers compete with the wild populations. Hence, in every generation, the proportion of sterile males to wild insects increase, making the control method more effective and economical than the conventional methods used for killing the flies (Vreysen & Robinson, 2011).

This approach presents the advantages of being non-intrusive to the environment, species-specific and having no adverse effects on non-target organisms. Moreover, the method can also be integrated with other control methods such as pathogens, predators and parasitoids. An example of the success of the method is the complete elimination of *Glossina austeni* from Unguja island of Zanzibar (Vreysen *et al.*, 2000).

However, the effectiveness of the technique depends on the low density of the target population and a detailed knowledge of the pest biology and ecology is required (Vreysen *et*

*al.*, 2012). Moreover, the success of the method also relies on the competitiveness of the released male insects relative to their wild counterparts as well as their ability to locate virgin females and copulate with them (Mutika *et al.*, 2012).

### 3. Paratransgenesis

Paratransgenesis is a control strategy for vector-borne diseases that exploits genetically modified endosymbiotic organisms expressing foreign genes that can block the development of pathogens or their transmission by vectors (Coutinho-Abreu & Ramalho-Ortigao, 2011). The development of a genetic transformation system for tsetse fly has been possible through the availability of an *in vitro* culture of *Sodalis glossinidus*, one of tsetse's commensal symbiotic microbes that can express trypanosome-resistance-conferring products (Aksoy, 2008).

The localization of *Sodalis* in the tsetse gut makes it suitable for delivering of trypanocidal molecules and drugs that directly target the trypanosomes (Hooper & Gordon, 2001).

Genetically transformed *Sodalis glossinidus* expressing an anti-trypanosomal factor are micro-injected into the thorax of fertile female tsetse flies, thus creating a hostile environment to trypanosomes. These recombinant *Sodalis* symbionts are successfully transmitted to the F1 and F2 progeny naturally, which continuously express the foreign gene product (Cheng & Aksoy, 1999). Modified *Sodalis* can actually be spread into the natural tsetse populations through cytoplasmic incompatibility (CI) driven by *Wolbachia* symbionts that tsetse flies also harbor, since both symbiotic systems are maternally transmitted in tsetse flies (Medlock *et al.*, 2013). Future studies will lead to the identification of novel and effector genes that can have adverse effects on pathogens while expressed in tsetse flies.

### 4. Attractive devices and live bait technique

This approach is based on the visual and odorant responses of tsetse flies to natural volatile and non-volatile cues (Obiero *et al.*, 2014). It aims at catching flies in a device to eventually kill them. The device consists of traps made of blue and black colored cloths that attract the flies (Vale, 1993). The traps are also baited with attractants including natural cow urine, acetone, octenol as well as other chemical attractants (Brightwell *et al.*, 1991). Once inside the trap, the flies are directed to a non-return cage where they are killed either by heat or starvation. On the other hand, targets are used to attract flies and kill them through tarsal contact with insecticides such as pyrethroids impregnated on the surface of the target

(Brightwell *et al*., 1991; Vale, 1993). This method has been widely used for the control of trypanosomiasis because it is relatively inexpensive, highly specific, unsophisticated, and suitable for farmer communities to protect small areas. It is also able to slow down the invasion of tsetse populations from adjacent areas (Vale, 1993). However, it cannot be applied for large areas and for long term management of the disease (Kappmeier & Nevill, 1999). In addition, traps and targets have successfully been used in sleeping sickness foci to suppress tsetse populations below the transmission threshold; but to date, the technique has never been effective for eradication of riverine tsetse species in West Africa or major vectors of HAT (Vreysen *et al.*, 2012).

Another similar method is the live bait technique, which uses the blood feeding behavior of male and female tsetse flies; it involves treatment of livestock with insecticides. While flies feed on the treated cattle or domestic livestock, they are killed by taking up a lethal deposit of insecticide that was applied on the ventral tarsal spines and on pre-tarsi whilst feeding (Vreysen *et al.*, 2012). The technique is also quite low-cost, rapid, and easy and does not require sophisticated equipment for insecticide application. However, eradication of riverine tsetse populations, vectors of sleeping sickness has never been achieved up to date using this technique (Bouyer *et al.*, 2007). Hence, improved knowledge of the molecular basis of olfactory responses of riverine species to natural cues is urgently required.

**5. Push-pull method**

Combinations of repellent and attractant chemicals have been used in push-pull for the control of trypanosomiasis. Tsetse flies have shown to feed preferably on several vertebrate animals and seem to exploit the push-pull tactics to evade some hosts and locate the preferred ones. The strategy uses chemical repellents (push) on the cattle to push the flies away and attractants (pull) in a trap to direct the flies away from hosts towards the baited traps and targets where they are killed by insecticides, heat or starvation (Hassanali *et al.*, 2008). Natural and synthetic repellents such as 2-methoxyphenol, a constituent of bovid odors have been used efficiently to protect the cattle (Saini & Hassanali, 2007). However, a repellent obtained from waterbuck, *Kobusdefassa*, is more effective and might be efficient for the suppression of tsetse populations (Hassanali *et al.*, 2008).

### 1.1.3 Tsetse biology

Tsetse flies belong to the genus *Glossina*, family *Glossinidae*, superfamily *Hippoboscidea* of order Diptera. The superfamily *Hippoboscidea* has four families namely *Glossinidae*,

*Hippoboscidae*, *Streblidae* and *Nycteribiidae* in which *Glossinidae* only contains one member, *Glossina* (Torp *et al.*, 2007).

The genus *Glossina* includes thirty-three species and subspecies that are commonly categorized into three distinct groups based on the ecological niche they occupy, namely *morsitans* for savannah, *fusca* for forest and *palpalis* for river (Gooding & Krafsur, 2005; Lindh *et al.*, 2009).

The *morsitans* group or savannah tsetse known as *Glossina* sensu stricto is mainly located in the East African woodland savannah. Species of this group include *Glossina morsitans*, *Glossina swynnertoni* and *Glossina pallidipes*, that are largely responsible for transmitting the cattle disease (Lindh *et al.*, 2009). Secondly, the *fusca* group or the forest tsetse known as *Glossina austenina* is represented by flies that live typically in primary forest belts and have not been reported to be vectors of HAT (Franco *et al.*, 2014). Finally, the riverine tsetse or *palpalis* group, known as *Glossina Nemorhina* is located in Western and Central Africa. This includes the species that are main vectors of sleeping sickness such as *Glossina palpalis palpalis* and *Glossina palpalis gambiense*, vectors of *T.b. gambiense* and *Glossina fuscipes*, vector of both *T.b. gambiense* and *T.b.rhodesiense* ( Lindh *et al.*, 2009; Franco *et al.*, 2014).

The most abundant *Glossina* species is *G. fuscipes* found in most parts of Central Africa, Uganda and Western Kenya. It is the preferential vector of both *T.b. gambiense* and *T.b. rhodesiense*. Being zoophagic; it is also a main vector of AAT (Krafsur *et al.*, 2008). In recent years, this species has been responsible for more than 90% of sleeping sickness cases (Dyer *et al.*, 2011).

### A. Life cycle of tsetse fly

Tsetse flies are an ancient and unique taxon of insects that reproduce by adenotropic viviparity. This means that the female produce a single egg at each gonotrophic cycle, which develops to a fully third-instar larvae in the intrauterine environment where it is supplied with all essential nutrients (Attardo *et al.*, 2012). Females usually mate only once in their lifetime, generally between 3 and 8 days after eclosion (emerging of the adult from puparium) while the males take several days to become sexually mature and can mate many times. During copulation, sperm are transferred to the uterus of the female and are stored in the spermathacae just after copulation. The stored sperm is usually sufficient for the female reproductive life (Leak, 1999).

In the female fly, once the egg released from the ovary descends into the uterus, opposite the spermatecal ducts, it is fertilized by the sperm. Following fertilization, embryogenesis proceeds for nearly 3-4 days in most tsetse species (Tobe & Langley, 1978). Afterwards, the developed embryo hatches into a first instar larva (Figure 1.2) that remains in the uterus where it is nourished on milk secretions from the mother's modified accessory gland until it fully develops to the third-instar larvae (Attardo *et al*., 2006b). Through this whole 6-day larval period, the nutrients as well as bacterial symbionts are transmitted to the progeny through the mother's milk secretions (Attardo *et al.*, 2006b; Aksoy & Rio, 2005).

The larva is then deposited on soft moist shaded soil (larviposition) where it does not feed but immediately burrows itself into the ground within 1-2 hours (Tobe & Langley, 1978; Attardo *et al*., 2006a). This contrasts significantly with other insects' larvae that intensively feed and store lots of food before pupating. Hence, in tsetse fly, all the nutrients essential for the growth of the embryo up to the adult stage result from the mother (Tobe & Langley, 1978).

After approximately 30 days of puparial development, the adult fly emerges with the aid of the ptilinum (a structure at the front of the head) in a temperature dependent fashion. Warmer temperatures generally increase the rate of metabolism and shorten the puparial duration; consequently the time for the adult to emerge is also reduced. However, very high and sublethal temperatures can lead to a delay in the time between the completion of development and the eclosion (Leak, 1999).

Almost 9-10 days after female adult emergence, the first oocyte is ovulated from the ovary and this marks the start of the first pregnancy cycle. Upon ovulation, the sperm from the spermathecae fertilize the egg and the cycle begins again. Hence, the female gives birth to young offspring at regular 9- to 10-day intervals of her adult life (Tobe & Langley, 1978). Typically, they generate only 8 to 10 offsprings in their lifespan of 3-4 months (Attardo *et al.*, 2006a; Attardo *et al.*, 2006b). An overview of the tsetse life cycle is shown in figure 1.2.

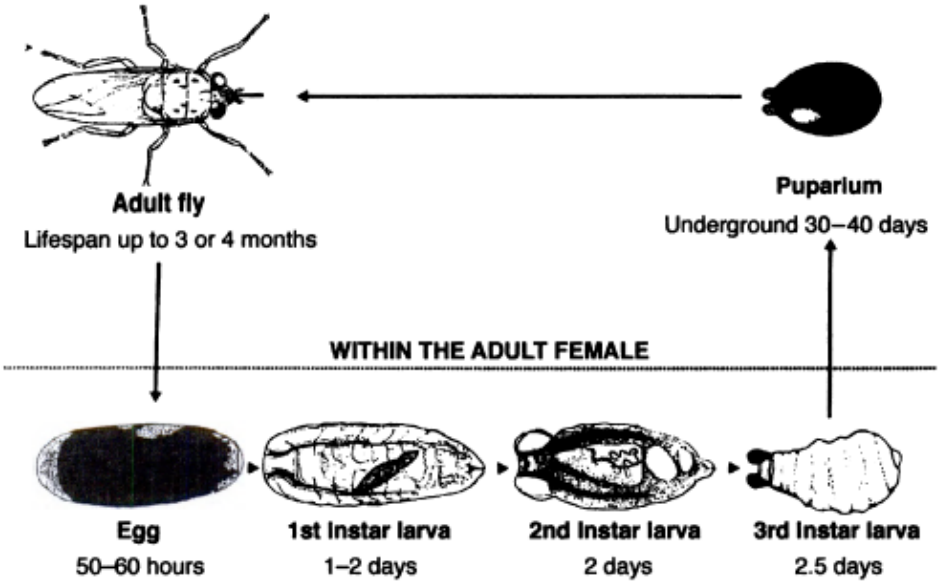


**Figure 1.2. Life cycle of a tsetse fly.** A mature female produces an egg that is fertilized and develops in utero into 1st, then 2nd and 3rd instar larva. This latter is then deposited on the ground where it burrows immediately and develops into a pupa. Subsequently, the pupae mature into an adult fly and the cycle begins again (Leak, 1999).

### B. Tsetse fly feeding

The tsetse fly has a very limited food regime, the male and female adult flies are exclusively hematophagous while the young larva depends completely on the milk secretions from the mother. The newly emerged adult fly has fat reserves from its previous stage, but it immediately looks for a blood meal before it can mate (Tobe & Langley, 1978). The exclusive blood diet of tsetse fly is deficient in nutrients such as B-complex vitamins and thiamine monophosphate, which are possibly supplemented by its microbial obligate endosymbiont *Wigglesworthia* (Aksoy & Rio, 2005; Sassera *et al.*, 2013).

Unlike many other blood-sucking Diptera, tsetse adult flies are pool feeders; they penetrate the mammalian host tissue with their proboscis, also called haustellum, forming a pool of blood on the skin sub-surface from which they suck blood. To prevent the host blood from coagulating on the site of infection, saliva of the fly is usually channeled into the wound, enabling the fly to feed longer. It is usually during this event that an infected fly transmits trypanosomes and an uninfected fly acquires trypanosomes from an infected mammalian host (Lehane, 2005). The female usually acquires blood meals weighing several times their own weight. This higher volume meal is likely to be required for the production of eggs, leaving them less energy for flight than males (Lehane, 2005).

Tsetse larvae exclusively depend on the nutrients from their mother's milk secretions. The milk is rich in lipids in early development of the larva, then consists of a combination of proteins and lipids in late larval periods (Attardo *et al.*, 2012). This is thought to be the route of transmission for bacterial endosymbionts during the intrauterine stage (Denlinger & Ma, 1975). Since the larva depends solely on this milk, it is possible that it acquires some of its nutrients from these microbes. Once deposited by the mother, tsetse larva do not feed like other insects do, but instead they pupate almost immediately; implying that all nutrients are acquired from the milk secretions and hence the mother's blood meal with contributions from the endosymbionts she harbors (Tobe & Langley, 1978).

### 1.1.4 Tsetse genome

The International Glossina Genome Initiative annotated the *Glossina morsitans morsitans* genome and found that its size is 366 megabases (Attardo *et al.*, 2014); which is more than three times that of *Drosophila melanogaster* (116.8 Mbp) (Misra *et al.*, 2002) and approximately one and a half times that of the malaria vector *Anopheles gambiae* (278 Mbp) (Holt *et al.*, 2002). This large genome provides some insight into unique tsetse features such as its obligate hematophagous diet, its viviparous reproduction and the requirement of the mother's milk secretion for larval survival.

The genome has shown a relative reduction of genes associated with carbohydrate metabolism. In contrast to mosquitoes and sandflies, tsetse utilizes a proline-alanine system for energy supply as well as triglycerides and diglycerides for milk secretion and fat body storage (Attardo *et al.*, 2014). The chemoreceptor repertoire in *G. m. morsitans* was found to be smaller than other Dipterans; tsetse has lost gustatory receptors involved in sweet taste but has expanded those associated with $CO_2$ detection. This is likely to be linked to its restricted blood-meal diet for both sexes (Obiero *et al.*, 2014).

### 1.1.5 Olfactory System

Insects constitute the earth's most diverse organisms, comprising approximately five million species, representing about half of the living organisms species and almost three-quarters of the animal kingdom (Wijesekara & Wijesinghe, 2003; Fan *et al.*, 2011).

This great success in diversity is related to their incredible adaptability to various environments. One such adaptation is their ability to detect, perceive and respond to external biological compounds as well as volatile and non-volatile cues through a chemical sensor

(Huebner & Strittmatter, 2009). Their sophisticated olfactory system senses odorants derived from individuals, host plants, and prey; enabling them to spot foods, locate hosts, mating partners or prey and find suitable larviposition sites (Cardé & Willis, 2008; Sánchez-Gracia *et al.*, 2009). The tsetse fly finds its suitable host beyond its visual range upwind, through odor-mediated chemical signals and cues (Gikonyo *et al.*, 2002; Klug *et al.*, 2014).

Olfactory organs of adult insects include antennae as well as maxillary palps for some other species (Carey & Carlson, 2011). The surface of these olfactory organs is covered by numerous sensilla, each of which is filled with a potassium-rich lymph that houses several (one to four) dendrites of a few olfactory receptor neurons (ORNs) also called olfactory sensory neurons (OSNs) (Huebner & Strittmatter, 2009; Fan *et al.*, 2011; Leal, 2011). In a typical *D. melanogaster* adult, each antenna contains approximately 1200 OSNs while each maxillary palp has about 120 OSNs (Hallem *et al.*, 2006).

In *Drosophila, the* third segment of antenna, or sensilla, is the major olfactory organ. Three main classes of sensilla have been characterized based on their morphology-basiconic, coeloconic and trichoid. Basiconic sensilla are club-shaped and carry dendrites of olfactory neurons detecting food odorants. Trichoid sensilla are long, needle-shaped and thick-walled and are specialized for pheromone reception (Laughlin *et al.*, 2008; Ronderos & Smith, 2009). Coeloconic sensilla, fin-shaped, contain neurons tuned to aldehydes and organic acids. However, the olfactory sensilla of the maxillary palp contain exclusively basiconic sensilla (Hallem *et al.*, 2006).

Like in other insects, chemosensation in *Glossina* is initiated immediately after chemical signalling molecules (volatiles) are taken up from the external environment and transported through the sensory hair and sensillum fluid to interact with specific chemoreceptors of the sensory neurons (Masiga *et al.*, 2014). The binding of a ligand to the olfactory receptor (Figure 1.3) represents the key event in olfaction as it initiates a cascade of olfactory transduction events that convert the extracellular chemical signal into an intracellular electronic signal, which results in perception of the chemical stimulus in the brain and generates the specific behavioural response (Masiga *et al.*, 2014).

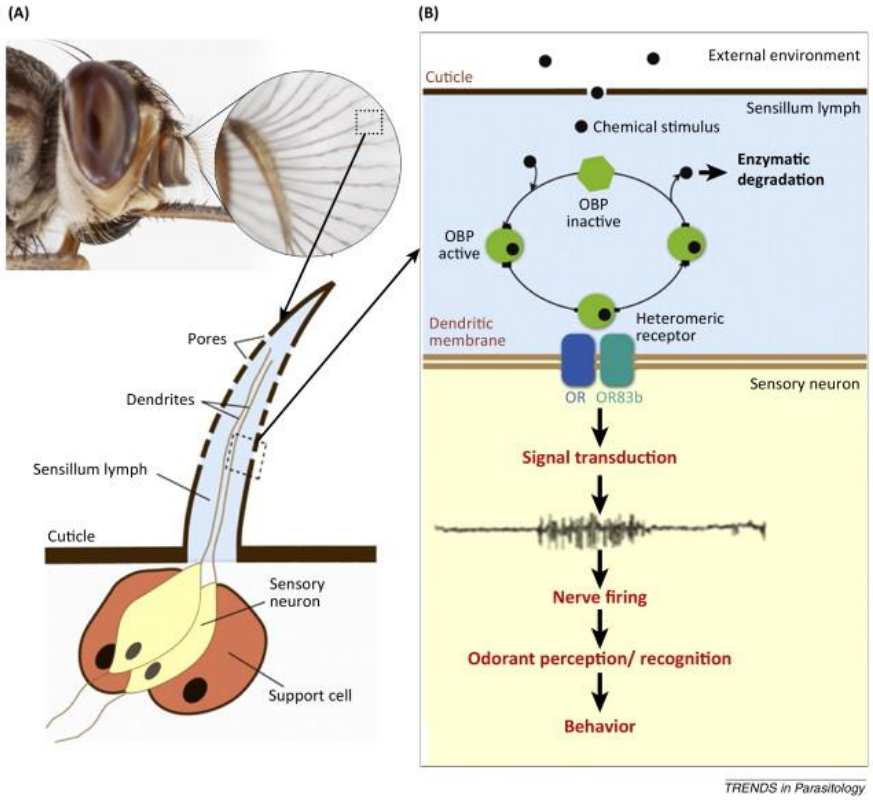


**Figure 1.3: Schematic representation of insect olfaction.** (A) Antennae of tsetse fly. Sensilla have hemolymph, dendrites, sensory neuron and pores through which odorants enter in the cell (B) Transport of odorants through sensillum lymph to the ORs in neurons where a specific receptor and a common receptor Or83b are needed. The binding of the odorant triggers signal transduction cascade which triggers the appropriate behaviour response (Masiga *et al.*, 2014).

### A. Chemosensory proteins

The olfactory perception involves specific multigene families that encode for chemosensory proteins (CSPs), odorant-binding proteins (OBPs), sensory neuron membrane proteins (SNMPs), and a chemoreceptor superfamily composed of gustatory receptors (GRs), ionotropic receptors (IRs) and olfactory receptors (ORs) (Sánchez-Gracia *et al.*, 2009; Leal, 2011).

The OBPs and CSPs are specific small soluble carrier proteins that recognize, bind and solubilize ligands, hydrophobic odorants and pheromones; transporting them through the sensillar hemolymph and shuttling them to the underlying sensory receptors (Kulmuni & Havukainen, 2013). These proteins are both characterized by the presence of a signal peptide and α-helices joined by disulphide bonds (Ozaki *et al.*, 2008).

OBPs are highly diverse and globular proteins that have typically a set of six conserved cysteine residues and are known to bind several odorants including pheromones (Ozaki *et al.*, 2008). Expressed at high levels in the olfactory sensilla, OBPs act as selectors, solubilizers, transporters of specific ligands but they are also deactivators after triggering olfactory signal transduction (Fan *et al.*, 2011). Hence, they contribute to the sensitivity and most probably to the selectivity of the insect olfactory system (Leal, 2011).

Based on the number of conserved cysteine residues, OBPs are divided into four different sub-groups in *Drosophila*: (i) Classic OBPs which contain six conserved cysteines with three disulphide bridges (ii) Minus-C OBPs that have lost two conserved cysteine residues (iii) Plus-C OBPs that harbor additional conserved cysteine residues with a conserved proline (iv) Classic-dimer OBPs harboring two of the six-cysteine signatures (Hekmat-Scafe *et al.*, 2002).

Although most OBPs have been shown to work by releasing their bound odorants at the end of the journey to directly activate their respective receptors; Lush, a specific OBP from *Drosophila melanogaster* (DmelOBP76a) actually forms an OBP.odorant complex that activates the receptor, Or67d (Hekmat-Scafe *et al*., 2002).

CSPs are small soluble proteins with hydrophobic binding pockets but they have an average length of 130 amino acids (Kulmuni & Havukainen, 2013; Sánchez-Gracia *et al.*, 2009). CSPs are more conserved than OBPs; they are characterized by a specific domain of four conserved cysteines that form two disulphide bridges between neighboring residues. They are secreted onto the insect sensilla lymph where several are highly expressed and are capable of binding diverse pheromonal blends (Sánchez-Gracia *et al.*, 2009).

However, there is no clear evidence showing that CSPs are involved in olfactory and gustatory functions. Moreover, it should also be emphasized that not all CSPs are restricted to chemosensory organs. Some are involved in larval development and brood pheromone transportation (Liu *et al.*, 2012); others in leg regeneration and carbon dioxide detection (Wanner *et al.*, 2004), nestmate recognition and behavioral shift from gragarization to solitarization (Kulmuni & Havukainen, 2013). In *Glossina morsitans morsitans*, the expression of CSPs has been related to host adult female seeking behavior (Liu *et al.*, 2012).

Another class of proteins involved in olfaction is the sensory neuron membrane proteins (SNMPs) which are homologs of the CD36 superfamily. In humans, they act as scavenger receptors, mediating the uptake of lipoprotein complexes (Ronderos & Smith, 2009). A

recent study showed that SNMP expression in T1 neurons of *Drosophila* is required for 11-cis-vaccenyl acetate pheromone detection in the antennae and for deactivation of the pheromone responses once initiated. However, SNMP is not involved in sensitivity of most odorants (Jin *et al.*, 2008).

### B. Chemosensory receptors

Receptors from three divergent multigene families are expressed in insect ORNs namely ionotropic receptors (IRs), gustatory receptors (GRs) and the odorant receptors (ORs) also called olfactory receptors (ORs) (Andersson *et al.*, 2013).

In the process of chemosensation, olfactory receptors are fundamental as they mediate the transduction of external signals upon binding of the ligand (Andersson *et al.*, 2013). Unlike their mammalian counterparts that have been classified as G-protein coupled receptors (Gaillard *et al.*, 2004), additional evidence showed that the insect ORs are heteromeric ligand-gated ion channels (Sato *et al.*, 2008). They are extremely diverse and are characterized by the presence of seven transmembrane domains with a reversed N-terminal membrane topology (Benton *et al.*, 2006). In each ORN, a specific OR that underlies the specific response spectrum of the ORN is co-expressed with the olfactory co-receptor, Orco (Or83b in *D. melanogaster*, Or2 in *Bombix mori* and Or7 in *Anopheles gambiae*) (Bellmann *et al.*, 2010; Leal, 2011). These heterodimers are required for transduction of odour-evoked signals (Mamidala *et al.*, 2013). Orco, expressed in OSNs that house ORs, is highly conserved across insects species (Neuhaus et al., 2005). It is required for localization and trafficking of the conventional OR to the ORN dendrites membranes as well as for olfactory responses; it increases the functionality of the OR by enhancing odorant responsiveness (Larsson *et al.*, 2004; Benton *et al.*, 2006).

ORs respond to a wide number of volatile chemicals (Hallem & Carlson, 2006; Carey *et al.*, 2010); including plant or microbe derived compounds (Stensmyr *et al.*, 2012) and pheromones (Sakurai *et al.*, 2004; Kurtovic *et al.*, 2007).

The genome content of olfactory receptors of *Glossina morsitans morsitans* revealed a total of 46 ORs (Obiero *et al.*, 2014), these are less than the number (60) identified in *Drosophila* (Hallem & Carlson, 2006). In addition, Obiero *et al*., 2014 also showed that six ORs (GmmOr41-46) were homologs of a single *Drosophila melanogaster* OR (DmelOr67d), that act as a receptor for a male-specific pheromone, 11-cis-vaccenyl acetate, a signal molecule necessary for mate seeking (Ha & Smith, 2006). However, the function of this expansive

receptor in *Glossina* is not well known. Insights into the role of this olfactory receptor in tsetse fly will be investigated in this study.

Gustatory receptors are distantly related to the Orco family (Dunipace *et al.*, 2001) but together with ORs, they belong to the same superfamily of insect chemoreceptors (Robertson *et al.*, 2003) based on a few amino acid residues conserved within transmembrane domain 7 (Scott *et al*., 2001). Few GRs are expressed on ORNs of the antenna, suggesting they have a role in olfaction (Robertson *et al.*, 2003). Two members of the GR family expressed on ORNs, Gr21 and Gr63a, are involved in $CO_2$ detection (Kwon *et al.*, 2007). However, most GRs are expressed in gustatory receptor neurons of different taste organs (mouthparts, proboscis, pharynx and leg) and function broadly in contact chemoreception (chemosensation) ( Robertson *et al.*, 2003; Andersson *et al.*, 2013). They are responsible for distinguishing odour tastes, detecting sugar and bitter compounds as well as contact pheromones (non-volatile pheromones) that promotes courtship and mating with females (Montell, 2009). Compared to *D. melanogaster* and other Diptera, fewer GRs (14) were identified in *Glossina* (Obiero *et al.*, 2014). Receptors for sugars were not found in *G. m. morsitans*, possibly because of the restricted blood-meal diet of the insect. However, GRs for $CO_2$ detection were conserved relative to *D. melanogaster* (Obiero *et al.*, 2014).

Ionotropic Receptors (IRs) are another class of divergent insect chemosensory receptors that have been recently reported (Croset *et al.*, 2010). Although they are related to ionotropic glutamate receptors (iGluRs) involved in synapse communication, IRs present atypical binding domains (Andersson *et al.*, 2013). They function in complexes formed with three subunits including odour-specific receptors and one or two co-receptors (Ir8a and Ir25a) (Abuin *et al.*, 2011). These functional heterodimers are required for distinct odour perception.

Unlike ORs that are expressed in olfactory neurons innervating basiconic and trichoid sensilla, IRs are expressed in coelonic olfactory neurons that lack Or83b or members of GR and OR gene families (Benton *et al.*, 2009). Two major groups of IRs have been characterized in insects; the antennal IRs, conserved across insects and involved in olfaction and the divergent IRs that are species-specific and are thought to have a role in taste (Croset *et al.*, 2010). In *Drosophila*, antennal IRs display higher specificity to chemicals than ORs and they respond to a wide number of odours including nitrogen-containing compounds (amines and ammonia), acids, aromatics and aldehydes (Abuin *et al.*, 2011). In *Glossina* species, Macharia *et al.*, 2016 identified homologs of the *Drosophila*-specific ionotropic receptor Ir84a, a potential candidate receptor of phenylacetaldehyde, that promotes male

courtship in *Drosophila*. These results suggest that male courtship is a conserved trait in tsetse fly species.

### 1.1.6 Functional characterization of olfactory receptors

The entire olfactory system heavily depends on the types of receptors expressed on the surface of ORNs (Leal, 2011).

*In silico* identification of odorant receptor genes in different insect species is the starting point of downstream functional characterization of ORs. These downstream studies, in which ORs are functionally characterized according to the ligands they are tuned to, are known as "deorphanization" (Clyne *et al.*, 1999). Insect ORs deorphanization is achieved through expression of the OR genes in different systems, followed by a process in which the expressed OR proteins response spectrum is tested toward odourant compounds (Gonzalez *et al.*, 2016).

Expression of individual OR genes has been carried out in different systems, *in vitro* and *in vivo*, by the aid of several techniques, such as immunohistochemistry, *in situ* hybridization, and reporter gene assays that are primarily based on the GAL4/UAS system (Hallem *et al.*, 2006). These methods revealed that ORs are expressed in spatially defined populations of OSNs (Clyne *et al.*, 1999). *In situ* hybridization coupled with immunocytochemistry showed that about 32 OR genes of *Drosophila* are restricted to the antenna while 7 are solely expressed in the maxillary palp (Vosshall *et al*., 2000).

*In vitro* systems entail heterologous expression of ORs genes in cell culture systems such as the Sf9 insect cell lines derived from *Spodoptera frugiperda* (Matarazzo *et al.*, 2005; Kiely *et al.*, 2007; Anderson *et al.*, 2009; Jordan *et al.*, 2009), human embryonic kidney cells ( Große-Wilde *et al.*, 2006; Corcoran *et al.*, 2014) and also *Xenopus laevis* oocytes (Sakurai *et al.*, 2004; Mitsuno *et al.*, 2008; Leary *et al.*, 2012; Liu *et al.*, 2013; Zhang & Löfstedt, 2013).

In the case of *in vivo* systems, the expression of ORs has been achieved through the use of the mutant ab3A antennal neuron, the empty neuron system of *D. melanogaster*. This system contains an OSN that lacks its endogenous OR, thereby is unresponsive to odours. Transgenic ORs are specifically expressed in the ab3A empty neuron using the GAL4/UAS system (Dobritsa *et al.*, 2003). Odorant responses conferred by transgenic ORs can then be screened electrophysiologically by means of single sensillum recordings (SSR), a method that consists of presenting different stimulus set in Pasteur pipettes to the sensillum lymph of the OSN

containing a recording electrode. The spontaneous activity of the OSN is then recorded through the action of an electric supply (Pellegrino *et al.*, 2010).

For deorphanization of pheromone receptors (PRs), heterologous expression targets the trichoid sensillum T1 of *D. melanogaster*, which contains a single receptor (Or67d) in the wild-type flies. This receptor responds to the pheromone 11-cis-vaccenyl acetate (Kurtovic *et al.*, 2007).

### 1.1.7 *Drosophila* empty neuron system

Mechanisms of insect olfaction over the past decades have been elucidated for most of the cases through the genetic tractability of *Drosophila melanogaster* (Carey & Carlson, 2011).

This fruit fly provides an excellent and sophisticated olfactory model system for the odour coding study. The organization of its odorant system is very similar to that of other insects and vertebrates. *Drosophila* are small in size but easily amenable to molecular, genetic and electrophysiological analysis (Hallem & Carlson, 2006).

The functions of antennal odour receptors in *D. melanogaster* have been examined using an *in vivo* expression system, the empty neuron system. The system is centred on a mutant antennal neuron Δab3A that lacks responses to odours due to loss of its endogenous receptors, Or22a and Or22b genes (Dobritsa *et al.*, 2003). This Δhalo deletion mutation in the empty neuron has allowed characterization of these two genes and showed that they are specifically coexpressed in the ab3A antennal neuron. By introducing and expressing another odorant receptor Or47a into this empty neuron, it has been possible to functionally characterize this gene as well (Dobritsa *et al.*, 2003).

The empty neuron system has shown to be a high fidelity expression platform for antennal *D. melanogaster* ORs genes (Hallem *et al.*, 2004) and for *Anopheles gambiae* OR repertoire as well (Carey *et al.*, 2010).

To functionally characterize ORs, targeted olfactory receptors are specifically introduced in the empty neuron and expressed using the GAL4/UAS system in which an Or22a-GAL4 promoter construct drives expression of a receptor from a UAS-OR construct (Goldman *et al.*, 2005). The GAL4/Upstream Activating Sequence (UAS) is among the most powerful tools for targeted function and expression of genes and cells *in vivo*. It has been widely used for genetic studies in *Drosophila* (Asakawa & Kawakami, 2008). The system is based on two

components, the yeast transcriptional activator protein, the GAL4 and the UAS sequence, an enhancer to which GAL4 binds in cis-regulatory sites to activate transcription of targeted genes (Busson & Pret, 2007).

In *Drosophila*, the components of the system are carried in separate lines, enabling a great number of combinatorial possibilities as well as expression of transgenes. The driver lines encode for GAL4 expression in a tissue specific manner and the responder lines contain the coding sequence of the gene of interest or transgene under control of UAS sites in which GAL4 will be bound (Busson & Pret, 2007). The two components are brought together in a simple genetic cross. In the progeny of the cross, the transgene is only transcribed in cells or tissues expressing the GAL4 protein (Elliott & Brand, 2008). To express transgenes of targeted ORs in the *Drosophila* empty neuron, we use driver lines containing the Or22a-GAL4 promoter construct (Figure 1.4). In addition, the responder lines carry the coding sequence of the OR of interest that is expressed under control of UAS, (Figure 1.4) which binds to and is activated by GAL4 (Goldman *et al.*, 2005).

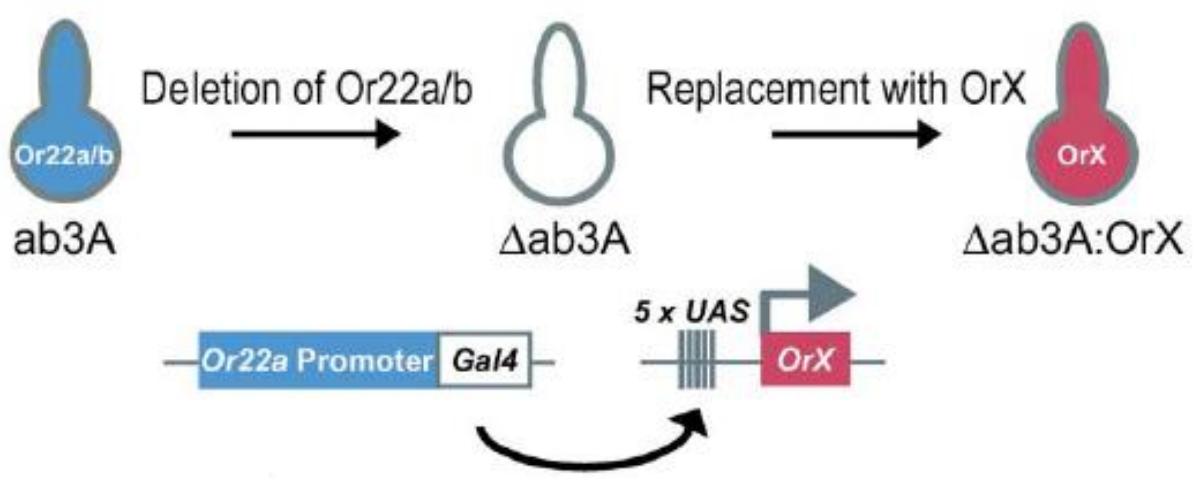


**Figure 1.4: The *Drosophila* empty neuron system** in which Δhalo mutation removes endogenous receptors Or22a and Or22b, hence the empty neuron lacks odour responses. Olfactory receptors are introduced in the empty neuron using GAL4/UAS system. The promoter Or22a-GAL4 drives expression of a receptor from a UAS-Or construct (Hallem *et al.*, 2004).

## 1.2 Statement of the problem

African trypanosomiasis is considered as a major threat to public health and economic growth in most parts of sub-Saharan Africa. In spite of its wide occurrence and implications, trypanosomiasis is still considered a neglected tropical disease by the WHO, largely due to the lack of a successful treatment (Barrett *et al.*, 2007). Presently, there are no HAT vaccines and treatment is hampered by high cost, drug resistance and severe adverse side effects of the available drugs (Benoit *et al.*, 2014). Hence, reduction of the vector populations remains the primary cornerstone of trypanosomiasis control (Aksoy *et al*., 2014). The tsetse vectors are attracted to the hosts by a wide range of olfactory cues that represent critical determinants responsible for disease transmission (Tegler *et al*., 2015). However, the molecular mechanisms by which the flies sense, convert and process sensory cues are still not well understood. Thus, there is a need to understand the molecular mechanisms of olfaction in tsetse flies and the role played by olfactory receptors in mediating transduction of odorants in cells. ORs are potential candidates for guiding discovery of novel attractants and repellents. They therefore merit further investigation for better control of the vector and the disease.

## 1.3 Justification

The economic and public health importance of tsetse flies derives from the fact that they are the only cyclical vectors of African trypanosomes which cause trypanosomiasis. Investigation towards a better understanding of the molecular mechanisms of olfaction in tsetse flies, in order to better define the role played by odorant receptors, for development of strategic interventions to control the vector and the disease is crucial for numerous reasons. Firstly, olfactory responses of the vector to odours represent the basis of disease transmission and are of potential application in vector control due to the designed technologies that are highly specific, affordable, environmentally friendly and applicable for riverine and savannah tsetse (Obiero *et al.*, 2014). However, research in this area is limited and poorly developed. So far, nothing is known about the role of specific odorant receptors mediating transduction of external signals; hence the significance of this study. This limits progress toward identification of novel ligands that could be applied for tsetse fly control. This work serves as a model for the functional characterization of olfactory receptors gene repertoire in tsetse flies.

Secondly, this work is relevant in light of finding that *Drosophila melanogaster* is an efficient expression system for olfactory receptors of tsetse fly. This study serves as a model

for functional characterization of a large number of olfactory receptors in *Glossina* species. Hence, the results of this study complement existing knowledge and open up new strategies for olfactory-based tsetse control and for the development of specific repellents and compounds that could possibly prevent the tsetse fly from mating.

## 1.4 Hypothesis

*Drosophila melanogaster* system is an efficient expression system for *Glossina* odorant receptors genes.

## 1.5 Objectives

### 1.5.1 Main objective

This study aimed to establish the expression of *Glossina fuscipes fuscipes* olfactory receptor Or67d in *Drosophila melanogaster* for further characterization.

### 1.5.2 Specific objectives

1. To identify different copies of the olfactory receptor gene Or67d of *Glossina fuscipes fuscipes.*
2. To amplify the most conserved copy of the gene Or67d of *Glossina fuscipes fuscipes.*
3. To establish the expression of *Glossina fuscipes fuscipes* Or67d using the *Drosophila melanogaster* system.

# CHAPTER TWO

# MATERIAL AND METHODS

## 2.1 Study site

This study was conducted at the Molecular Biology and Biotechnology Unit (MBBU) laboratory at ICIPE Duduville Campus.

## 2.2 Bioinformatics analysis

The cDNA sequences of the copies of Or67d gene in *G. f. fuscipes* were retrieved from VectorBase (Megy *et al.*, 2012) using their VectorBase IDs as annotated in Macharia *et al.*, 2016. Sequences were translated using the online tool Emboss transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). The amino acid sequences were found in NCBI using BLASTp algorithm with an e-value cut-off of $1e^{-5}$ to determine percentage identity with Or67d of *Drosophila melanogaster* (Dmel.), *Musca domestica* (Md) and *Lucilia cuprina* (Lc) whose genome has recently been released (Anstead *et al.*, 2015). The retrieved copies were validated through sequence-based search for the presence of the specific 7tm_6 odorant receptor domain (Robertson *et al.*, 2003) against the Conserved Domains Database (CDD) (Marchler-Bauer *et al.*, 2014). The sequences with conserved domains were subjected to multiple sequence alignment using the MUSCLE algorithm (Edgar, 2004) on the online EBI web server (http://www.ebi.ac.uk/Tools/msa/muscle/) with default settings. A phylogeny tree for the aligned sequences was constructed using PhyML (ref) with a bootstrap of 100. *Drosophila melanogaster* Or67d gene was used as an out-group for rooting the tree.

## 2.3 Production of blunt-end PCR products

The most conserved sequence of Or67d of *G. f. fuscipes* was synthesized from GenScript Hong Kong according to the manufacturer descriptions (GenScript®, Inc., USA). The sequence was cloned in the vector pUC57 by *Eco*RV at the multiple cloning sites 429-434 (GAT▼ATC).

To produce blunt-end PCR products, the plasmid DNA was amplified using the designed primers (Forward primer 5'-CACCGACATGGTCATGAAACGACTTGAAAGGTG-3' and Reverse primer 5'-TTATTTTAATTCTCTCTTTAATCCCACCAC-3') that contain CACC

and kozak sequences necessary for proper directional cloning and translation initiation of the PCR product, respectively.

The polymerase chain reaction (PCR) was performed using the thermostable proofreading Phusion™ Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific, Inc., USA) which possesses the 5' to 3' DNA polymerase activity and 3' to 5' exonuclease activity, generating blunt ends in the amplification product. The amplification was done in a programmed thermal cycler Applied Biosystems® ProFlex™ PCR System (Life Technologies, CA, USA). The reaction mix consisted of 0.4 µl of 10mM dNTPs mix, 2.0 µl of 5X Phusion GC buffer, 1.0 µl of 10 µM of each primer, 0.2 µl of Phusion DNA polymerase, 1µl of DNA template and 4.4 µl of nuclease free water to give a total volume of 10 µl. Cycling conditions involved initial denaturation at 98 ºC for 1 minute followed by 35 cycles of denaturation at 98 ºC for 15 seconds, annealing at 60 ºC for 30 seconds, elongation at 72 ºC for 1 minute then final extension at 72 ºC for 15 minutes.

Subsequently, PCR products were gel electrophoresed on 1.5% w/v agarose gel using 1X TAE buffer (appendix 1) run for 1h 30 minutes at 70 volts, then viewed under UV-light transilluminator (Eastman Kodak Company, NY, USA) and gel purified as described below.

**2.4 Gel extraction of PCR products**

Following gel electrophoresis, the gel was viewed under UV-light using the Kodak gel logic 200 UV transilluminator (Eastman Kodak Company, NY, USA) and the amplified gene products were subsequently excised using sterile sharp blades. Gel purification was carried out using QIAquick® Gel Extraction Kit (QIAGEN Group, USA) as described below, for removal of all sources of nuclease contamination. The excised gel bands were transferred into sterile 1.5 ml microcentrifuge tubes and weighed. One volume of the gel slices was dissolved in three volumes of the solubilization buffer QG. The mixture was incubated at 50ºC in a water bath for about 10 minutes with occasional vortexing to ensure complete dissolution and melting of the gel. One volume of isopropanol to one volume of the gel was added to the sample and thoroughly mixed. The mixture was applied to a QIAprep spin column and centrifuged at 13,000 rpm (17,900x g) for 1 minute and the flow-through was then discarded.

Thereafter, 500 µl of the resuspension buffer QG was added to the same QIAprep column and centrifuged at 13,000 rpm for 1 minute; and the flow-through discarded.

Subsequently, 750 µl of the wash buffer PE was added to the same QIAquick column and left to stand for 5 minutes. This was followed by centrifugation at 13,000 rpm for 1 minute and the flow-through discarded. The QIAquick column was centrifuged again at 13,000 rpm for 1 minute to remove the residual buffer PE. Lastly, to elute the amplicon, the QIAprep column was transferred to a clean 1.5 ml microcentrifuge tube and 30 µl of elution buffer EB was added to the center of the QIAprep spin column and left to stand for 5 minutes. The column was then centrifuged at 13,000 rpm for 2 minutes. The flow-through containing the amplification product was retained for subsequent cloning.

Afterwards, the extracted PCR product (3 µl) was gel electrophoresed on a 1.5% w/v agarose gel prepared with 1X TAE buffer (appendix 1) run for 1h 30 minutes at 70 volts; to determine the success of the extraction. Finally, the product was also sent for sequencing.

Sequences were analyzed and edited with Bioedit version 7.2.5.0 software (Hall, 1999). The consensus sequence resulted from the analysis was translated using the online tool expasy (http://web.expasy.org/translate/). The complete open reading frame was found on NCBI using BLASTp algorithm with an e-value cut-off of $1.0e^{-0.5}$ to find similar hits to Or67d.

### 2.5 Gateway cloning technology for expression of gene

The Gateway® Cloning is a universal cloning method that relies on the site-specific recombination properties of bacteriophage lambda which integrates into *Escherichia coli*. It provides a rapid and efficient way to move DNA sequences into a great number of vector systems for functional and protein expression analysis (Landy, 1989).

#### 2.5.1 TOPO® cloning reaction

Directional TOPO® cloning allows cloning of blunt-end PCR products into a vector for entry into the gateway system at greater than 90% efficiency with no ligase, post PCR procedures or restriction enzymes required.

Hence, the blunt-end purified PCR product was directionally TOPO® cloned into the pENTR™/D- TOPO® vector (Thermo Scientific, Invitrogen, Inc., USA) to generate an entry clone, with the gene of interest flanked by attL1 and attL2 sites of the vector.

The ligation reaction was done at a molar ratio of 3:1 of PCR product: TOPO® vector. The mix consisting of 1 µl of sterile water, 1 µl of salt solution, 1 µl of TOPO® vector and 3 µl of purified PCR product was prepared on ice, mixed thoroughly and incubated at room

temperature (28 ºC) for 30 minutes. Thereafter, the construct was transferred on ice before proceeding to transformation into high efficiency TOP 10 chemically competent *E. coli* cells.

### 2.5.2 Transformation

The pENTR™/D- TOPO® construct (ligation mixture) was used to transform high efficiency One Shot® TOP10 chemically competent *E. coli* cells included in the pENTR™/D- TOPO® cloning kit (Invitrogen, Inc., USA) as follows. For each reaction, a sterile 1.5 ml microcentrifuge tube was pre-chilled on ice, 3 µl of the ligation reaction mix was aliquoted and added to the empty pre-chilled tube. Then, a vial (50 µl) of One shot® TOP10 chemically competent *E. coli* was added to the ligation reaction mix and gently mixed by tapping. The mixture was incubated on ice for 20 minutes. Subsequently, without shaking, the transformation was carried out by heat-shocking the mixture at 42 ºC for 45 seconds in a water bath and thereafter the tubes were immediately returned on ice for 2 minutes. Subsequently, 750 µl of room temperature S.O.C. medium (appendix 2) was added to the transformation mixture. Afterwards, the cells were incubated for 1.5 hours at 37 ºC with horizontal shaking (150 rpm). The cells were concentrated by centrifuging at 3000 x g for 5 minutes and 250 µl of the broth was retained for resuspension of the pellet. The 250 µl resuspended cells were plated on LB agar plates containing kanamycin at a final concentration of 50 µg/ml (appendix 2) and incubated overnight at 37 ºC.

### 2.5.3 Selection of positive colonies

Single white colonies were randomly selected and picked from the LB agar plates with a micropipette tip and diluted in nuclease free water to serve as template DNA for the PCR reaction. The amplification was carried out using the thermostable proofreading Phusion™ Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific, Inc., USA) and previously designed primers that anneal to the Or67d gene region. The reaction was done in a programmed thermal cycler Applied Biosystems® ProFlex™ PCR System (Life Technologies, Inc., USA).

The reaction mix consisted of 2.0 µl of 5X Phusion GC buffer, 0.4 µl of 10mM dNTPs mix, 1.0 µl of 10 µM of each primer, 0.2 µl of Phusion DNA polymerase, 1 µl of DNA template and 4.4 µl of nuclease free water to give a total volume of 10 µl. Cycling conditions involved initial denaturation at 98 ºC for 3 minutes followed by 35 cycles of denaturation at 98 ºC for 30 seconds, annealing at 60 ºC for 1 minute, elongation at 72 ºC for 1 minute then final

extension at 72 ºC for 15 minutes. Subsequently, amplification products were electrophoresed on 1.5 % w/v agarose gel prepared with 1X TAE buffer (appendix 1), run for 1.5 hours at 70 volts and the positive colonies identified.

### 2.5.4 Plasmid purification

Plasmids from the transformed positive colonies were purified using QIAprep® Spin Miniprep Kit (QIAGEN Group, USA) following the manufacturer's instructions. Single positive white colonies were picked from the LB agar plates using sterile micropipette tips and used to inoculate 5 ml of LB broth (appendix 2) containing kanamycin at a final concentration of 50µg/ml. The cells were grown overnight at 150 rpm in a shaking incubator at 37 ºC.

The bacterial cultures were pelleted by centrifugation at 8000 rpm for 5 minutes at room temperature (25 °C). The pelleted cells were resuspended in 250 µl of resuspension buffer, Buffer P1, by flicking the tubes. Then, the cells were lysed by adding 250 µl of lysis buffer, Buffer P2 and were gently mixed by inverting the tube 4 to 6 times. Afterwards, the mixture was neutralized by addition of 350 µl of neutralization buffer, Buffer N3 followed by inversion of the tubes for 4-6 times. The cells were then centrifuged at 13,000 rpm for 10 minutes and the supernatants were applied into QIAprep spin columns. The spin columns were centrifuged for 1 minute at 13,000 rpm and the flow-through discarded. Then, 750 µl of wash buffer, Buffer PE, was added to each column and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded and the spin columns were centrifuged again at the same conditions to remove the residual wash buffer. Thereafter, the columns were transferred to new sterile 1.5 ml microcentrifuge tubes and the plasmids were eluted with 30 µl of elution buffer, Buffer EB, added to the center of the column and left to stand for 5 minutes before centrifugation at 13,000 rpm for 1 minute. The recovered plasmids were gel electrophoresed on a 1.5 % w/v agarose gel prepared with 1x TAE buffer (appendix 1) run for 1.5 hours at 70 volts and then viewed under UV-light transilluminator (Eastman Kodak Company, NY, USA).

### 2.5.5 Analyzing constructs by PCR

To further confirm the presence of the gene insert, the recombinant purified plasmids were analyzed by PCR using the previously designed primers specific for Or67d gene with the same conditions as described in section 2.3. The PCR products were gel electrophoresed on

1.5% w/v agarose gel using 1X TAE buffer run for 1h 30 minutes at 70 volts, and then viewed under a UV-light transilluminator (Eastman Kodak Company, NY, USA).

### 2.5.6 Sequencing

To determine whether the cloned gene Or67d is in the correct orientation and in its complete open reading frame, the purified constructs were sent to Macrogen Inc. (South Korea) for sequencing using M13 forward (5´-GTAAAACGACGGCCAG-3´) and M13 reverse (5´-CAGGAAACAGCTATGAC-3´) primers. Sequence analysis was done using Bioedit version 7.2.5.0 software (Hall, 1999)**.**

### 2.5.7 LR recombination reaction

The LR (recombination reaction between attL and attR sites) recombination reaction was performed to transfer the gene of interest Or67d flanked by attL1 and attL2 of the pENTR™/D- TOPO® entry vector into the attR-containing destination vector ptW to create an attB-containing expression vector. The reaction was carried out by mixing 1.5 µl of the purified entry clone with 0.5 µl of the destination vector ptW. The volume was topped up to 8 µl with TE buffer. 4 µl of the Gateway® LR Clonase® II enzyme briefly vortexed was added to the sample and to the positive control (pENTR™-gus). The mixture was vortexed twice for 2 seconds each time and incubated at 25 ºC for an hour. 2 µl of proteinase K was then added to each reaction sample and incubated for 10 minutes at 37 ºC.

### 2.5.8 Transformation

The expression clone obtained from the LR recombination reaction was used to transform Library Efficiency® DH5α™ Competent *E. coli* cells (Invitrogen, Inc., USA). For each reaction, a sterile 1.5 ml microcentrifuge tube was pre-chilled on ice, 3 µl of the expression vector was aliquoted and added to the pre-chilled eppendorf tube.

50 µl of DH5α™ Competent *E. coli* cells was added to the expression vector and gently mixed by tapping. Afterwards, the mixture was incubated on ice for 20 minutes, without shaking, and the transformation performed by heat shocking the mixture at 42 ºC for exactly 45 seconds in a water bath. The tubes were immediately returned on ice for 2 minutes after heat shock, then, 750 µl of pre-warmed S.O.C. medium (appendix 2) added to the transformation mixture. Thereafter, the cells were incubated for an hour and half at 37 ºC in a

shaking incubator at 150 rpm. Cells were concentrated by centrifugation at 3000 x g for 5 minutes and 250 µl of the broth used to resuspend the pellet. The resuspended cells were plated on LB agar plates containing 50 µg/ml of ampicillin (appendix 2) and incubated at 37 ºC overnight.

### 2.5.9 Selection of positive clones

To select positive clones, single white colonies were picked randomly from the LB agar plates with a micropipette tip and diluted in nuclease free water to serve as template DNA for the PCR reaction. The amplification was carried out using the thermostable proofreading Phusion™ Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific, Inc., USA) and the previously designed primers annealing the Or67d gene region as described in section 2.5.3. Thereafter, the amplicons were gel electrophoresed on 1.5 % w/v agarose gel, run for 1.5 hours at 70 volts and the positive colonies identified.

### 2.5.10 Plasmid purification

Plasmids from the transformed positive colonies were purified using QIAprep® Spin Miniprep Kit (QIAGEN Group, USA) as described in section 2.5.4. Subsequently, the recovered plasmids were gel electrophoresed on a 1.5 % w/v agarose gel prepared with 1X TAE buffer (appendix 1) run for 1.5 hours at 70 volts and then viewed under UV-light transilluminator (Eastman Kodak Company, NY, USA).

### 2.5.11 Analyzing constructs by PCR

To further confirm the presence of the insert Or67d, the recombinant purified plasmids were analyzed by PCR using the previous designed primers specific for Or67d gene with the same conditions as described in section 2.3. The PCR products were gel electrophoresed on 1.5% w/v agarose gel prepared with 1X TAE buffer (appendix 1) run for 1h 30minutes at 70 volts, and then viewed under a UV-light transilluminator (Eastman Kodak Company, NY, USA).

### 2.5.12 Sequencing

To determine whether the cloned gene Or67d is in the correct orientation and in its complete open reading frame, the purified constructs were sent to Macrogen Inc. (South Korea) for sequencing using M13 forward (5´-GTAAAACGACGGCCAG-3´) and M13 reverse (5´-

CAGGAAACAGCTATGAC-3´) primers. Sequencing analysis was done using Bioedit version 7.2.5.0 software (Hall, 1999).

### 2.6 Transgenic expression of GffOr67d4 in *Drosophila* OSNs

The purified recombinant attB-expression vector ptW containing the transgene (GffOr67d4) cloned downstream of the upstream activating sequence (UAS) sequence was injected in *Drosophila melanogaster* embryos $w^{118}$ flies that are homozygous recessive for the *w*- allele giving them white eye color.

The injections were carried out by Fly Genetic Services in France (http://www.geneticservices.com/injectionservices.htm). The recombinant plasmid contains a marker gene, the dominant $w^{+}$ allele which is expressed as red eye colour in the flies; hence it is used to identify transgenic flies in which the plasmid DNA has been successfully transposed into a recipient chromosome of the fruit fly. This donor plasmid was injected together with another plasmid that carries the P element encoding the transposase, required to catalyze the transposition of the donor plasmid into the fly genome. Following injections, crosses were established to confirm that fly lines were homozygous for the insertion, depicted by a strong red eye color.

The obtained transgenic strains UAS-GffOr67d flies were crossed with Or22a-Gal4 strains (from *Drosophila* Stock Center Bloomington, IN). These flies expressing the yeast transcription factor Gal4 drive expression of Or67d cloned downstream of the UAS sequences by binding to the UAS sequences (Dobritsa *et al.*, 2003; Syed *et al.*, 2006).

The expression of the gene in the flies was assessed by a two-step RT-qPCR (quantitative reverse transcription polymerase chain reaction) analysis as follows.

The RNA was extracted using the direct-zol™ RNA MiniPrep (ZYMO Research, US) as described below. 5 to 10 live-flies males and females were homogenized in 600 µl of Trizol reagent with a pestle in an RNase-free tube. The sample was subjected to centrifugation at 13000 x g for 30 seconds at 4 ºC. The supernatant was transferred into a new RNase-free tube. RNA purification was carried out by transferring 600 µl of 100% ethanol to the supernatant and mixed thoroughly. Then, the mixture was transferred into a Zymo-Spin™ IIC Column in a collection tube and centrifuged at 13000x g, at 4 ºC for 1 minute. The flow-through was discarded and the column transferred into a new collection tube.

The DNase treatment was then performed by addition of 400 µl of RNA Wash buffer to the column followed by centrifugation at 13000x g for 1 minute. The flow-through was discarded and the column transferred into a new collection tube. A mix of 75 µl of DNA digestion buffer and 5 µl of DNaseI was directly added to the column matrix and incubated at room temperature for 15 minutes. 400 µl of Direct-zol™ RNA PreWash was added to the column prior to centrifugation at 13000x g for 1 minute. The flow-through was discarded, and the column transferred into a new collection tube and the procedure repeated. Then, 700 µl of RNA Wash buffer was added to the column and centrifuged for 2 minutes; after which the flow-through was discarded and the column transferred into a new collection tube. The column was dried by centrifugation at 13000x g for 1 minute then transferred carefully into an RNase-free tube.

The RNA was eluted by adding 30 µl of DNase/RNase-Free water directly to the column matrix and centrifuged at 13000x g for 1 minute. The isolated RNA was quantified using the Nanodrop reader (Thermo scientific, UV-VIS spectrophotometer, California, USA). Blanking was done with 1 µl of nuclease free water prior to loading 1 µl of RNA sample. Data was exported onto a Microsoft Excel spreadsheet for further analysis. Then, the RNA was stored at -70 ºC before proceeding to cDNA construction.

The generation of cDNA was undertaken using a High-capacity cDNA Reverse Transcription (RT) kit (Applied Biosystems, USA) following the manufacturer's instructions. The 2X RT master mix consisted of 2.0 µl of 10X RT buffer, 0.8 µl of 25X 100 mM dNTPs mix, 2.0 µl of 10X RT Random primers, 1.0 µl of MultiScribe™ Reverse Transcriptase, 1.0 µl RNase Inhibitor, 3.2 µl of nuclease-free water. The reaction was gently mixed and placed on ice.

The cDNA RT reaction was prepared by adding 10 µl of 2X RT master mix to each individual tube containing 1 ng of RNA sample. The tubes were sealed and briefly centrifuged to spin down the contents. Cycling conditions consisted of one step at 25 ºC for 10 minutes, followed by a second step at 37 ºC for 120 minutes, a third step at 85 ºC for 5 seconds and then a final step of holding at 4ºC. The cDNA generated was quantified using Nanodrop reader (Thermo scientific, UV-VIS spectrophotometer, California, USA). Blanking was done with 1 µl of nuclease free water prior to loading 1 µl of each cDNA sample. Then, the samples were stored at -20 ºC ready for conventional PCR and qPCR.

The transcription levels of the transgene, which give an indication of expression levels, were first evaluated by reverse transcription polymerase chain reaction (RT-PCR) using the

previous designed primers specific for GffOr67d4 gene with the same conditions as described in section 2.3. The same conditions were applied for amplification of the internal reference gene (alpha-tubulin 84B, size 96bp) using universal primers (forward primer 5'-TGTCGCGTGTGAAACACTTC-3' and reverse primer 5'-AGCAGGCGTTTCCAATCTG-3') as described in Ponton *et al.*, 2011.

The amplicons were gel electrophoresed on 1.5% w/v agarose gel using 1X TAE buffer run for 1h 30 minutes at 70 volts, and then viewed under a UV-light transilluminator (Eastman Kodak Company, NY, USA). The gel products were purified as described in section 2.4. The purified products were then sent to Macrogen for sequencing and sequences analysed with Bioedit version 7.2.5.0 software (Hall, 1999). The expression of the gene in wild *Drosophila melanogaster* was also investigated using the previously designed primers with the same conditions as described in section 2.3 to confirm that the primers used were species-specific.

Furthermore, the expression of GffOr67d4 gene in *Drosophila melanogaster* was quantified with Fast SYBR® green detection dye using Stratagene Mx3005P real-time qPCR system (Agilent technologies, USA). Amplification of the aforementioned gene was performed using the previous designed oligonucleotide primers (section 2.3). Reactions were performed in a total volume of 20 µl containing 12.5 µl of SYBR® green master mix (Thermo scientific, USA) in the presence of 1 µl of each primer (10µM), 9.5 µl of water and 1µl of cDNA (0.1µg). Each reaction was run in triplicates. The same reaction conditions were applied to alpha-tubulin 84B that was used as an internal reference gene. Cycling conditions involved initial denaturation at 95 ºC for 10 minutes followed by 40 cycles of denaturation at 95 ºC for 15 seconds, annealing at 65ºC for 45 seconds, elongation at 72 ºC for 1 minute; this was followed by 1 cycle of 95 ˚C (30 sec), 55 ˚C (1 minute) and 95 ˚C (30 sec) for all sample genes.

The expression levels of the transgene GffOr67d4 in male and female transgenic flies were determined as the number of cycles required for the amplification to reach the defined threshold in the exponential phase of the qPCR reaction known as Ct (threshold cycle) values. As the Ct value is inversely proportional to the logarithm of the initial amount of the amplicons, transcription was shown as the inverse logarithm of Ct values as described in Wade *et al.*, 2005.

# CHAPTER THREE

# RESULTS

## 3.1 Bioinformatics analysis

Of the five copies of *G. f. fuscipes* Or67d gene present in the fly genome, only sequences of the four complete copies were retrieved from VectorBase (vectorbase.org) and analysed (Table 3.1).

All the gene copies contained the definitive olfactory receptor domain (7tm_6) except the first gene copy GffOr67d1 that does not have the domain signature, and therefore was discarded for further analysis (Table 3.1). GffOr67d4 presented the highest percentage homology to DmelOr67d (34.08%) and to LcOr67 (40.26%) while GffOr67d5 displayed the highest percentage homology to MdOr67d (39.77%) (Table 3.1).

**Table 3.1: *G. f. fuscipes* Or67d gene copies information**

| GffOr67d Copies | | | | Percentage homology | | |
|---|---|---|---|---|---|---|
| Gene ID | Vectorbase ID (Transcripts IDs) | Length | Conserved domain (7tm_6) | DmelOr67d | LcOr67d | MdOr67d |
| GffOr67d1 | GFUI007388-RA | 654 bp | No | 32.41% | 32.39% | 26.73% |
| GffOr67d4 | GFUI043789-RA | 1095 bp | Yes | **34.08%** | **40.26%** | 34.84% |
| GffOr67d5 | GFUI036188-RA | 1062 bp | Yes | 33.90% | 39.94% | **39.77%** |
| GffOr67d6 | GFUI022534-RA | 1107 bp | Yes | 33.54% | 36.94% | 32.08% |

The abbreviation ID means identity. The percentage in bold shows the highest percentage identity of GffOr67d4 to DmelOr67d (34.08%) and to LcOr67d (40.26%) as well as the highest percentage identity of GffOr67d5 to MdOr67d (39.77%).

A phylogenetic tree of the GffOr67d gene copies against DmelOr67d depicted a closer relationship between GffOr67d4 (GFUI043789-RA), GffOr67d5 (GFUI036188-RA) and DmelOr67d compared to GffOr67d6 (Figure 3.1). All results pointed towards GffOr67d4 as the most conserved copy of the gene in *Glossina fuscipes fuscipes*, hence it was used for this study.

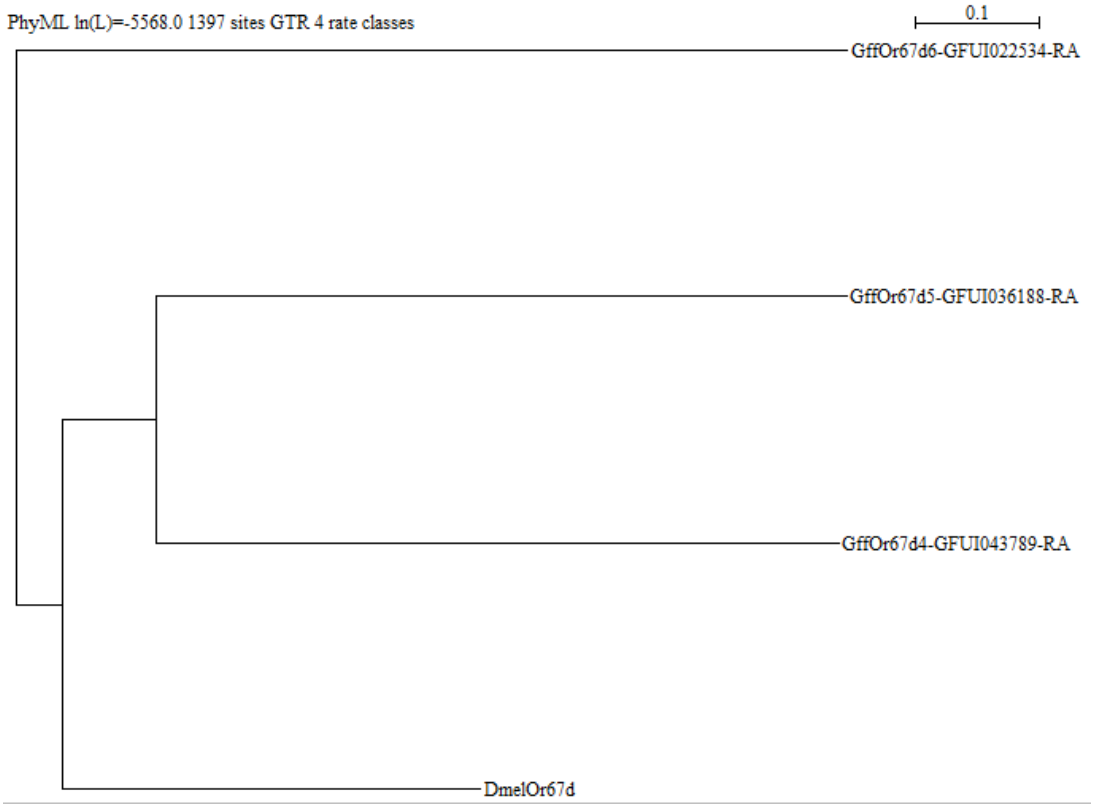


**Figure 3.1: Phylogenetic tree of GffOr67d gene copies (GffOr67d4, GffOr67d5 and GffOr67d6) together with DmelOr67d used as an out-group.**

### 3.2 Generation of UAS-Gffor67d4 construct

The amplification of GffOr67d4 gene from the pUC57 vector, where it was cloned, using the designed gene-specific primers showed DNA bands of approximately 1.1 kb in a 1.5% w/v ethidium bromide stained agarose gel (Figure 3.2 A). The amplicons were gel purified (Figure 3.2 B), prior to sequence analysis. Sequence analysis resulted in a consensus sequence of about of 1,182 base pairs (including flanking regions). The alignment of the translated consensus sequence using the online tool, BLASTp, showed hits to Or67d of *Drosophila melanogaster*, *Lucilia cuprina*, *Musca domestica* and showed 95% identity to the gene of interest.

After directional cloning of the insert into pENTR™/D- TOPO® vector, transformation of the ligation mixture resulted in fewer white colonies (10-20) on the plate. Amplification of the gene from the purified constructs was successful as confirmed by agarose gel electrophoresis of amplified products with bands at approximately 1.1 kb (Figure 3.3 A); revealing the presence of the insert inside the construct. Sequencing results revealed the total length of the

gene inserted between attL1 and attL2 sites of the pENTR™/D-TOPO® vector (Figure 3.3 C), as expected.

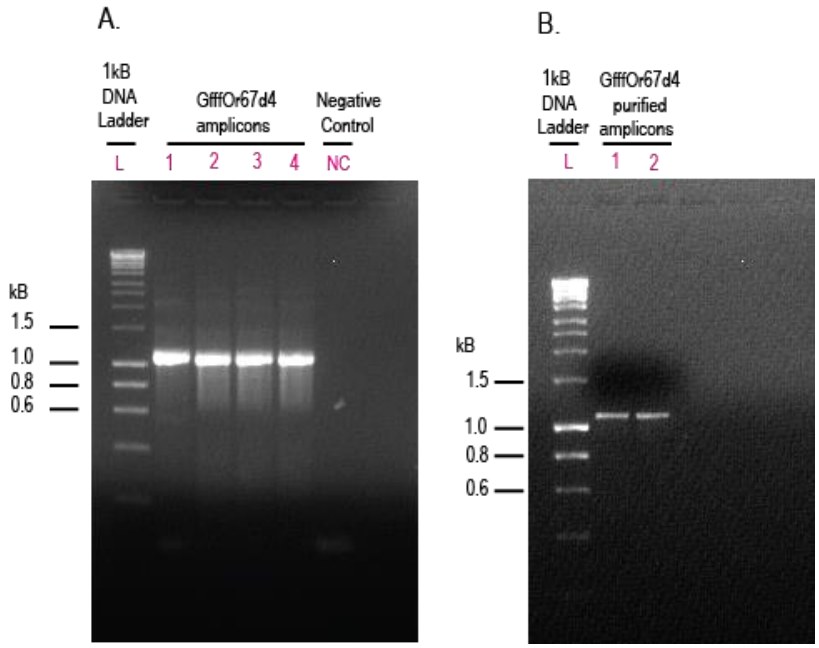


**Figure 3.2: Agarose gel pictures showing DNA bands of GffOr67d4 PCR amplification products and DNA bands of GffOr67d4 purified amplicons.** The negative control (NC) is nuclease free water. (A) Amplification of GffOr67d4 from pUC57 vector using the designed primers necessary for directional cloning producing 1.1 kb products (B) Gel purification of GffOr67d4 amplicons.

The LR recombination reaction performed between the recombinant pENTR™/D- TOPO® vector and the destination vector pTW was successful, resulting in attB-containing expression vector. Transformation of the construct into DH5α competent cells yielded hundreds of recombinant cells. The amplification of GffOr67d4 gene from the purified recombinant pTW vector (attB-expression vector) revealed bands at approximately 1.1 kB, in an ethidium-bromide stained agarose gel; the size of GffOr67d4 gene being 1095bp (Figure 3.3 B). Sequence analyses through Bioedit v.7.2.5.0 and by Expasy translate tool confirmed the successful cloning of GffOr67d4 gene into pTW vector downstream of UASt promoter between attB1 and attB2 sites (Figure 3.3 D).

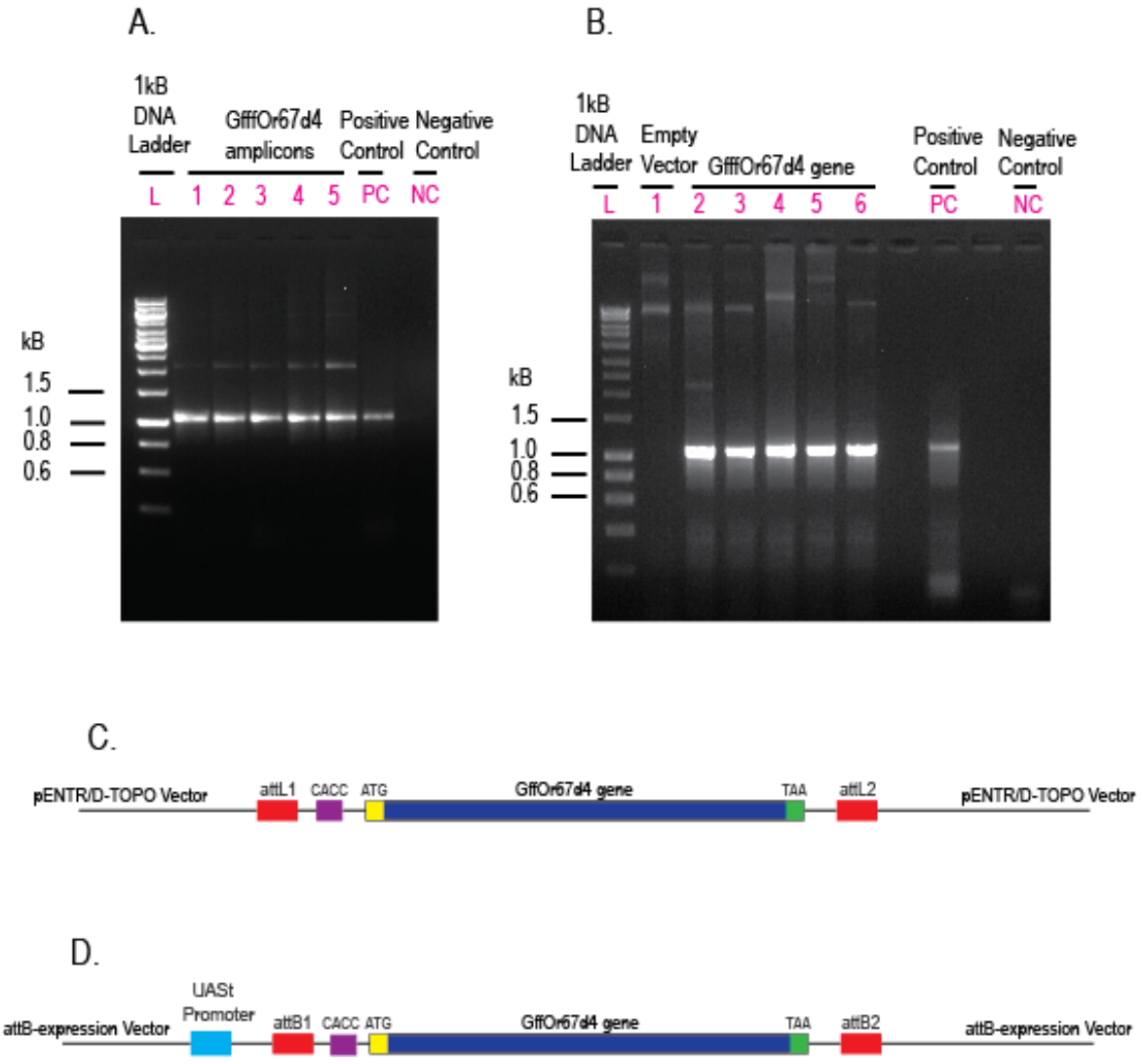


**Figure 3.3: Agarose gel pictures showing DNA bands of GffOr67d4 amplification products after subsequent cloning and bioinformatics analysis.** (A) Gels showing GffOr67d4 amplification products from recombinant pENTR™/D- TOPO® vector. (B) Gels showing GffOr67d4 amplification products from recombinant pTW plasmid. The positive control (PC) is GffOr67d4 gene and the negative control (NC) is nuclease free water. (C) Schematic representation of recombinant pENTR™/D- TOPO® vector after sequence analysis. (D) Schematic representation of recombinant attB-expression vector (pTW vector) after sequence analysis.

### 3.3 Transgenic expression of GffOr67d4 in *Drosophila* OSNs

The purified recombinant attB-expression vector was injected into the fly strain mutant for the *white* gene (hence having white eyes) by Fly genetic services in France.

Crosses carried out to ensure that transgenic fly stocks were homozygous for the insertion resulted in flies having very strong red eyes, a marker of successful transposition of the gene into the fly genome and flies with white eyes in which the gene was not inserted.

The presence of the transgene in transgenic flies both males and females was confirmed by agarose gel electrophoresis of RT-PCR products, that revealed bands at approximately 1.1 kb (Figure 3.4 A). The amplification of the control gene, alpha-tubulin 84B, from the same transgenic flies was also successful, revealing bands of approximately 100 bp in an 1.5 % w/v ethidium-bromide stained agarose gel; the size of the gene being 96 bp (Figure 3.4 B).

To verify that the gene being amplified from transgenic *Drosophila* was not DmelOr67d, amplification of the transgene from wild-type *Drosophila melanogaster* was performed and no products were obtained as shown in Figure 3.4 C. These results confirmed that the primers were specific to GffOr67d4 and that the gene being expressed is not a *Drosophila melanogaster* homolog.

In addition to RT-PCR, the specificity of the transgene amplification was confirmed by the presence of a single, sharply defined peak obtained in the dissociation curve of qPCR analysis (Figure 3.5). The curve showed that melting point of GffOr67d4 and alpha-tubulin 84B occurred at 79.75ºC and 86.35ºC, respectively. The Ct values for GffOr67d4 and alpha-tubulin 84B (Table A5.1 and A5.2 in Appendix 5) were obtained from the amplification curves (Figure 3.6). The internal gene (alpha-tubulin 84B) was shown to be uniformly expressed in male and female samples (Table A5.1), proving to be an ideal control gene. GffOr67d4 gene was expressed in both male and female *Drosophila* flies (Figure 3.7).

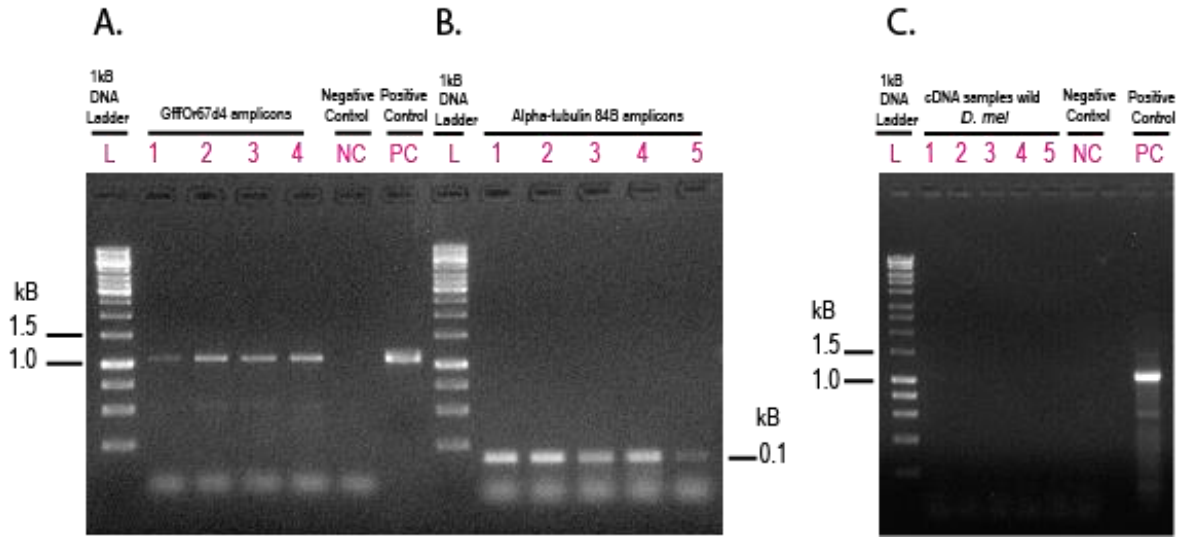


**Figure 3.4: Agarose gel images showing DNA bands of GffOr67d4 and alpha tubulin 84B RT-PCR products from cDNA of transgenic flies and wild type flies.** (A) Amplification of GffOr67d4 from cDNA of transgenic flies both females (1, 3) and males (2, 4) (B) Amplification of reference gene alpha-tubulin 84B from transgenic flies both females (1, 3, 5) and males (2, 4) (C) Amplification of GffOr67d4 gene from wild-type *Drosophila melanogaster* females (1,3,5) and males (2,4) showing no products. The negative control (NC) is nuclease free water and positive control (PC) is GffOr67d4 gene.

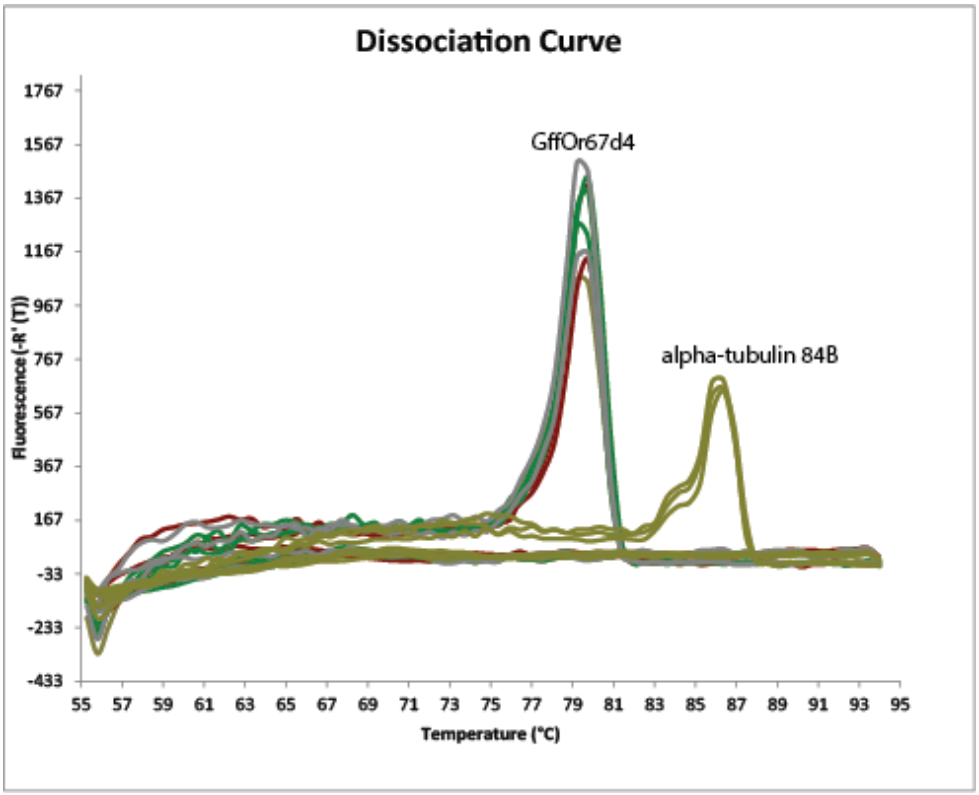


**Figure 3.5: Dissociation curves of GffOr67d4 and alpha-tubulin 84B showing single amplification peaks at 79.75ºC and 86.35ºC respectively, as an indication of specific amplification**.

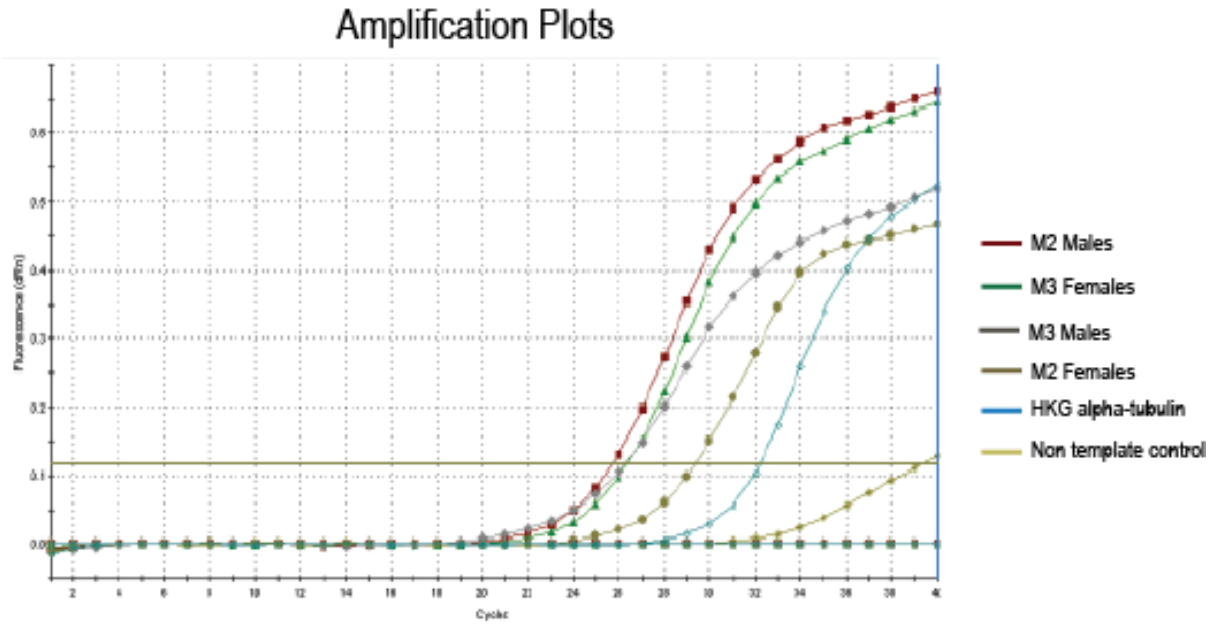


**Figure 3.6: Amplification curves of GffOr67d4 and alpha-tubulin 84B in all transgenic fly samples (M3 and M2).** The threshold line intercept the log phase of the amplification curve and the intersection point defines the Ct value, the cycle at which the fluorescence resulting from amplification is detected by the instrument.

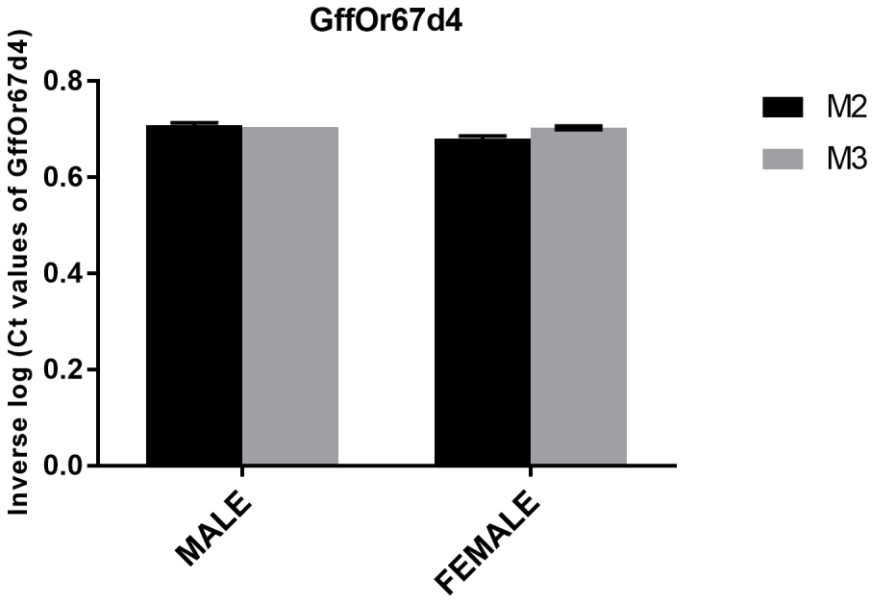


**Figure 3.7: Expression levels of GffOr67d4 gene in males and females transgenic *Drosophila* flies, as inverse log of Ct values. M2 and M3 are the different samples.**

# CHAPTER FOUR

# DISCUSSION

## 4.1 Bioinformatics analysis

The genome content of ORs in tsetse flies showed that six putative ORs were homologous to the single *Drosophila melanogaster* Or67d gene, revealing an expansion of the gene in the *Glossina morsitans morsitans* genome (Obiero *et al.*, 2014) and also in the *G. fuscipes fuscipes* genome (Macharia *et al.*, 2016).

However, of the five gene copies present in *G. f. fuscipes*, four were complete and already annotated by Macharia *et al.*, 2016. The incomplete copy was not considered in our study for bioinformatics analysis due to additional annotations required. One copy of the gene (GffOr67d1) did not contain the specific 7tm_6 odorant receptor domain. This could be linked to the fact that chemosensory genes in tsetse genome are distributed across distant regions in the genome, probably due to transposition events that might have led to the loss of gene function over the course of evolution. This is in contrast with other insects such as *D. melanogaster* whose genes occur in clusters and are less exposed to a loss of their function.

Based on the phylogenetic reconstruction of GffOr67d gene copies (GffOr67d4, GffOr67d5 and GffOr67d6) together with DmelOr67d used as an out-group, we suggested that GffOr67d4 (GFUI043789-RA) and GffOr67d5 (GFUI036188-RA) are more recently emerged gene copies (created by a gene duplication event after the split between the lineages leading to *Drosophila* and *Glossina*). These genes have a higher level of sequence conservation with DmelOr67d, however, GffOr67d6 (GFUI022534-RA) was shown to diverge more from the other copies during evolution. An assessment of its function should be carried out in further studies. Hence, from our data, we implied that GffOr67d4 is the most conserved copy of the DmelOr67d in the Tsetse fly.

## 4.2 Generation of UAS-GffOr67d4 construct

The synthesis of GffOr67d4 gene inserted into the pUC57 plasmid by GenScript saved the time-consuming and labour intensive steps of RNA extraction and cDNA synthesis of our gene of interest. The use of proofreading thermostable Phusion Taq polymerase which

contains 3' to 5' exonuclease function as recommended by Gonzalez *et al*., 2016 reduces the likelihood of incorrect sequence and resulted in complete sequence of the gene being amplified, containing CACC sites and kozak sequences for correct directional cloning (approximately 1.1 kB).

The directional cloning of our gene of interest into the pENTR™/D- TOPO® vector yielded fewer recombinant white colonies (10-20) than normally expected (several hundred colonies). This proves that the ligation was not so efficient most probably due to length of the insert. GffOr67d4 being 1095 bp, greater than 1kB, it is considered as "long insert" and the TOPO cloning has been shown to be significantly less efficient with long inserts (Litterer, 2009). Despite this, the TOPO cloning method saved the labour-intensive and time-consuming procedures of classical plasmid construction, since no restriction enzyme and further ligation step were needed. With this method, the whole cloning process was done two days faster than it would have been done with traditional cloning methods (Patel, 2009).

In contrast, the high number of colonies obtained after transformation of the ligation mixture of the pENTR™/D-TOPO® vector containing GffOr67d with destination vector pTW revealed the high efficiency of the LR recombination reaction. Since the ccdB gene is present in the pTW destination, it was used as a negative selectable marker to interrupt growth of cells that did not take up the gene (Bernard, 1996). All the cells that grew on the plate are believed to be recombinants and to have successfully taken the gene of interest (Xu *et al.*, 2008). However, this was not the case in our experiments since one of the plasmids purified from recombinant colonies did not contain the gene of interest (Fig 3.3 B). We probably unintentionally did not add the template DNA that is the plasmid during the amplification reaction.

### 4.3 Transgenic expression of GffOr67d in *Drosophila*

The *white* mini-gene present in the UAS-GffOr67d4 construct was found to be a good marker in this study since transgenic flies were distinguishable by their strong red eyes, as described in Edwards *et al*., 1989; compared to non-transgenic flies who present typical white eyes. The advantage of such a marker gene which confers a visible phenotype to the transformants, is that it can be easily followed in subsequent generations. This makes the *white* mini-gene the most convenient and frequently used marker gene.

Both RT-PCR and qPCR revealed that the transgene was expressed in *Drosophila melanogaster* relative to the internal gene alpha-tubulin 84B. The strength of bands displayed in the ethidium-bromide stained gel from reverse transcription PCR was correlated to Ct values obtained from quantitative PCR, with M2 females giving the weakest band and the highest Ct value. However, the expression in all samples was not significantly variable in both males and females (Figure 3.7). These results imply that the expression of the gene was independent of sex, like the case of the gene homolog of *D. melanogaster* (Kurtovic *et al*., 2007a).

Moreover, the absence of products in amplification of GffOr67d4 from non-transgenic flies confirmed that the primers were specific to the transgene and were not annealing to the *D. melanogaster* Or67d. Nevertheless, this can also be due to the low homology identity (34.08%) obtained between GffOr67d4 and DmelOr67d. Hence, these results confirmed that the expression being monitored was of GffOr67d4, and not that of DmelOr67d4. The uniformity of alpha-tubulin 84B expression across male and female flies confirmed its choice as an ideal reference gene for gene expression profiling in *Drosophila melanogaster* (Ponton *et al.*, 2011).

# CHAPTER FIVE

# CONCLUSION AND RECOMMENDATIONS

## 5.1 Conclusion

The *G. f. fuscipes* genome contains five homologues of the Or67d gene showing that there has been an expansion of this gene family in the *Glossina* lineage. Three out of the four complete copies considered in this study have the specific 7tm_6 odorant receptor domain and might therefore maintain their function in the fly. In the current work, the most conserved copy of the gene was amplified and expressed in the *Drosophila* system for further functional characterization.

## 5.2 Recommendations

Observations made in this study suggest that the *Drosophila* system is an effective expression system to study olfactory receptors of insects and in this case, those of tsetse flies. This was carried out by limited wet lab experiments including molecular cloning and injections of the plasmid in *Drosophila* embryos.

Moving forward, a larger panel of odorant receptors of *Glossina fuscipes fuscipes* and of other tsetse species should be assayed to confirm the assumption that this method should be applicable to ORs across insect species (Hallem *et al.*, 2004; Carey *et al.*, 2010). Secondly, expression of tsetse odorant receptors in *Drosophila* empty neuron system and T1 system should be established through sequential genetic crossings of the transgenic flies with specific promoters in ORNs of ab3 and T1 antennal sensilla as described by Gonzalez *et al.*, 2016. Confirmation of expression can also be achieved through qPCR and western blot analysis.

Functional characterization of expressed transgenic ORs must be carried out by single sensillum electrophysiological recordings in order to identify chemical signals that induce responses to individual ORs. Understanding the molecular basis of *Glossina* olfaction will provide more insights into identifying target molecules that can be used in traps, live bait and push-pull methods for better management of trypanosomiasis.

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## APPENDICES

**Appendix 1:** PCR and Gel Electrophoresis Buffers

a) **50X TAE Buffer:** For preparation of 1000 ml of 50X TAE buffer, 242 g of Tris base were transferred into a 2 L beaker and were dissolved completely in about 600 ml of distilled water with a stir bar. 100 ml of 0.5 M of EDTA (Ethylenediaminetetraacetic acid) solution (pH 8.0) and 57.1 ml of glacial acetic acid were added to the solution and mixed thoroughly. Subsequently, the solution volume was adjusted to 1000 ml with distilled water. The solution was finally mixed and stored at room temperature.

b) **1X TAE Buffer:** To prepare 1000 ml of 1X TAE buffer, 20ml of 50X TAE buffer was aliquoted into a 2L beaker and the solution volume was adjusted to 1000 ml with distilled water**.**

**Appendix 2:** Transformation media

a) **Luria-Bertani (LB) medium:** 10 g Bacto-Tryptone, 5 g NaCl and 5 g Bacto-Yeast extract were dissolved in 950 ml of distilled water. Afterwards, its pH was adjusted to 7.0 using NaOH and then the volume was topped up to one liter with distilled water. The solution was autoclaved and left to cool down at room temperature before use.

b) **LB plates with ampicillin:** 15 g of Agar were added to 1 liter of LB medium then autoclaved. The medium was allowed to cool to 50 ºC before ampicillin was added at a final concentration of 50 µg/ml. 30 to 35 ml of LB Agar medium were poured onto 8 mm petri dishes and left to harden. The plates were then used immediately or kept at 4 ºC for later use.

c) **LB plates with kanamycin:** 15 g of Agar were added to 1 liter of LB medium then autoclaved. The medium was allowed to cool to 50 ºC before kanamycin was added at a final concentration of 50 µg/ml. 30 to 35 ml of LB Agar medium were poured onto 8 mm petri dishes and left to harden. The plates were then used immediately or kept at 4 ºC for later use.

d) **Super Optimal Broth with Catabolite repression (SOC):** 100 ml of SOC were prepared by mixing 2 g of Bacto-Tryptone, 0.5 g of Bacto-Yeast extract, 1 ml of 1 M

NaCl, 0.25 ml of 1M KCl, 1 ml of 2 M $Mg^{2+}$stock (filter sterilized), 1 ml of 2 M glucose (filter sterilized) and distilled water up to a final volume of 100 ml.

**Appendix 3:** Vectors created

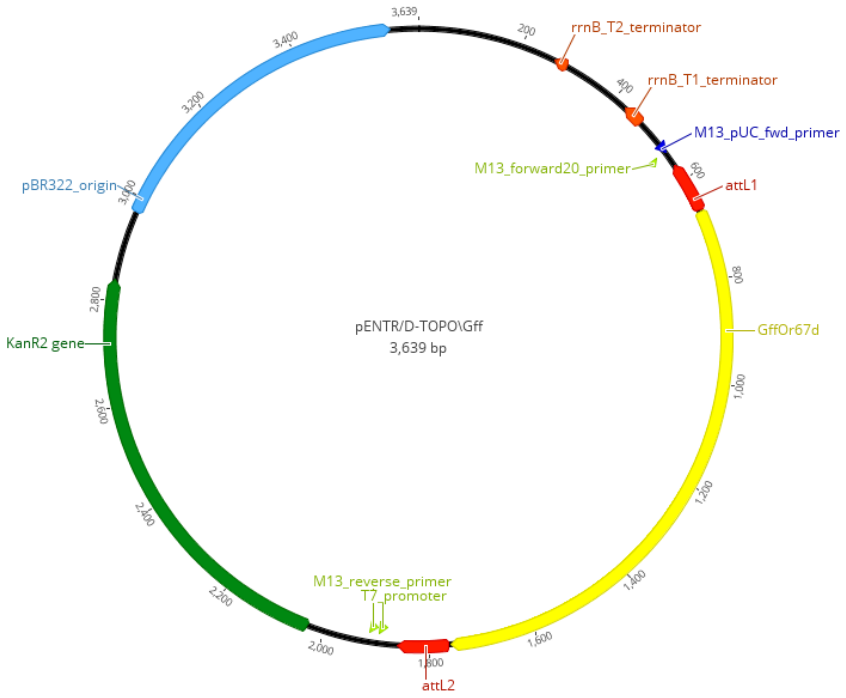


**Figure A3.1: Construct pENTR™/D-TOPO® Vector + GffOr67d4 gene**


**Figure A3.2: attB-expression vector (pTW vector + GffOr67d gene)**

**Appendix 4:** Supporting information to the results of RT-qPCR analyses

**Table A4.1: Ct values of reference gene (alpha-tubulin) in male and female transgenic flies**

| Samples | Replicates | Ct values | Average of CT values | Standard deviation |
|---|---|---|---|---|
| HKG M2 Females | 1 | 33.33 | 33.31667 | 0.023094 |
| HKG M2 Females | 2 | 33.33 | | |
| HKG M2 Females | 3 | 33.29 | | |
| HKG M3 Males | 1 | 32.81 | 32.68667 | 0.115902 |
| HKG M3 Males | 2 | 32.58 | | |
| HKG M3 Males | 3 | 32.67 | | |
| NTC | 1 | 39.14 | 39.14 | 0.01 |
| NTC | 2 | 39.13 | | |
| NTC | 3 | 39.15 | | |

The abbreviations HKG and NTC mean housekeeping gene and non-template control respectively. M2 and M3 just represent different sample names.

**Table A4.2: Ct values of GffOr67d4 and alpha-tubulin 84B in male and female transgenic flies samples**

| Samples | Replicates | Ct values | Average of CT values | Standard deviation |
|---|---|---|---|---|
| M2 Females | 1 | 28.72 | 29.4633 | 0.856524 |
| M2 Females | 2 | 29.27 | | |
| M2 Females | 3 | 30.4 | | |
| M2 Males | 1 | 26.37 | 25.80333 | 0.591383 |
| M2 Males | 2 | 25.19 | | |
| M2 Males | 3 | 25.85 | | |
| M3 Females | 1 | 25.89 | 26.40333 | 0.445009 |
| M3 Females | 2 | 26.68 | | |
| M3 Females | 3 | 26.64 | | |

| | | | | |
|---|---|---|---|---|
| M3 Males | 1 | 26.27 | 26.29333 | 0.020817 |
| M3 Males | 2 | 26.31 | | |
| M3 Males | 3 | 26.3 | | |
| NTC | 1 | 39.36 | 39.29333 | 0.065064 |
| NTC | 2 | 39.29 | | |
| NTC | 3 | 39.23 | | |
| HKG M3F | 1 | 32.16 | 32.22667 | 0.133167 |
| HKG M3F | 2 | 32.14 | | |
| HKG M3F | 3 | 32.28 | | |

The abbreviations HKG and NTC mean housekeeping gene and non-template control respectively. M2 and M3 just represent different sample names.