

**COMPARATIVE AND FUNCTIONAL ANALYSIS OF TSETSE FLY  
AQUAPORINS**

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degree of Master of Science (Biochemistry) in the School of Pure and Applied  
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## DECLARATION

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

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## **DEDICATION**

This work is dedicated to my family and especially my dad, James Aroko for your determination to see me through my studies.

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## ABBREVIATIONS

<b>AAT</b>	Animal African trypanosomiasis
<b>AQP</b>	Aquaporin
<b>Bp</b>	Base pairs
<b>cDNA</b>	Complementary DNA
<b>DNA</b>	Deoxyribonucleic acid
<b>DRIP</b>	Drosophila integral protein
<b>dsRNA</b>	Double stranded RNA
<b>EDTA</b>	Ethylenediaminetetraacetic acid
<b>GAPDH</b>	Glyceraldehyde-3-phosphate dehydrogenase
<b>HAT</b>	Human African trypanosomiasis
<b>LB</b>	Luria- Bertani
<b>mM</b>	Millimolar
<b>M</b>	Molar
<b>NFW</b>	Nuclease free water
<b>NPA</b>	Asparagine-Proline-Alanine
<b>NTD</b>	Neglected tropical disease
<b>ORF</b>	Open Reading Frame
<b>PBS</b>	Phosphate buffered saline
<b>PCR</b>	Polymerase chain reaction
<b>RNA</b>	Ribonucleic acid
<b>RNAi</b>	RNA interference
<b>SIT</b>	Sterile insect technique

<b>TAE</b>	Tris acetate EDTA
<b>UV</b>	Ultra violet
<b>°C</b>	Degrees celcius
<b><i>g</i></b>	Gravitational force
<b>μg</b>	Microgram
<b>μL</b>	Microlitre
<b>μM</b>	Micromolar

## ABSTRACT

Tsetse flies (*Glossina sp*) are the vector of African trypanosomes, the causative agents of sleeping sickness in man and Nagana in cattle. The insect is strictly hematophagous, taking in significantly high quantities of water during bloodmeals that present a considerable osmotic challenge. This necessitates machinery for eliminating excess water. One potential candidate is a group of membrane channel proteins called aquaporins (AQPs) that are responsible for the movement of water across cell membranes. In other insect species, aquaporins have been shown to facilitate physiological processes including desiccation and freeze tolerance. *Gmmdripa* aquaporin has been shown to play an important role in diuresis and larval development in *Glossina morsitans morsitans*. This study sought to determine presence, tissue distribution and function of *gmmdripa* homologs proteins in three tsetse fly species. Using bioinformatics approach, three putative aquaporin genes named *GfmAQP1*, *GffAQP1* and *GLAQP1* from *Glossina fuscipes martini*, *Glossina fuscipes fuscipes* and *Glossina longipennis* respectively, were identified. Multiple sequence alignment and phylogenetic analysis using Neighbour-Joining method showed that these AQPs had high homology to *Glossina morsitans morsitans* integral protein A (*DripA*) and belonged to the water-specific *Drosophila* integral protein (DRIP) group of aquaporins. Further amino acids multiple alignment revealed that the AQPs had two asparagine-proline-alanine (NPA) motifs and six transmembrane domains that are evolutionarily conserved in most AQPs. Semi-quantitative RT-PCR showed higher levels of AQP expression in midgut as compared to proboscis, legs and head. In addition, the genes are expressed during all life stages with higher levels in newly emerged teneral flies as compared to larvae, pupae and adult. Functional analysis using RNA interference of *Gfm* AQP1 in *G. f. martini* showed decreased rate of diuresis after a blood meal but no significant effect on the fly survival ( $p < 0.655$ ). However, differences on the feeding success between the test and uninjected control groups were significant ( $p < 0.0113$ ), indicating the negative effects of injury inflicted by the needle during injections. These findings indicate an important role of AQP1 in regulation of water in tsetse.

## CHAPTER ONE

### 1. INTRODUCTION

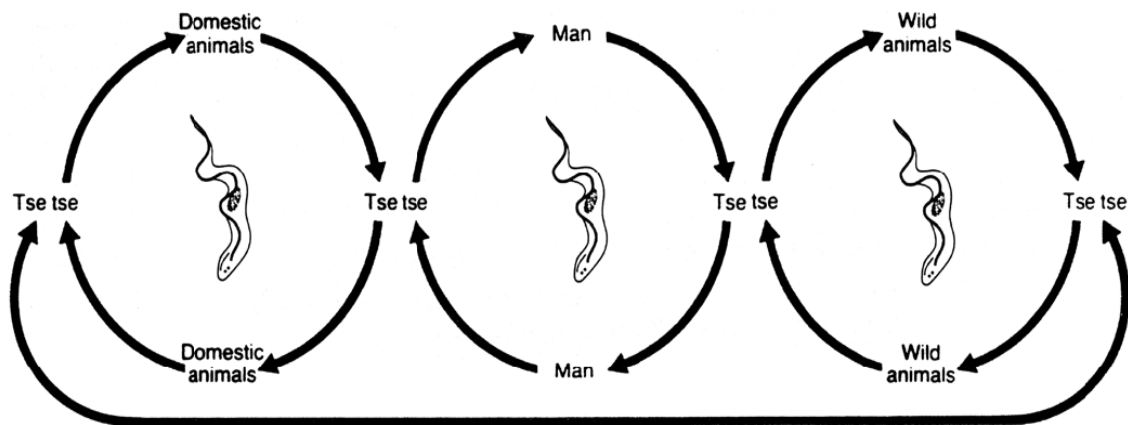
#### 1.1 Background information

African trypanosomiasis is a tropical disease afflicting humans and animals in many countries in sub-Saharan Africa. The disease is commonly referred to as animal African trypanosomiasis (AAT) or nagana in cattle, while in humans it is referred to as human African trypanosomiasis (HAT) or sleeping sickness. Scottish missionary David Livingstone was the first to report death of his cattle as a result of tsetse fly bites in 1852 (Sterveding, 2008). However, it was until 1894 that David Bruce first observed trypanosomes in an infected cow blood (Baker, 1995). David Bruce later confirmed that trypanosomes were the causative agents of both nagana and sleeping sickness (Sterveding, 2008).

Tsetse flies (Diptera: Glossinidae) are divided into several groups. The palpalis group including *Glossina palpalis* and *Glossina fuscipes* are mainly limited to humid areas in Africa, the mangrove swamps, rain forests and lake shores. The morsitans group including *Glossina morsitans* and *Glossina pallidipes* is mainly present in Africa's wooded savanna and grasslands (FAO, 1992). In 2003, 17000 HAT cases were reported to the World Health Organization (WHO) with a majority (80%) of the cases being reported from Angola and the Democratic Republic of Congo (WHO, 2005). In 2006, 12,000 new cases were reported (Simarro *et al.*, 2008) while below 10,000 cases were reported in 2009. This reduction in HAT cases was attributed to concerted efforts

including enhanced diagnosis, strengthened control strategies and increased surveillance by organizations like FAO and WHO (Simarro *et al.*, 2011).

Both animal and human trypanosomiasis are obstacles of development in rural sub-Saharan Africa. HAT reduces labour resources, while AAT causes reduction in milk and meat production, deprives farmers of draught power and thus reducing crop production. Therefore, HAT and AAT are the major factors hindering the establishment of flourishing agriculture to provide food security and lead to sustainable economic growth and healthy populations in sub-Saharan Africa (Simarro *et al.*, 2008). The spread of trypanosomosis depends on four interacting organisms; the insect vector, the human host, the wild and domestic animals reservoirs and the pathogenic parasites (Aksoy and Rio, 2005) (Figure 1.1).



**Figure 1.1** HAT Transmission Cycle. Simarro *et al.*, 2008.

West and Central African HAT, which accounts for more than 90% of the reported HAT cases, are caused by the parasite *T. b. gambiense*. These pathogenic parasites are

transmitted to humans and animals by the bite of a tsetse fly which has acquired the infection from animals or human beings harboring the parasites (Simarro *et al.*, 2008). This multiplicity of factors gives us a chance to explore different avenues for control of trypanosomiasis.

Aquaporins (AQPs) are membrane channel proteins involved in the movement of water molecules across plant and animal cells (Agre *et al.*, 1993). Studies on various insect species show that insects have varying numbers of AQP genes. The model organism *D. melanogaster* and the Rift valley virus vector *Aedes aegypti* both have eight aquaporin genes distributed in different tissues (Drake *et al.*, 2010). Malaria parasites vector *Anopheles gambiae* has seven AQPs of which down-regulation of AgAQP1 has been shown contribute to desiccation resistance of female adult *A. gambiae* (Liu *et al.*, 2011).

*G. m. morsitans* has ten putative aquaporin genes, two more than that reported for other insects (Benoit *et al.*, 2014). One of these AQPs called *gmmdripa* clusters together with *Drosophila DRIP* which is water channel protein. Multiple AQPs play critical roles in tsetse flies including dehydration/heat tolerance, diuresis after a bloodmeal, while both *gmmdripa* and *gmmdripb* are important during pregnancy (Benoit *et al.*, 2014). The higher number of aquaporins and their significance in tsetse fly physiology indicates that they are ideal candidates to be evaluated for control initiatives.

## **1.2 Statement of the problem**

Tsetse flies are the vectors of African trypanosomes which are the causative agents of HAT and AAT. Efforts to control trypanosomiasis including chemotherapy have been



hampered by issues of toxicity and drug resistance while tsetse fly population reduction strategies, which still is the most appropriate strategy for trypanosomiasis control, have not been sufficient due to socio-economic reasons. Therefore, there is urgent need for development of alternative vector control techniques to supplement the already existing ones. Aquaporins have been shown to play various roles in insects including dehydration and heat tolerance, lactation and diuresis. Although there is demonstrated expression data on aquaporins in other insect species including *G. m. morsitans*, none exist for the species under this study. Comparative and functional studies are therefore important to validate tsetse fly aquaporins as possible targets for designing control strategies.

### **1.3 Justification**

The success of using aquaporins in the control of trypanosomiasis requires a detailed understanding of tsetse fly genetics and characterization of aquaporins is one approach. Evaluation of the roles of aquaporins in enhancing vector fitness and tsetse fly survival with potential for development of vector control tools is important due to several reasons. First, despite huge impact HAT and AAT, the current control strategies suffers several limitations hence need for identification and validation of novel control approaches. Investigation into the role of aquaporins in tsetse fly, with recent data indicating significant involvement in various biological processes in most organisms including insects of medical importance, will therefore provide knowledge that allows development of novel vector control strategy. Secondly, vector control initiatives like spraying using insecticides and use of odor baited traps are not environmentally friendly and affect many other beneficial organisms such as bees. Insect-specific approach is therefore preferable, and investigation of tsetse aquaporins can allow development of such strategies. Thirdly

development of a control initiative targeting the vector will reduce both HAT and AAT as transmission of trypanosomes to their reservoirs will be reduced. Therefore a strategy that is environmentally friendly and tsetse-specific, which this tsetse fly aquaporins potentially provides is much needed. In addition, this approach can be applied in other insects of medical and agricultural importance.

#### **1.4 Hypothesis**

*Glossina* species have differentially expressed aquaporins in their tissues which play an important role in osmoregulation and water balance.

#### **1.5 Objectives**

##### **1.5.1 General objective**

To generate genomic data on *Glossina* species aquaporins that can be applied in trypanosomiasis disease control.

##### **1.5.2 Specific objectives**

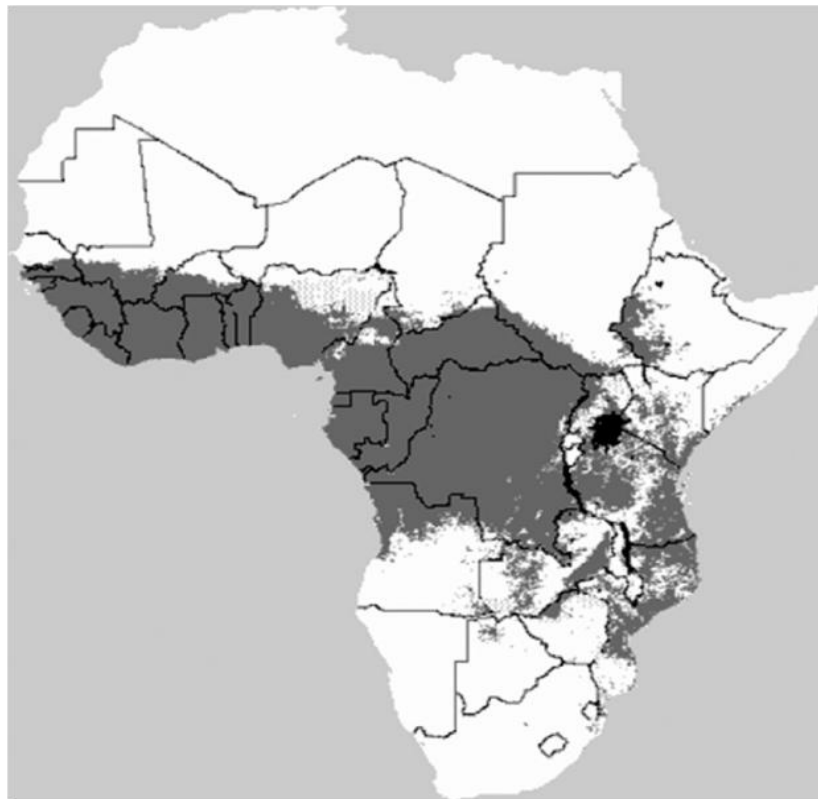
- i. To determine tissue expression patterns of *Drip*-like AQPs in *G. longipennis*, *G. f. martini* and *G. f. fuscipes*.
- ii. To evaluate the life stage expression pattern of *Drip*-like AQPs in *G. f. martini* and *G. f. fuscipes*.
- iii. To determine the effect ablation of *Drip*-like AQP has on *G. f. martini* feeding, survival and diuresis.

## CHAPTER TWO

### 2. LITERATURE REVIEW

#### 2.1 Medical and economic significance of tsetse fly

Tsetse flies are of concern in much of sub-Saharan Africa because of their blood feeding habits that cause pain and their role as vectors of pathogenic trypanosomes. Approximately 60 million people are at risk across 37 countries covering about 40% of Africa (WHO, 2002) (Figure 2.1).



**Figure 2.1** Tsetse fly distribution in Africa. Grey shading indicate countries infested by tsetse flies (adapted from Abd-Alla *et al.*, 2013).

Different species of trypanosomes are transmitted by tsetse flies including; *Trypanosoma b. brucei*, *Trypanosoma b. rhodesiense*, and *Trypanosoma b. gambiense*. *T. b.*

*rhodensiense* and *T. b. gambiense* are highly pathogenic to man being the agents of ‘sleeping sickness’ or human African trypanosomiasis (HAT). Infection with these two forms of parasites is called HAT however, the infections differ markedly in terms of the geographical distribution of the parasites, the clinical symptoms of the infections and the control strategies employed in each instance (Fevre *et al.*, 2006).

Tsetse flies also transmit livestock pathogens that cause Nagana. These include *T. vivax*, *T. congolense*, *T. simiae*, and *T. godfreyi* (Stevens and Brisse, 2004). *T. evansi* is pathogenic although it’s not transmitted by tsetse flies. Nagana is of much economic significance because where it is prevalent; meat, milk, dung, and draught power production are greatly reduced or lost altogether (Krasfur, 2009).

Four geographic locations have been mapped in Africa where significant HAT cases are reported. In the Western Africa region significant cases of HAT are reported in Ivory Coast, Nigeria and Guinea. Between 2000 and 2009 the total number of reported cases in the three countries was around 1,600 (Simarro *et al.*, 2010). The Central African region countries that reported higher cases between 2000 and 2009 include Congo, Central African Republic, Uganda, Sudan and Angola totaling around 34,700. Western and Central African HAT are caused by *T. b. gambiense* (Kennedy, 2004). The other HAT foci are the Eastern and South-eastern Africa regions. In this region *T. b. rhodensiense* is the causative agent of HAT and the disease is linked with livestock reservoir (Simarro *et al.*, 2010). *T. b. brucei* occurs throughout sub-Saharan Africa wherever its vectors may be

found. It is not infective to humans due to the presence of trypanolytic factor apolipoprotein L1 in the human serum (Pays *et al.*, 2006).

There are no distinctive signs and symptoms associated with trypanosomiasis, therefore, serological tests are normally carried out to detect the presence of trypanosomes antibodies in the blood, serum or plasma of HAT patients. The Card Agglutination Test for Trypanosomiasis (CATT), developed in the 1970s is commonly used for the detection of *T. b. gambiense*-specific antibodies in HAT patients (Magnus *et al.*, 1978).

The Latex/ *T. b. gambiense* test is also used to screen for HAT (Busher *et al.*, 1999). This is an indirect agglutination assay of specific antibodies with antigens coupled to the surface of latex beads. Molecular methods including polymerase chain reaction (Moser *et al.*, 1989) and loop mediated isothermal amplification (LAMP) (Kuboki *et al.*, 2003) have also been used for detection of African trypanosomes.

## **2.2. Control of trypanosomiasis**

Several approaches are used in the control of trypanosomiasis. There are those strategies that target the vector (tsetse fly) population and those that target the parasites.

### **2.2.1 Use of Trypanocides/Drugs**

Despite many years of research no vaccine has been developed for human and animal trypanosomiasis. This is majorly due to antigenic variation of the surface glycoproteins of trypanosomes while in the insect vector and in the mammalian host (Aksoy and Rio, 2005). Infections are therefore normally treated by administration of drugs after parasitaemia is confirmed. In the first stage of HAT, infection is restricted to the blood

and lymph. The drug administered at this stage is pentamidine (Yun *et al.*, 2010). In the second stage of HAT the infection spreads to the brain and until 2009 melarsoprol or eflornithine was used. Eflornithine therapy is cumbersome as it requires 56 infusions administered after 6 hours within 14 days (Schmid *et al.*, 2012). Melarsoprol is highly toxic and can cause severe post-treatment reactive encephalitis which may lead to death of the patient (Kennedy, 2008). A new treatment regime called nufurtimox-eflornithine combination therapy (NECT) was added to the WHO List of Essential Medicines in 2009 for the treatment of second stage *T. b. gambiense* HAT (WHO, 2010). However, NECT also has limitations including vomiting at the onset of administration and the need for trained personnel to deliver the infusions (Schmid *et al.*, 2012).

In the case of animals, only three drugs have been used over the years to treat trypanosomiasis. These include isometamidium chloride, diminazene aceturate and homidium bromide. Diminazene aceturate is a curative drug mainly used in cattle and in small ruminants administered at a dosage rate of 3.5-7 mg/Kg (FAO, 1998). Isometamidium chloride is used mainly in cattle as a curative at lower dosage of 0.25-0.5mg/Kg and for prophylaxis at a higher dosage of 1.0 mg/Kg. Suramin is used in camels at a dosage of 10 mg/Kg for treatment of *T. evansi* infection (FAO, 1998). However, drug resistance has been observed in HAT and AAT due to misuse and inefficient application of these drugs.

### **2.2.2 Tsetse fly control**

The high costs of HAT and AAT treatment, toxicity of some of the drugs, drug resistance and lack of a vaccine makes vector control the most reliable way of lowering

transmission rates (Abd-Alla *et al.*, 2013). On this front several strategies of tsetse fly control have been developed and these include;

#### **2.2.2.1 Use of baits and traps**

Different kinds of traps and baits have been developed. Tsetse flies are attracted to specific colours, particularly black and blue, and to certain shapes (Vale, 1982). Chemical attractants like cow urine, acetone and butanone have also been shown to draw the flies to the traps. These traps are simple and relatively cheap and can be managed by the local communities. *G. morsitans morsitans* and *G. pallidipes* populations were reduced by up to 99% in 1984 in the Zambezi valley of Zimbabwe using baited traps employed at 3-5/km<sup>2</sup> (Vale *et al.*, 1988). In Kenya, low-cost and highly efficient odour-baited NGU traps developed by ICIPE scientist were deployed in Nguruman. This lowered *G. pallidipes* population by 90.99% (Williams *et al.*, 1992). Due to its success, the same traps were employed in the Lambwe valley by the Kibwer and Samba communities between 1992 and 1996. The mean number of flies per trap per day decreased from 12.7 in 1993 to nil from august 1996 to December 1997 when the initiative stopped (UNDP, 2006). For the savanna group of flies, the recommended density is four traps per square kilometer while for riverine flies the number of traps is about ten per square kilometer. This is because attractants are known for the savanna flies unlike for the riverine flies (Lindh *et al.*, 2009). For better performance, other important aspects including regular repair or replacement of torn traps, appropriate siting of the traps and periodic replenishment of the odours (Vreysen, 2001) must be considered.

#### **2.2.2.2 Use of insecticides**

This involves spraying of insecticides like dieldrin and endosulfan selectively on livestock or through several sequential spraying of the insecticides on the ground or vegetation in tsetse infested area. The principle is to kill all the adult flies that come into contact with the insecticides and all the emerging flies by subsequent spraying cycles (Vrysen, 2012). In Kenya, concerted efforts by FAO, WHO and Ministry of livestock involving aerial and ground spraying of dieldrin and endosulfan between 1960s and 1981 brought down *G. palidippes* populations in the Lambwe valley and adjacent areas (Otieno *et al.*, 1990). This was followed by increase in the fly population in the subsequent years due to factors like the high costs involved, inadequate knowledge of *G. pallidipes* ecology and behaviour. Sequential aerial spraying of deltamethrin at 0.26-0.3g/ha in the Okavango delta in Botswana in 2001 and 2002 reduced the population of *G. morsitans centralis* until no flies were detected in surveys carried out between 2002 and 2005 (Kgori, 2006). However this approach has several draw backs: prolonged use of large insecticide volumes causes environmental pollution, kills other organisms that would otherwise predate on pests, leads to development of insecticide resistance and is injurious to the health of people handling the insecticides (Vreysen, 2012).

#### **2.2.2.3 Sterile insect technique (SIT)**

SIT involves production of large numbers of target flies followed by sterilization of males or both sexes either chemically or by ionizing radiation. The sterile males are then systematically released to the environment in large numbers in relation to the wild male population so as compete for the wild females (Vreysen *et al.*, 2000). Females are inseminated once in their lifetime, therefore when mated with sterile males they become



unable to produce offspring (Hargrove, 2003). This approach was applied in Zanzibar in between 1994 and 1999 resulting in the successful eradication of *Glossina austeni* from the island (Vreysen *et al.*, 2000). Prior to the release of sterile males to the environment it is important to bring down the male population by use of other vector control strategies like spraying or use of traps so as to increase the probability of a male mating with a female (Vreysen *et al.*, 2000). To achieve the intended purpose, the sterile males should be released in large numbers over time sufficient to cover three to four generations. This leads to reduced fertile mating and consequently the population will be eliminated (Aksoy, 2003). The disadvantage of this method is that large numbers of sterile males are required and it is therefore expensive to implement.

#### **2.2.2.4 Other tsetse fly control strategies**

Tsetse fly feed on blood which contains a lot of water. The fly must therefore have a clear mechanism of eliminating this excess water to maintain its gut osmotic gradient. Since AQPs have been shown to play a big part in water movement it is possible that they are involved in elimination of this excess water. Ten putative aquaporins have been identified in *G. m. morsitans* genome (Benoit *et al.*, 2014) where they are involved in important physiological processes including heat/dehydration tolerance, diuresis after a bloodmeal and milk production. A putative aquaporin (APAQP1) isolated from the gut of pea aphid *Acyrtosiphon pisum* which is a phloem-feeder, and expressed in *Xenopus* oocytes showed that it was involved in water movement (Shakesby *et al.*, 2009). The importance of tsetse AQPs makes them ideal for consideration as targets for vector control.

## **2.3 Aquaporins discovery and functions**

Aquaporins are transmembrane channel proteins -belonging to the major intrinsic protein (MIP) family- that mediates movement of water across cell membranes (Agre *et al.*, 1993; Agre, 2004). These water channel proteins are conserved and widely distributed across the plant and animal kingdoms and in lower organisms like bacteria and protists (Verkman, 2012). They have traditionally been classified into two broad categories based on substrate selectivity: the classical aquaporins, which are selectively permeable to water, and the aquaglyceroporins, which are permeable to water and multiple uncharged solutes such as glycerol and urea (Agre, 2004). Some MIPs can also transport ammonia, anions and possibly carbon dioxide (King *et al.*, 2004; Rojek *et al.*, 2008).

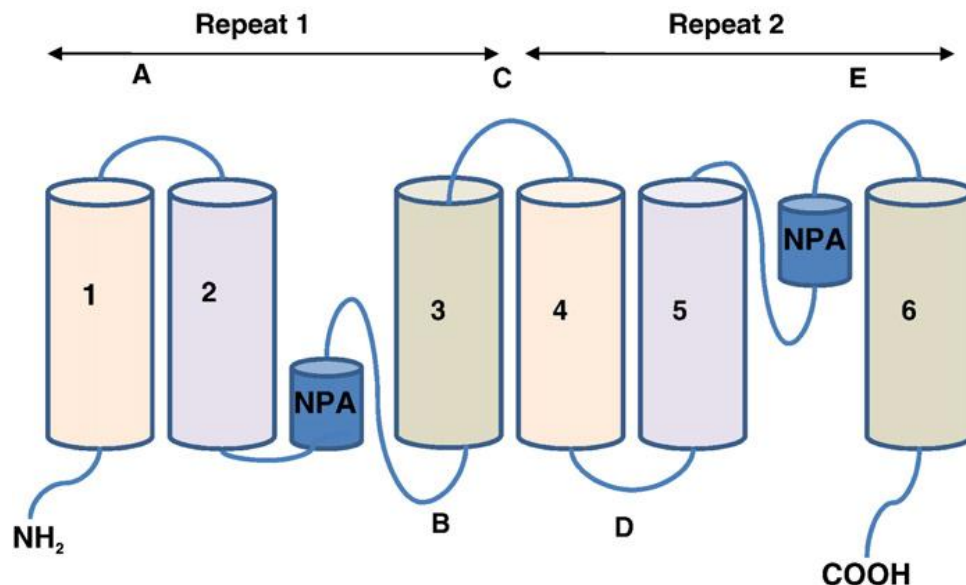
Aquaporins have been found to have some common features relating to their permeability. In most AQPs, movement of water molecules and other solutes is driven by a concentration gradient eliminating the need for energy to facilitate transport (Kaufmann *et al.*, 2005). Mutations in the side chains adjacent to the conserved regions have been implicated in determining the solute selectivity and transport efficiencies of AQPs. The selectivity filter in water specific AQPs is smaller and more polar while in aquaglyceroporins it is large and polar (Park and Saier, 1996).

### **2.3.1 Aquaporin structure**

The aquaporin molecule structure is highly conserved in animals, plants, yeast and bacteria. Most aquaporins have six transmembrane domains joined by five loops A-E (Figure 2.2). The N- and C- terminals are intracellular, facing the cytosol (Zhao *et al.*,

2008). The conserved three amino acid sequence of Asparagine-Proline-Alanine (NPA) is found on both the C- and N- terminals.

Mutations in the amino acid residues adjacent to the conserved NPA motifs in the lipid bilayer reduce water movement through the pores indicating that indeed these regions contribute to formation of the aqueous pore (Jung *et al.*, 1994). A cysteine residue at position 189 near the C-terminal NPA motif confers sensitivity to mercurial compounds. Mercuric chloride binds to this cysteine residue resulting in the blockage of the pore and thereby hindering water transport. Despite the conserved features, there are functional differences within the AQP families brought about by differences in the pore side-chains. In aquaporins that strictly facilitate movement of water, the region is smaller and more polar with a conserved histidine while in aquaglyceroporins this region is large and hydrophilic with two conserved aromatic residues (Savage and Stroud, 2007).



**Figure 2.2** MIP topology. The Primary structure of MIPs consists of six transmembrane domains (1-6), five loops (A-E) and two conserved NPA motifs (Gomes *et al.*, 2009).

### 2.3.2 Insect aquaporins

Insect AQPs are widely distributed in different tissues and play an important role in various physiological processes including freeze tolerance and desiccation which requires removal of water from the cell (Spring *et al.*, 2009). AQPs play an important role in water facilitation and movement in the alimentary canal of sap-feeding insects where there is need for elimination of excess water, concentration of nutrients and osmoregulation (Mathew *et al.*, 2011). Phloem and xylem-feeding insects must therefore have specific ways of eliminating the excess water that comes with their food. So far, around seven insect AQPs have been classified and shown to play an important role in osmoregulation. Two aquaporins *pvAQP1* and *pvAQP2* have been functionally characterized from the sleeping chironomid, *Polypedilum vanderplanki*, which inhabits temporary rock pools in semi-arid regions in Africa. A study on the larvae of *P. vanderplanki* by Kikawada *et al.* (2008) revealed that these AQPs play a critical role in anhydrobiosis in the insect.

*AQPcic* has been isolated from the filter chamber of the leafhopper *Cicadella viridis* which is a homoptera feeding on large volumes of xylem (Spring *et al.*, 2009). *AQPcic* has been localized in the filter chamber of other xylem feeders but not in phloem feeders like aphids (LeCaherec *et al.*, 1997). Expression of *AQPcic* in *Xenopus* oocytes showed that it had a higher water permeability rate compared to the human AQP1 (LeCaherec *et al.*, 1996).

Blood feeding insects also experience the same problems similar to sap feeding insects and also have defined systems for osmoregulation and elimination of excess water. In the yellow fever mosquito vector *Aedes aegypti*, four AQPs have been identified. Duchense *et al.* (2003) functionally characterized *AeaAQP* and showed that it is located in the end cells of tracheoles associated with the malpighian tubules. Expression studies in *Xenopus* oocytes showed increased water permeability compared to *AQPcic* and comparisons studies with *E. coli* aquaglyceroporins GlpF shows that *AeaAQP* is a strict water channel. A single water channel protein (*RpMIP*) has also been isolated from the hematophagous assassin bug *Rhodnius prolixus*. Studies by Eschevarria *et al.* (2001) showed that this protein is distributed in the proximal and distal tubules. Functional expression studies in *Xenopus* oocytes showed that it was permeable to water. *Drosophila* AQPs (DAQPs) have been proposed to maintain fluid homeostasis, which is a particularly daunting task because flies are at constant risk of dehydration as a result of their high surface area-to-volume ratio (O'Donnell and Maddrell, 1995). Furthermore, *Drosophila* undergoes significant morphological changes during metamorphosis, so their fluid needs change considerably.

Dow *et al.* (1995) previously reported the cloning of the first putative *Drosophila* AQP, *drip*, from an adult *Drosophila* malpighian tubule cDNA library. At that time, the cellular localization of *drip* had not been determined, and it was not known whether *drip* was expressed in the tubule epithelium or in associated tissue, as had been found for the *Aedes aegypti* putative aquaporin *AeaAQP* (Pietrantonio *et al.*, 2000; Duchesne *et al.*, 2003). Since the publication of the original report, five additional putative *Drosophila* AQPs

have been cloned (Rubin *et al.*, 2000) and, on the basis of micro array studies, it appears that two of these are also highly expressed in the adult MT (Wang *et al.*, 2004). Notably, of the seven DAQPs, the *drip* sequence is the most similar to *hAQP4*, a water-specific human AQP exhibiting the highest transport rates of any AQP (Chou *et al.*, 1998).

*Drosophila drip* is most closely related to putative AQPs cloned from *Aedes aegypti* and the malaria parasites carrier *Anopheles gambiae*, suggesting that the pore properties determined for *drip* may be relevant to its dipteran relatives (Kaufmann *et al.*, 2005). Thus a detailed characterization of *drip* is of great importance and may provide one means to fight the spread of disease. *DRIP* is expressed at many stages during development, suggesting that this protein play important roles throughout the organism's life cycle (Kaufmann *et al.*, 2005). Analysis of *Gfm* AQP1 gene function was done through RNAi in *Glossina f. martini* in this study.

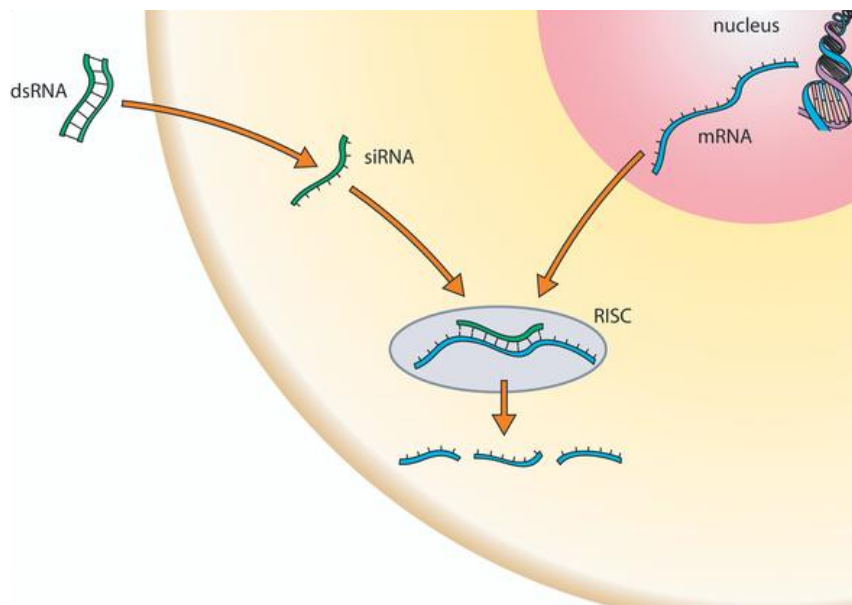
#### **2.4. RNA interference (RNAi)**

RNAi is a post-transcriptional sequence-specific gene silencing mechanism initiated by double stranded RNA (dsRNA). This technique is of great value as it enables the study of specific gene functions. Fire *et al.* (1991) successfully disrupted the expression of two genes *unc-22* and *unc-54* encoding body wall muscles in the nematode *Caenorhabditis elegans* using antisense RNA. This led to the initial assumption that RNAi was initiated by single stranded antisense and sense RNA (Montgomery and Fire, 1998). In subsequent work to determine the requirements for delivery of interfering RNA in *C. elegans*, Fire *et al.* (1998) discovered that indeed dsRNA was more effective in gene silencing and disruption of protein expression than sense and antisense RNA separately. The effect of RNAi is heritable only up to the first generation progeny. RNAi has been described in

many organisms including insects (Hoa *et al.*, 2003; Ghanim *et al.*, 2007; Xue *et al.*, 2012) and in plants (Baulcombe, 2004).

### 2.4.1 RNAi mechanism

When dsRNA is injected into a cell, it is cleaved by an enzyme called dsRNA endonuclease (dicer) into small fragments - approximately 22 nucleotides- called short interfering RNA (siRNA). The siRNA is then bound to the RNA-induced silencing complex (RISC), which then binds to any matching mRNA sequence (Figure 2.3). The mRNA is degraded by the enzymatic activity of RISC effectively silencing the gene from which it came (Robinson, 2004).



**Figure 2.3** RNAi mechanism, borrowed from Robinson (2004). Once the dsRNA is inside the cell, it is cleaved by the dicer into small fragments called the siRNA in the cytosol. The siRNA bind to the cellular enzyme (RISC) which uses one strand of siRNA to bind to a complementary single stranded mRNA. The nuclease activity of RISC then degrades the mRNA leading to gene suppression.

Various mechanisms have been employed to introduce the dsRNA into the target cells. Amine based and liposomal transfection has been used and in some cases electroporation has been used (Dexburry *et al.*, 2004). Insects can take up dsRNA through feeding (Huvenne and Smagghe, 2010) or the dsRNA can be injected directly between the abdomen and thorax of the insect (Takahashi *et al.*, 2009). RNAi mechanism can be utilized for insect pests and vector control.



## CHAPTER THREE

### 3. MATERIALS AND METHODS

#### 3.1 Tsetse flies

*Glossina f. fuscipes* and *Glossina f. martini* colonies were obtained from the tsetse insectary at ICIPE, Duderuville. *G. f. fuscipes* was originally collected from Mbita in western Kenya; *G. f. martini* was from the IAEA colony in Czech Republic. *Glossina longipennis* was also obtained from Nguruman in Kenya's south Rift, and reared for purposes of this study. The flies were maintained at 26 °C and 60-70% RH with membrane feeding on porcine blood after every 48 hours.

#### 3.2 Total RNA extraction

Tsetse flies were collected from the insectary and subsequently anaesthetized by chilling on ice for 30 minutes. The flies were then placed into separate autoclaved 1.5 ml Eppendorf tubes. (Eppendorf tubes, plastic pestles, and pipette tips were autoclaved prior to use). 550 µL of phosphate buffer saline (PBS) pH 7.4 was added to each of the tubes and using clean autoclaved pestles the samples were homogenized. The lysate was centrifuged (Eppendorf AG 5417R centrifuge, Hamburg, Germany) at 12,000 *xg* for 5 minutes at 4°C. The supernatant was transferred into a clean 1.5 ml Eppendorf tube.

Two hundred and fifty microlitres of the supernatant was transferred into a clean 1.5 ml Eppendorf tube containing 750 µL of TRIzol<sup>®</sup> LS reagent (Invitrogen, Carlsbad CA, USA). The remainder of the supernatant was kept at -80°C for long term storage. This was left to stand at room temperature for 10 minutes. Subsequently, 200 µL of

chloroform (reagent grade) was added to the sample and vortexed for 1 minute. The sample mixture was again left to incubate at room temperature for a further 10 minutes. After the incubation, the sample mixture was centrifuged (Eppendorf AG 5417R centrifuge, Hamburg, Germany) at 12,000  $xg$  for 10 minutes and at 4°C. (This centrifuge was used for all steps requiring centrifugation).

Approximately 500  $\mu L$  of the upper aqueous phase was transferred into a clean autoclaved 1.5 ml micro-centrifuge tube carefully using a pipette. Twenty micrograms of glycogen was added to the sample to maximize precipitation and increase the RNA mass followed by 500  $\mu L$  of isopropanol (reagent grade). This was mixed thoroughly using a vortex machine for 1 minute and incubated overnight at -20°C for maximum RNA precipitation. The sample was then centrifuged at 12,000  $xg$  for 10 minutes at 4°C. A white pellet was apparent at the bottom of the tube. The supernatant was discarded then 500  $\mu L$  of 75% ethanol was added and the tube was capped and gently inverted to wash the insides of the tube. The tube containing the pellet was centrifuged at 12,000  $xg$  for 10 minutes at 4°C. The supernatant was discarded and the centrifugation step repeated once. Traces of the supernatant were removed using a pipette with fine tip. The pellet was resuspended in 15  $\mu L$  of nuclease-free water and left to stand at room temperature for 5 minutes before mixing using a vortex machine. An aliquot of this sample was stored at -20°C. The rest of the RNA was used for quantification and cDNA synthesis.

### **3.2.1 Removal of genomic DNA from RNA preparation**

To remove any residual DNA from the RNA preparation, RNase-free DNase I (Fermentas, Life Sciences) was used to treat the samples as per the manufacturer's

protocol. The following reaction components were added into a clean micro-centrifuge tube; RNA sample (2 µg), 2 µL of 10X reaction buffer and 1 unit of DNase I, RNase-free. This was incubated in a water bath at 37°C for 30 minutes. To the sample mixture, 2 µL of 50 mM EDTA was added and incubated at 65°C for 10 minutes. The DNA free RNA was then quantified and used as template for cDNA synthesis.

### **3.2.2 Determination of RNA yield and purity**

Total RNA concentration was determined by diluting an aliquot of the sample in a ratio of 1:10 in nuclease-free water. The concentration and absorbance at 260 nm was measured by spectrophotometry (Shimadzu BioSpec-mini, Kyoto, Japan). The purity of the isolated RNA was determined from the absorbance ratio at 260 nm and 280 nm. Pure RNA samples exhibit  $A_{260\text{nm}}/A_{280\text{nm}}$  ratios of 2.0. Ratios lower than 1.7 indicates contamination of the sample, particularly with proteins, thus prompting for further purification.

## **3.3 Reverse transcription-Polymerase chain reaction (RT-PCR)**

### **3.3.1 Complementary DNA (cDNA) synthesis**

The first strand cDNA reaction was synthesized using oligo(dT)<sub>18</sub> primer. Into a sterile microfuge tube, an equivalent of 2 µg of total RNA was added followed by 5 µM oligo(dT)<sub>18</sub> primer. Water was added to bring the volume to 12 µL. The tube contents were mixed gently and centrifuged at 15,400 *xg* for 1 minute at 4°C, followed by incubation at 65°C in an oven for 5 minutes. The mixture was spun down and chilled on ice and the following reagents added to bring the volume to 20 µL according to the manufacturer's protocol (RevertAid<sup>TM</sup> H Minus First Strand cDNA synthesis kit,

Fermentas, Lithuania); 1X reaction buffer (250mM Tris-HCl, pH 8.3 at 25°C), 250mM KCl, 20mM MgCl<sub>2</sub>, 50mM DTT), 20 units of RiboLock™ RNase inhibitor (20 U/μL), 20 mM dNTP mix and 200 units of RevertAid™ H Minus M-MuLV reverse transcriptase (200 U/μL). The tube contents were vortexed for 30 seconds and centrifuged at 12,000 *xg* for 30 seconds at 4°C. This was then incubated in 9800 Fast Thermal Cycler (Applied Biosystems, Carlsbad, California) at 42°C for 60 minutes and consequently heated at 72°C for 5 minutes to terminate the reaction.

### 3.3.2 Primer design

Putative *gmmdripa* aquaporin sequence from *G. m. morsitans* was retrieved from GeneDB (<http://www.genedb.org/genedb/glossina/index.jsp>). In order to select the longest Open reading frame including the start and stop codons, the DNA sequence was first translated into the amino acid sequence using the ExPasy translate tool (<http://web.expasy.org/translate/>). Primers for GfmAQP1, GffAQP1 and GIAQP1 were then designed manually based on the DNA sequence of *gmmdripa*. Primer parameters were checked by primer stats hosted by the sequence manipulation suite ([http://www.bioinformatics.org/sms2/pcr\\_primer\\_stats.html](http://www.bioinformatics.org/sms2/pcr_primer_stats.html)).

### 3.3.3 Polymerase chain reaction (PCR) amplification of first strand cDNA

The first strand cDNA synthesis product was used directly as the template for AQP amplification. The *gmmdripa* gene specific primers used (Table 3.1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as internal control during the expression studies.

**Table 3.1** Primer sequences

<b>Primer code</b>	<b>Primer sequence 5' to 3'</b>
<i>gmmdripa</i> -Fw	GGA TTC ATG CTA TTT GCT GAG CTT GCG
<i>gmmdripa</i> -Rv	TTC GAA AAA ATC GTA GGA GTT CGT GTC
RNAi-Fw <i>gmmdripa</i>	AGC GTG CCT CAA ATA GCA TTC
RNAi-Rv <i>gmmdripa</i>	TTA AAA ATC GTA GGA GTT CGT
GAPDH Fw	TAA AAT GGG TGG ATG GTG AGA GTC
GAPDH Rv	CTA CGA TGA AAT TAA GGC AAA AGT
M13 Fw	GTA AAA CGA CGG CCA G
M13 Rv	CAG GAA ACA GCT ATG AC
dsRNA-Fw <i>Gfm</i> AQP1	TATAGAATTCCATGCTATTTGCTGAGCTTGCG
dsRNA-Rv <i>Gfm</i> AQP1	TATAAAGCCTCCGAATGAGCGTGCTGGGTTTC

Amplification was done using Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). To set up a 20  $\mu$ L PCR, the following components are added in sterile PCR tubes; 13  $\mu$ L nuclease-free water, 1X Phusion Reaction Buffer HF, 1 mM MgCl<sub>2</sub>, 4mM dNTPs, 0.25 mM of primers (*gmmdripa*-Fw and *gmmdripa*-Rv for AQP; GAPDH Fw and GAPDH Rv for internal control), 0.25 mM of reverse primer, 0.4 units of Phusion polymerase enzyme and 2 $\mu$ g of the DNA template. The following cycling conditions were used; initial denaturation at 98°C for 30 seconds, 35 cycles of subsequent denaturation at 98°C for 15 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 45 seconds and final elongation at 72°C for 8 minutes. This reaction was carried out in a 9800 Fast Thermal Cycler (Applied Biosystems, Carlsbad, California). Negative control reaction was also set up and it included all the components above but excluding the template.

### **3.4 Agarose gel electrophoresis**

The PCR products were electrophoresed through a 1% agarose gel in 1X TAE (Tris Acetate EDTA) buffer. The sample was loaded on the gel and 70 volts of electric current was supplied to the gel tank for 1 hour 20 minutes from a Bio-Rad power pack (model 200/2.0). After the run was completed the gel was transferred for UV viewing in a Kodak Gel-Logic 200 transilluminator and photographs were taken.

### **3.5 DNA recovery from the gel**

A sharp sterile surgical blade was used to excise the gel with DNA bands of interest which were transferred into clean pre-weighed 1.5 ml Eppendorf tubes. The DNA was purified using QuickClean DNA Gel Extraction Kit (GenScript Corporation, Piscataway, NJ) as follows; the gel pieces placed in the Eppendorf tubes were weighed and recorded. Three volumes of binding solution II was added into the tubes containing the gel (100 mg = 100  $\mu$ L) and incubated in a water bath at 50°C for 10 minutes with occasional vortexing until all the gel was dissolved and the solution turned yellow. One volume of isopropanol was added to the tube contents and vortexed. This was then transferred into QuickClean column and centrifuged at 12,000  $xg$  for 30 seconds. The flow-through in the collection tube was discarded into a discard container and the column was washed twice by adding 500  $\mu$ L of washing solution and centrifugation at 12,000  $xg$  for 1 minute.

The column with bound DNA was then transferred into clean 1.5 ml Eppendorf tube. 30  $\mu$ L of the elution buffer was added carefully into the bottom of the column and incubated at room temperature for 1 minute. This was then eluted by centrifugation at 12,000  $xg$  for

1 minute at 4°C. Seven microlitres of the eluted DNA was run on 1% agarose ethidium-bromide stained gel to confirm DNA recovery.

### **3.6 Cloning of AQP gene in pGEM-T Easy vector**

#### **3.6.1 Preparation of LB-agar/ampicillin/ITPG/X-Gal media**

Luria-Bertani (LB) medium, growth media for microorganisms, was prepared by adding 10 g Bacto-tryptone, 5 g Bacto-yeast extract and 5 g NaCl into 1 L of distilled water containing 15 g of agar (Bertani *et al.*, 1951). The pH was adjusted to 7.0 with NaOH. The LB-agar media was autoclaved and allowed to cool down to 50°C before adding 100 µg/ml ampicillin, 0.5 mM Isopropyl-β-D-thiogalactopyranoside (IPTG), and 40 µg/ml chromogenic dye, 5-bromo-4-chloro-indolyl-D-galactoside (X-Gal) (Promega Corporation, Madison, WI). Into sterile 85 mm petri dishes, 30 ml of the LB-agar/ampicillin/ITPG/X-Gal media was poured and allowed to harden at room temperature. The plates were kept at 4°C for a maximum period of one month.

#### **3.6.2 A-tailing of PCR products**

A-tailing reaction adds 3' Adenine (A) overhangs to the blunt ended PCR products generated by thermostable polymerases as a result of their proof reading activity. This modification to introduce A-overhangs in PCR products allows for ligation into a T-vector (T=Thymine) thus making gene-cloning possible. For one reaction, 1X genescript Taq buffer (with MgCl<sub>2</sub>), 2 mM dATP, 5 units of Genescript DNA polymerase and 7µL of PCR product were added into a clean PCR tube and incubated at 70°C for 30 minutes in a 9800 Fast Thermal Cycler (Applied Biosystems, Carlsbad, California). This reaction

enabled introduction of A-tails to the blunt-ended AQP gene generated by Phusion polymerase (Finnzymes, Finland).

### **3.6.3 Ligation reaction and *E. coli* transformation**

A ligation reaction was set up to insert the gene of interest into the pGEM-T Easy vector (Promega Corporation, Madison WI). Into a 0.5 ml micro-centrifuge tube 1X Rapid ligation buffer, 2 µg of the PCR product, 1 unit of T4 DNA ligase (3 Weiss U/µL), and 50 ng of pGEM-T Easy vector was added and topped up with nuclease-free water to 10 µL. A positive control (supplied with the kit) and a background control (that lacked the DNA insert) were also included. The tube contents were mixed by gently pipetting up and down several times followed by brief centrifugation and overnight incubation at 4°C. High efficiency competent *E. coli* (JM109, Promega Corporation, Madison WI) were transformed with the ligation reaction. Into clean 1.5 ml Eppendorf tube, 5µg of the ligation reaction was added followed by 50 µL of the competent cells. The tube contents were mixed gently and chilled on ice for 20 minutes before heat shocking at 42°C for 1 minute in order for the cells to take up the foreign material.

The cells were supplied with 1 ml of SOC media (super optimal broth with glucose) to allow recovery. The transformation reaction and SOC mixture was incubated at 37°C for 1 hour with shaking at 150 rpm on a rotary shaker (Shellab Mini Shaker, US). Consequently, the LB-agar plates containing ampicillin, IPTG and X-Gal were transferred into the hood (Bellco Glass, Inc, Vineland, USA) so as to warm up at room temperature. After incubation in the SOC media, the transformed JM109 *E. coli* cells were pelleted by centrifugation at 5000 *xg* for 5 minutes at 37°C. The excess supernatant



was discarded and the pellet resuspended in 100  $\mu$ L of LB. These cells were plated on LB-agar/IPTG/X-Gal plates and incubated overnight at 37°C in a Binder oven (Binder Inc. North America).

#### **3.6.4 Colony PCR**

Screening by PCR was done to confirm the success of transformation. Colony PCR was set up using both gene-specific and vector-specific M13 primers as described in section 3.4 above. Sterile tips were used to pick a fraction of the selected colonies to act as the template for the reaction. The following conditions were used for amplification; 1 cycle of initial denaturation at 98°C for 5 minutes, followed by 35 cycles of subsequent denaturation at 98°C for 15 seconds, annealing at 56 °C for 45 seconds, and extension at 72°C for 45 seconds and final extension at 72°C for 8 minutes. The positive clones on the LB-agar plates were subsequently cultured in LB broth containing 100  $\mu$ g/mL of ampicillin in a shaker set at 150 rpm at 37 °C overnight.

#### **3.6.5 Plasmid purification**

Recombinant plasmid was isolated and purified using GeneJET™ Plasmid Miniprep Kit (Fermentas, Lithuania). One ml of recombinant *E. coli* culture was transferred into clean 1.5 ml Eppendorf tube and centrifuged, at 12,000  $xg$  for 1 minute at room temperature (Eppendorf centrifuge 5417R). The supernatant was discarded and the pelleted cells were re-suspended in 250  $\mu$ L of the resuspension solution supplied in the kit. The mixture was vortexed until no cell clumps were evident. This was followed by addition of 250  $\mu$ L of the lysis solution and mixed by inverting the tube 6 times until the solution became viscous. Three hundred and fifty microlitres of the neutralization solution was then added

and mixed again by inverting the tube 6 times. The tube contents were centrifuged at 12,000 *xg* at room temperature for 5 minutes to pellet the cell debris and chromosomal DNA. The supernatant was transferred into the GeneJET™ spin column and centrifuged at 12,000 *xg* for 1 minute at room temperature. The flow-through was discarded and the column placed back in the same collection tube. To the GeneJET™ spin column, 500  $\mu$ L of the wash solution was added and centrifuged at 12,000 *xg* for 1 minute at room temperature. The flow-through was discarded and the step repeated. Finally, the GeneJET™ spin column was transferred into a clean 1.5 ml Eppendorf tube and 35  $\mu$ L of the elution buffer was added to the center of the column membrane followed by incubation at room temperature for 2 minutes. The plasmid DNA was recovered by centrifugation at 12,000 *xg* for 2 minutes at room temperature. Purified plasmid DNA was sequenced using M13 Fw and M13 Rv primers (Table 3.1).

### **3.7 Tissue distribution and life stage expression of aquaporin**

Localization of AQP focused on two tsetse species; *G. f. martini* and *G. f. fuscipes*. However, AQP-knockdown experiment was conducted in *G. f. martini*. Tissues including proboscis, head, legs and midgut were dissected from ten male flies. The flies were first chilled on ice to immobilize before dissections were done. The specific tissues were pooled together in Eppendorf tubes. For life stage expression study, 2<sup>nd</sup> instar larvae dissected from pregnant females, 3<sup>rd</sup> instar larvae, 20 day old pupa, 1 day old teneral fly and adult fly were used. Total RNA was extracted from samples using the TRIzol® LS protocol described in section 3.2 and used for semi-quantitative RT-PCR described in section 3.4, to amplify AQP gene.

## **3.8 RNA interference**

### **3.8.1 RT-PCR for in-vitro transcription**

RT-PCR was done (as described in section 3.4) using *gmmdripa* primers (Bargul, 2011) with T7 promoter sequence on both forward and reverse primers. The PCR products were purified using QuickClean II PCR extraction kit (GenScript USA Inc. Piscataway, NJ). Two volumes of the binding buffer were added to 1 volume of the PCR products and mixed gently. This was transferred into a spin column and centrifuged at 6,000  $xg$  for 1 minute. The flow through was discarded and the column washed twice with 650  $\mu$ L of wash buffer. To the centre of the spin column, 35  $\mu$ L of the elution buffer was added incubated for 2 minutes at room temperature and then centrifuged at 12,000  $xg$ . The purified products were used as template for *in vitro* transcription using MEGAscript<sup>®</sup> T7 *in vitro* transcription Kit (Ambion, 2130 Woodward St. Austin TX 78744).

### **3.8.2 dsRNA synthesis and purification**

Transcription reaction was set up at room temperature. Into a sterile PCR tube, nuclease free water was added (to 20  $\mu$ L), followed by 2  $\mu$ L each of dNTPs (ATP, CTP, GTP, UTP), 1X Reaction Buffer, 2 $\mu$ g DNA template and 2 units of T7 enzyme mix. The reaction mixture was mixed gently by pipetting up and down and centrifuged briefly to collect the reaction mixture at the bottom of the tube. The tube contents were incubated at 37°C for 14 hours. To digest the template DNA, 1  $\mu$ L of TURBO DNase enzyme was added and incubated for 15 minutes at 37°C. Purification of the dsRNA was carried out using MEGAclean<sup>™</sup> Kit (Ambion, 2130 Woodward St. Austin TX 78744). The dsRNA binding mix was assembled as per the manufacturer's protocol and transferred into the

filter cartridge. The mixture was centrifuged at 15,400 *xg* for 2 minutes followed by two wash steps with 500  $\mu\text{L}$  of 2X wash solution. Recovery of the dsRNA was achieved by applying hot elution buffer at the centre of the filter cartridge and centrifuging at 15,400 *xg* for 2 minutes. The dsRNA was quantified by spectrometry (Shimadzu BioSpec-mini, Kyoto, Japan).

### **3.8.3 Concentration of dsRNA**

Ammonium acetate (5M) was mixed with purified dsRNA (section 3.9.2) in the ratio of 1:10 followed by addition of 2.5 volumes of 100% ethanol. The tube contents were mixed and incubated at  $-20^{\circ}\text{C}$  for 30 minutes followed by centrifugation ( $4^{\circ}\text{C}$  for 15 minutes at 15,400 *xg*) to pellet the RNA. The supernatant was discarded and the pellet washed using 500  $\mu\text{L}$  of 70% v/v ice-cold ethanol. The DNA was air dried and re-suspended in nuclease-free water to a final concentration of 5  $\mu\text{g}/\mu\text{L}$ .

### **3.8.4 Microinjection of tsetse fly**

A total of 96 male teneral *G. f. martini* flies (32 test, 32 controls and 32 uninjected controls) were used in this experiment. On the first day, the newly emerged flies were membrane-fed on porcine blood. Microinjections were done on the second day. The flies were chilled on ice for 30 minutes with the test flies being injected with 2  $\mu\text{L}$  (equivalent to 10  $\mu\text{g}$ ) of concentrated dsRNA corresponding to *GfmAQP1*, using a fine glass needle into the dorsolateral surface of the flies' thorax. The control flies were injected with 2  $\mu\text{L}$  of nuclease-free water and the last group was uninjected. The flies were then caged and kept at a controlled temperature of  $26\pm 1^{\circ}\text{C}$  and humidity of 60-70%. The flies received porcine blood via an artificial membrane system every 48 hours for ten days.

### **3.8.5 Verification of gene knockdown**

Semi-quantitative RT-PCR was used to measure the degree of *GfmAQP1* gene knockdown. The flies were chilled on ice prior to dissections to isolate midguts. Total RNA was extracted from the midguts using TRIzol® LS reagent as described in section 3.2, followed by removal of contaminating DNA as described in section 3.2.1. RNA yields were determined by spectrophotometry and the concentrations normalized to 0.4 µg/µL before first strand cDNA synthesis (Section 3.3.1). PCR was done using gene specific aquaporin RNAi primers (RNAi-Fw4886 and RNAi-Rv4886, Table 3.1) and the products were electrophoresed in ethidium bromide-stained 1% agarose gel. The gel image was acquired using KODAK Gel logic imaging system (Raytest GmbH, Straubenhardt).

### **3.8.6 Effect of AQP gene knockdown on tsetse fly survival and diuresis**

In each of the three groups (test, control and uninjected controls), the mortality rates were monitored and recorded on daily basis for 8 days. All flies were kept in the same controlled conditions as described in section 3.9.4. Dead flies were counted and removed from the cages daily. The effect of *GfmAQP1* knockdown on *G. f. martini* diuresis was monitored by measuring rates of the of abdomen reduction 20 minutes and 60 minutes after a bloodmeal using Leica EZ4 D stereo microscope (Leica Microsystems, Wetzler Germany).

### **3.8.7 Data analysis**

*GfmAQP1*, *GffAQP1* and *GIAQP1* DNA sequences were edited using BioEdit Sequence Alignment Editor version 7.1.3.0 (Hall, 1999) to remove and/or replace ambiguous bases.

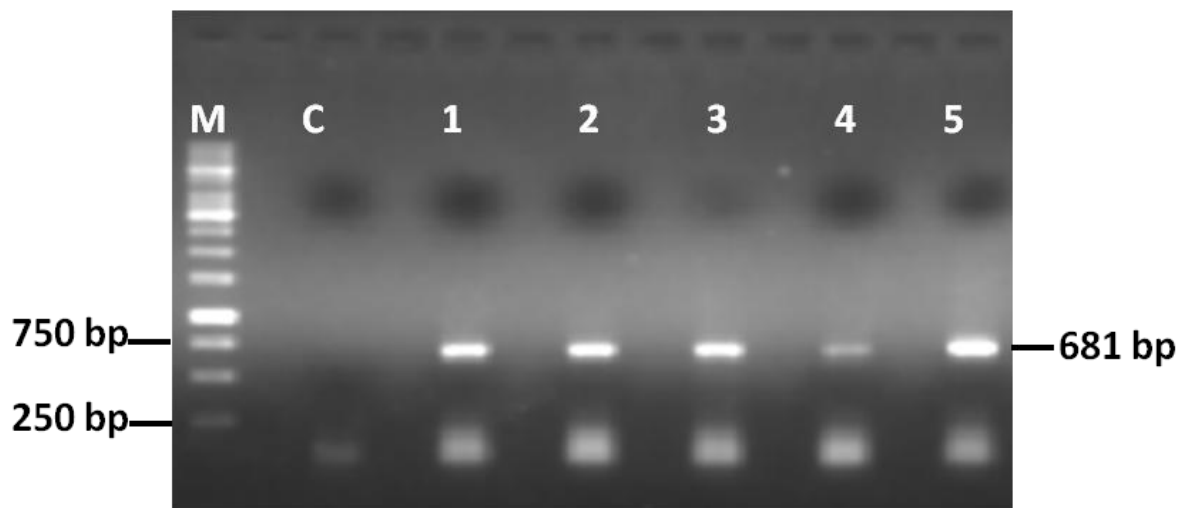
tBLASTx algorithm of NCBI GenBank was used to search for sequences related to the AQPs. Edited sequences were aligned by Clustal W and re-adjustments done manually. The sequences were also translated to their respective amino acid sequences using the translator algorithm of JustBio ([www.justbio.com](http://www.justbio.com)). Amino acid sequences were aligned using MUSCLE algorithm of the multiple sequence alignment tool hosted by the European Bioinformatics Institute (EBI) (<http://www.ebi.ac.uk/Tools/msa/muscle/>) and finally by ESPript version 2.2 software (<http://esprict.ibcp.fr/ESPript/ESPript/>). Phylogenetic and molecular evolutionary analyses were conducted using Molecular Evolutionary Genetic Analysis (MEGA) software version 6.0 (Tamura *et al.*, 2013). Structural prediction of the AQP protein was performed using TMHMM Server v. 2.0 ([www.cbs.dtu.dk/services/TMHMM](http://www.cbs.dtu.dk/services/TMHMM)) Chi square test was used to determine whether there was a significant difference in the feeding success of flies in the three groups of treatments (Water injected controls, dsRNA injected and uninjected controls). A negative binomial (NB) regression model was used to investigate the association between the treatments and the mortality rates of the flies.

## CHAPTER FOUR

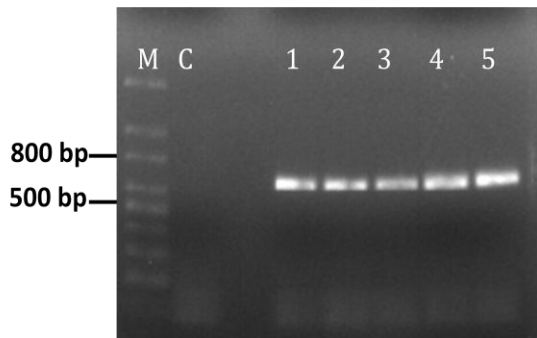
### 4. RESULTS

#### 4.1 Amplification of AQP genes

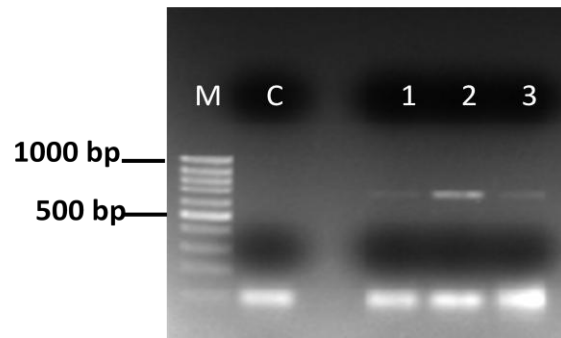
RT-PCR amplification of whole body tissue RNA from adult male *G. f. fuscipes*, *G. f. martini* and *G. longipennis* yielded product sizes of 681 bp, which were assigned the names *GffAQP1*, *GfmAQP1* and *GlaAQP1* respectively (Figure 4.1a, 4.1b and 4.1c respectively). Recovery of DNA fragments from 1% w/v agarose gel was successful. It yielded band fragments of 681 (Figure 4.2).



**Figure 4.1a** Gel image of *G. f. fuscipes* (*GffAQP1*) AQP RT-PCR DNA amplification **M**: 1kb DNA ladder (Fermentas), **C**: Negative control, **Lanes 1- 5**: *GffAQP1*.

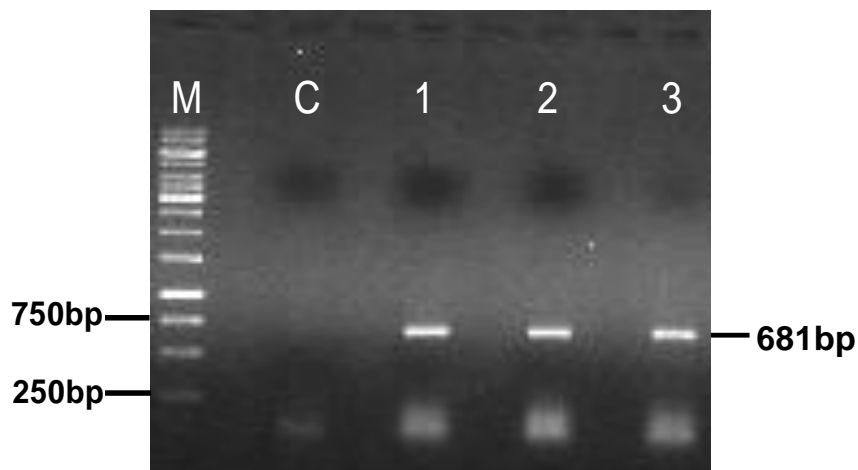


**Figure 4.1b**



**Figure 4.1c**

**Figure 4.1b** Gel images of *G. f. martini* AQP (GfmAQP1) DNA amplification and electrophoresis **M**: DNA marker 100bp, GenScript, **C**: Negative control, **Lanes 1-5**: *GfmAQP1*. **Figure 4.1c** *G. longipennis* AQP (GIAQP1) DNA amplification and electrophoresis 1hr **M**: DNA molecular marker 100bp, **C**: Negative control, **Lanes 1-3**: GIAQP1.

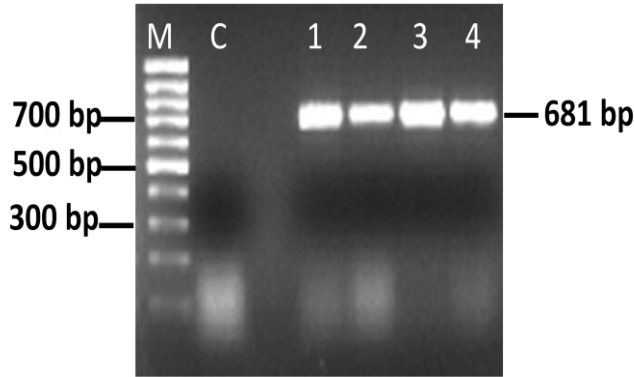


**Figure 4.2** Gel image of purified RT-PCR products electrophoresed in 1% w/v agarose **M**: 1kb DNA marker (Fermentas). **C**: Control **Lane 1**: *GfmAQP1*, **Lane 2**: *GffAQP1* **Lane 3**: *GIAQP1*.

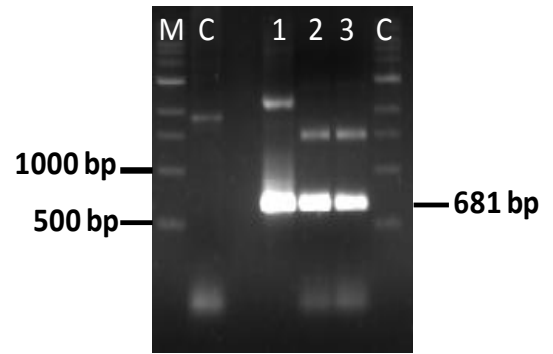
#### 4.2 Detection of AQP gene inserts

Colony PCR with AQP gene specific primers confirmed that the three AQPs (*GffAQP1*, *GfmAQP1* and *GIAQP1*) were successfully ligated into pGEM-T Easy vector and yielded fragments of 681 bp which were equivalent to the targeted AQP band size (Figure 4.3a and 4.3b).





**Figure 4.3a**



**Figure 4.3b**

**Figure 4.3a** Gel image showing fragments obtained from Colony PCR using gene specific primers and electrophoresis **M**: DNA molecular marker 100bp. **C**: Negative control (blue colony). **Lane 1 and 2**: white colonies with *GfmAQP1*. **Lane 3 and 4**: white colonies with *GffaQP1*. **Figure 4.3b**: Colony PCR using gene specific primers, **M**: 1kb DNA molecular marker, Biolabs. **C**: Empty plasmid, control. **Lane 1-3**: Plasmids with GIAQP1 insert.

### 4.3 AQP sequence analysis

#### 4.3.1 Sequence comparison

A BLAST search using blastp algorithm of NCBI GenBank database showed that *GfmAQP1* was 99% identical to *G. m. morsitans* integral protein a (*Dripa*) with E value of  $2.7e-157$ , while both *GffaQP1* and *GIAQP1* had 98% identities (E values  $7e-156$  and  $2.6e-157$ ) to *G. m. morsitans dripa*. CLUSTAL W alignment of the AQPs showed high degree of genetic conservation within the putative *Glossina* AQPs (Figure 4.4).



BLAST search of *Gfm*AQP1, *Gff*AQP1, and *GIA*AQP1 in the GenBank database also revealed identity to other insect AQPs: with *Drosophila melanogaster* integral protein *Drip* (NM 165833.3) showing 70% identity, the adult buffalo fly, *Haematobia irritans exigua* water channel 1 BFWC1 (Q25074.1) had 71% sequence identity while *Phormia regina* PregAQP1 (AB 713909.1) aquaporin had 70% identity and *Aedes aegypti* aquaporin (AF218314.1) with 59% identity (Table 4.1).

**Table 4.1** *Gff*AQP1, *Gfm*AQP1 and *GIA*AQP1 protein sequences similarity to other AQPs obtained from NCBI GenBank database using blastp algorithm.

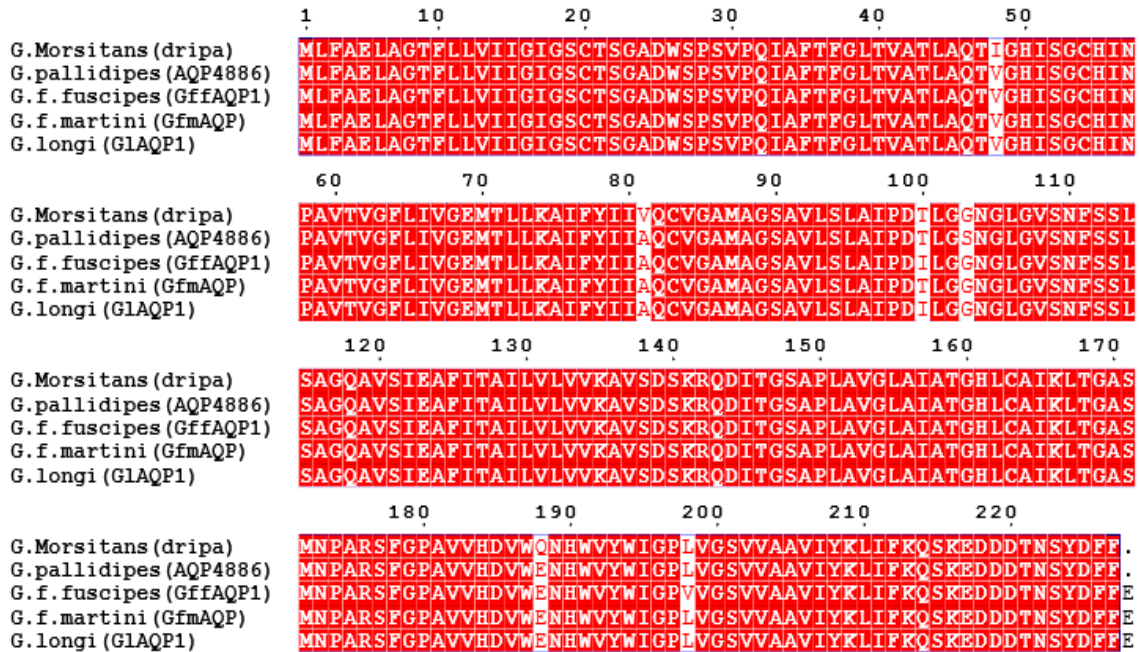
Accession No.	Description	E-value	% Identity	Reference
JN685581.1	<i>G. m. morsitans</i> integral protein a (Dripa) mRNA, complete cds	3e-157	99%	Benoit <i>et al.</i> , 2014
NM_165833.3	<i>Drosophila melanogaster</i> drip (Drip), isoform b, mRNA	7e-103	70%	Hoskins <i>et al.</i> , 2007
Q25074.1	<i>Haematobia irritans exigua</i> water channel protein BFWC1	2e-48	71%	Elvin <i>et al.</i> , 1999
AB713909.1	<i>Phormia regina</i> aquaporin PregAQP1	1e-112	70%	Ishida <i>et al.</i> , 2012
JN685582.1	<i>G. m. morsitans</i> integral protein b (Dripb)	6e-157	71%	Benoit <i>et al.</i> , <i>In press</i>
AF218314.1	<i>Aedes aegypti</i> aquaporin-1	2e-83	60%	Pietrantonio <i>et al.</i> , 2000
EU124628.1	<i>Lutzomyia longipalpis</i> pupative aquaporin, mRNA complete cds	1e-87	58%	Jochim <i>et al.</i> , 2008
AB433197	<i>Coptotermes formosanus</i> aquaporin CfAQP1	6e-78	57%	Kambara <i>et al.</i> , 2009
XM_319584	<i>Anopheles gambiae</i> str. PEST (AQP-ANOGA)	2e-57	55%	Mongin <i>et al.</i> , 2004
XM_624528	Predicted: <i>Apis mellifera</i> aquaporin AQPAn. G-like	5e-75	54%	INV, 2011
EU127479	<i>Bemisia tabaci</i> aquaporin 1 mRNA, complete cds	2e-66	50%	Fabrick, 2008
NM_001085862	<i>Xenopus laevis</i> aquaporin 2 (collecting duct) (aqp2),	9e-64	50%	Kubota <i>et al.</i> , 2006

Using multiple sequence alignments (CLUSTAL W 2.1), amino acid sequences of the studied AQPs were found to contain the hallmarks of orthodox aquaporins. There were

two asparagine-proline-alanine (NPA) motifs which are conserved in most aquaporins. The conserved aromatic/arginine constriction region (phenylalanine 56, histidine 180 and arginine 125) for channel selectivity in water specific AQPs such as human AQP1 was also present in *Gff*AQP1, *Gfm*AQP1 and *Gl*AQP1 (Figure 4.5).



Using MUSCLE software (Edgar, 2004), it was observed that *GpAQP1* and *GfmAQP1* had highest identity of 98.24% with reference to *G. m. morsitans Dripa*. *GIAQP1* and *GffAQP1* had a 100% similarity (Figure 4.6).



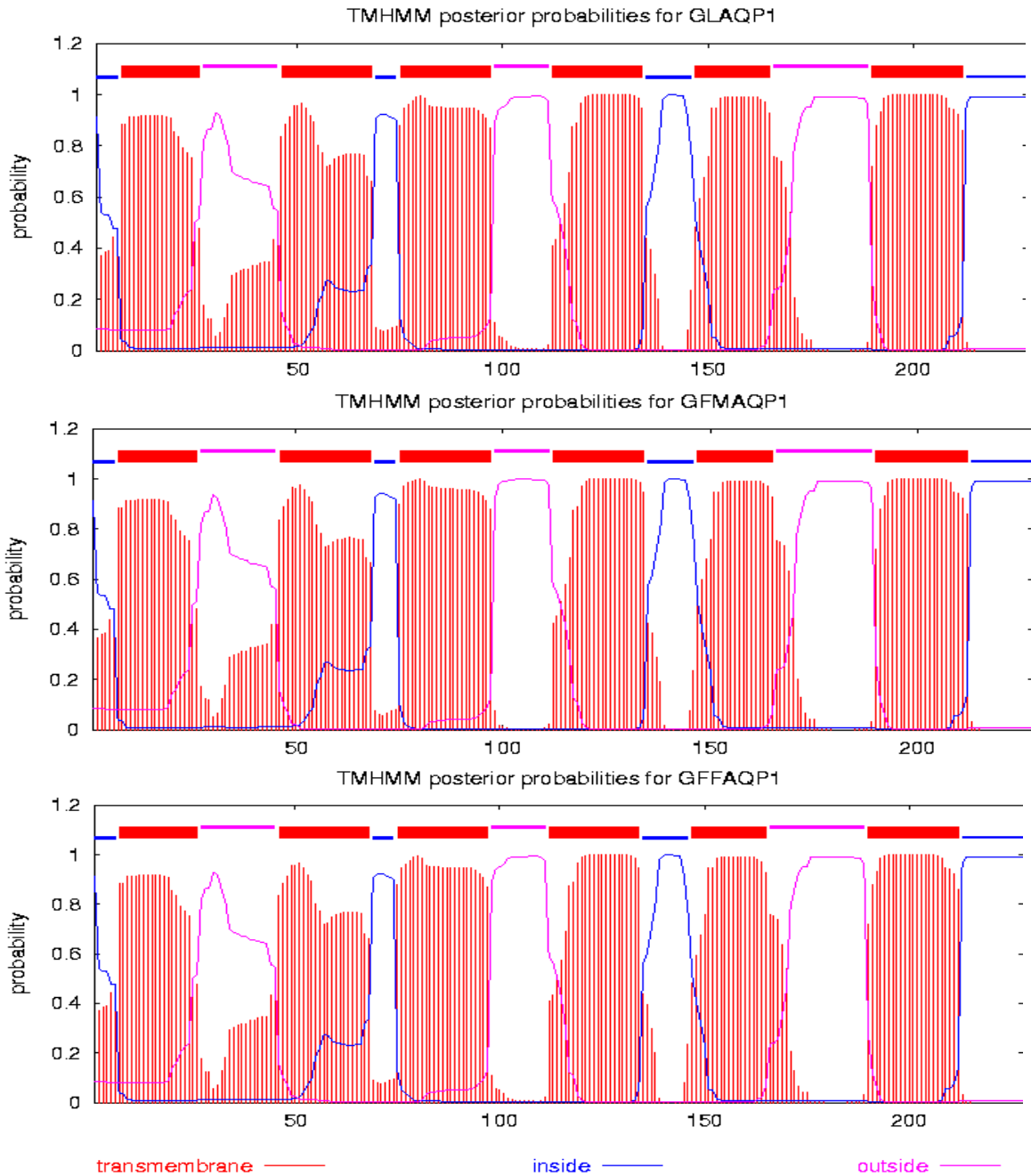
**Figure 4.6** MUSCLE amino acid sequence alignment of Glossina AQP genes and projection by ESPript sequence alignment formatting tool version 2.2. Identical residues are in red and different residues are in white.

The only difference between *GIAQP1* and *GffAQP1* amino acid sequence is at position 198 where the hydrophobic amino acid leucine in *GIAQP1* and the other AQPs is replaced by valine -which is also a hydrophobic amino acid- in *GffAQP1*. Valine 48 in *GfmAQP1*, *GffAQP1*, *GpAQP1* and *GIAQP1* is substituted by hydrophobic isoleucine in *G. morsitans drip a*. Another hydrophobic to hydrophobic amino acid substitution occurs at position 81 where alanine is replaced by valine in *G. morsitans Dripa*. Threonine 100 in *G. morsitans Dripa*, *GpAQP1* and *GfmAQP1* is replaced by isoleucine in *GIAQP1* and

*GffAQP1*. Glutamic acid at position 188 in *GfmAQP1*, *GffAQP1*, *GpAQP1* and *GIAQP1* is substituted by glutamine in *Dripa*.

#### **4.3.2 Membrane topology prediction.**

The amino acid sequences of *GfmAQP1*, *GffAQP1* and *GIAQP1* were submitted to TMHMM Server v. 2.0 ([www.cbs.dtu.dk/services/TMHMM](http://www.cbs.dtu.dk/services/TMHMM)) for prediction of transmembrane helices in proteins. The prediction showed that the three AQPs are membrane proteins with six transmembrane domains (Figure 4.7).



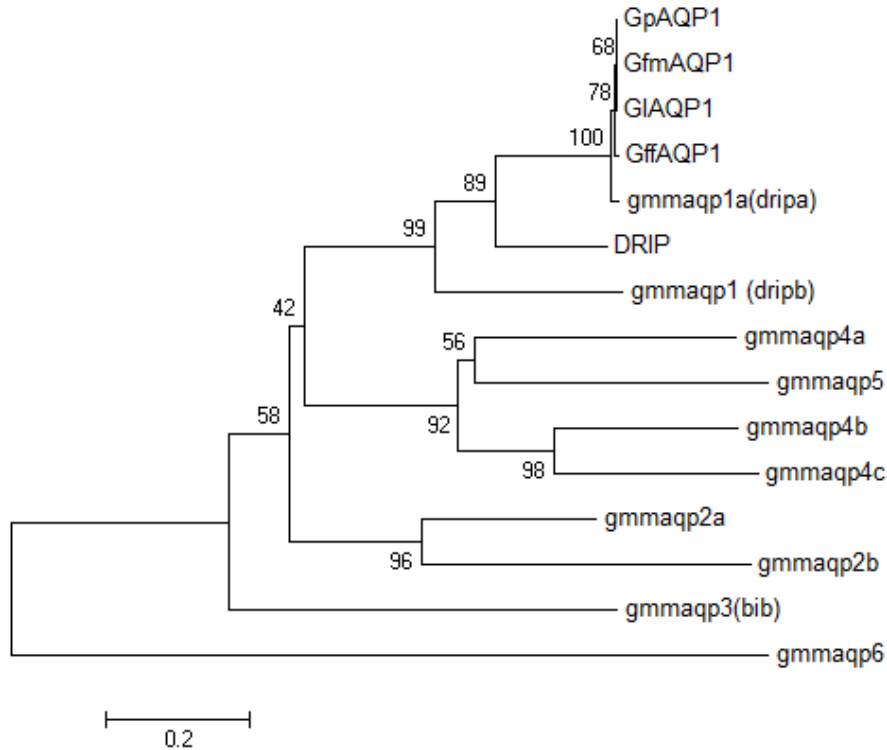
**Figure 4.7** TMHMM membrane topology predictions of GLAQP1, GfmAQP1 and GffAQP1. Transmembrane domains are shaded in red while the joining loops are in blue and pink.

### 4.3.3 Evolutionary relationships of AQP genes

Using MEGA version 6 (Tamura *et al.*, 2013), three distinct clusters were evident (Figure 4.8). It was observed that *GLAQP1*, *GfmAQP1*, *GffAQP1* and *GpAQP1* aquaporin genes all



clustered with *G. m. morsitans* (*gmmaqp1*) *dripa* aquaporin and *D. melanogaster* water specific *drip*.



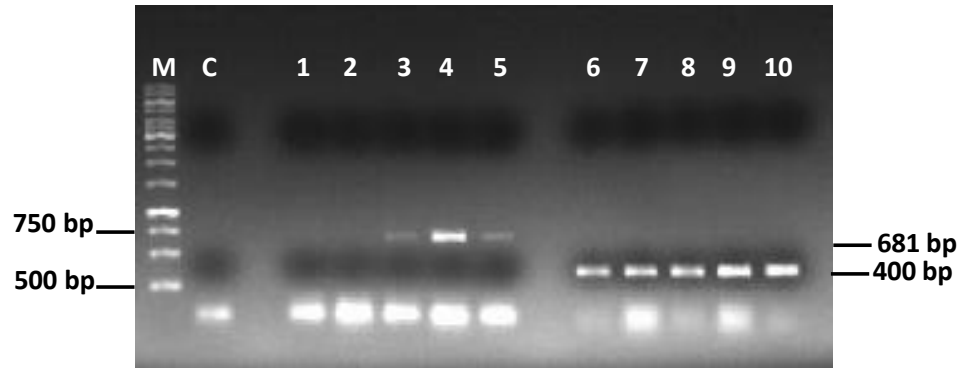
**Figure 4.8** A dendrogram showing the relationship between GIAQP1, GfmAQP1, GffAQP1 and GpAQP1 with the ten putative *G. m. morsitans* AQP genes.

The second cluster consisted of *gmmaqp4a*, *gmmaqp4b*, *gmmaqp4c* and *gmmaqp5* while the third cluster consisted of *gmmaqp2a*, *gmmaqp2b*, and the *G. m. morsitans* big brain (*bib*) aquaporin *gmmaqp3*. The most distant was *gmmaqp6* which clustered independently from all the other AQPs.

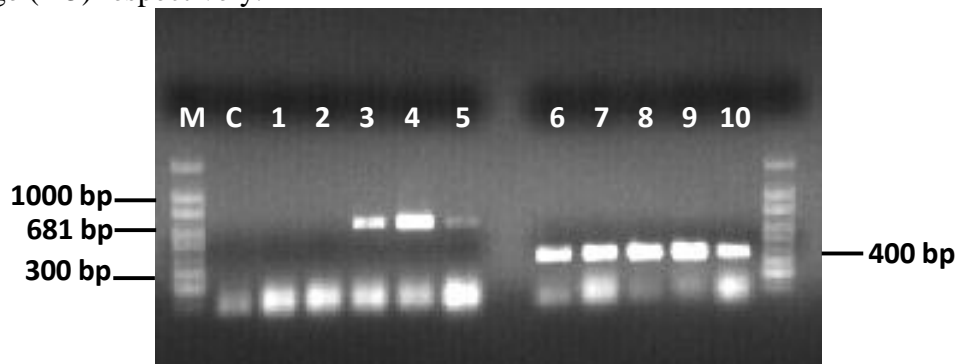
#### 4.4 Developmental stage expression of AQP

Semi-quantitative RT-PCR data showed that the expression levels of AQP in the larval stages of *G. f. martini* and *G. f. fuscipes* were low and amplification was achieved only after running a second PCR using the product of the first PCR as the template. Expression

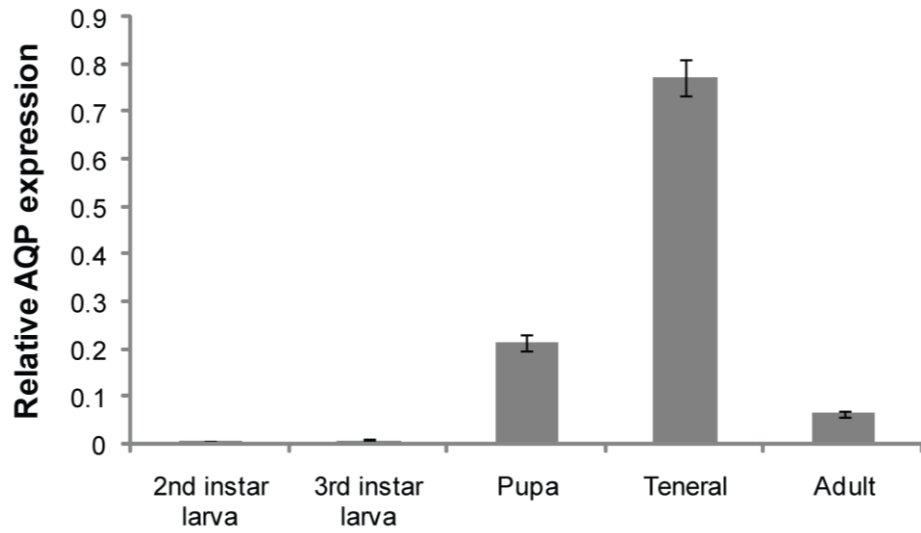
levels of the AQP gene in the pupa and adult stages was almost the same with the highest expression levels recorded in the teneral stages as shown in (Figure 4.9a and 4.9b; figure 4.10a and 4.10b). All tests were done in three replicates.



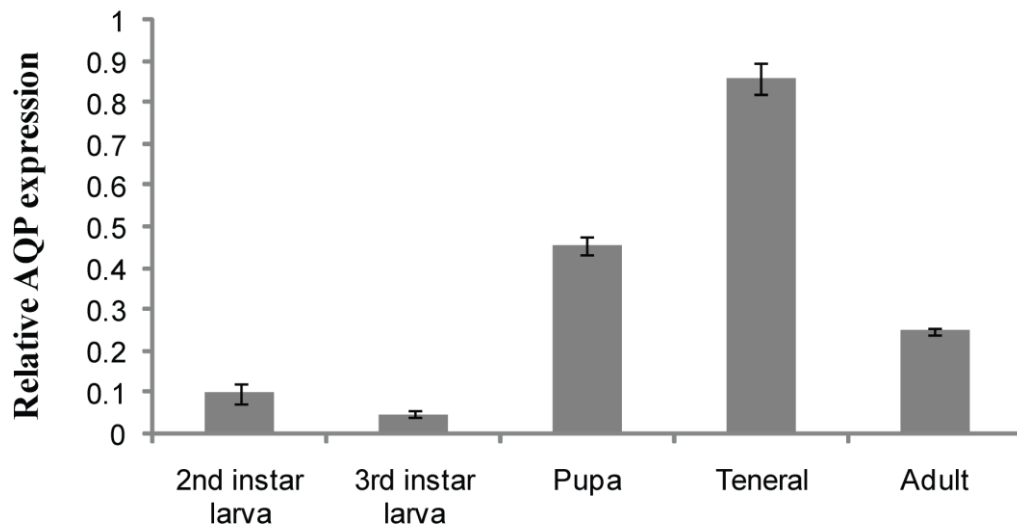
**Figure 4.9a** Gel image showing expression of GfmAQP1 in different life stages of *G. f. martini*. **M:** 1Kb DNA molecular ladder, GenScript. **C:** Negative control. **Lane 1:** second instar larvae. **Lane 2:** third instar larvae. **Lane 3:** pupa **Lane 4:** Teneral fly. **Lane 5:** Adult fly. **Lane 6-10:** GAPDH internal control for each life stage (1-5) respectively.



**Figure 4.9b** Gel image showing expression of GffAQP1 in different life stages of *G. f. fuscipes* **M:** DNA molecular ladder 100 bp, GenScript. **C:** Negative control. **Lane 1:** second instar larvae. **Lane 2:** third instar larvae. **Lane 3:** pupa **Lane 4:** Teneral fly. **Lane 5:** Adult fly. **Lane 6-10:** GAPDH internal control for each life stage (1-5) respectively.



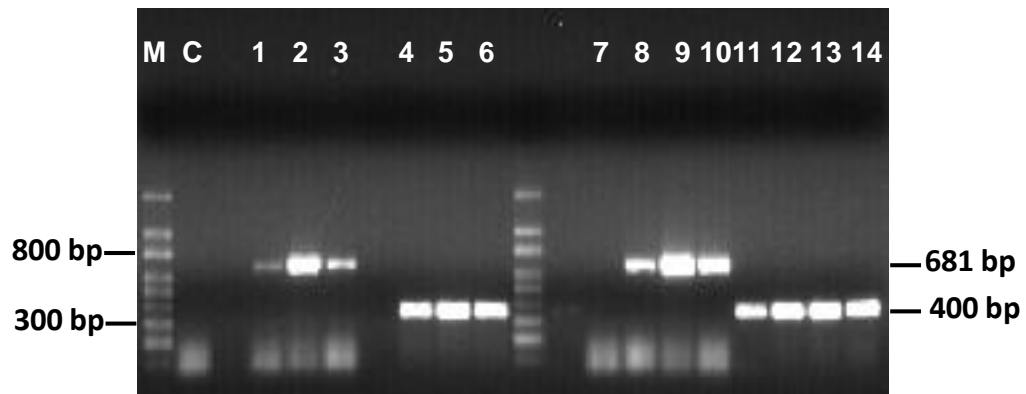
**Figure 4.10a** Life stage expression of *GffAQP1* in *G. f. fuscipes* normalized against GAPDH.



**Figure 4.10b** Life stage expression of *GfmAQP1* in *G. f. martini* normalized against GAPDH.

#### 4.5 AQP tissue distribution pattern

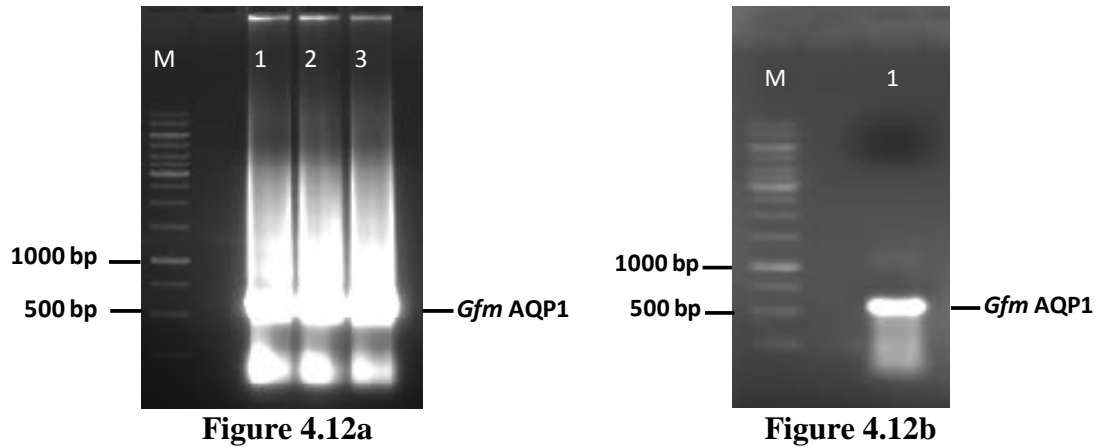
RT-PCR results showed that the AQP gene was expressed in different tissues in both *G. f. martini* and *G. f. fuscipes* (Figure 4.11). Since aquaporins are water-channel proteins, it was expected that their expression levels will be highest in tissues associated with higher rates of water flux. AQP was expressed in the head and legs albeit at low levels. In the proboscis, the expression level was much lower.



**Figure 4.11** Gel image showing tissue expression patterns of *GfmAQP1* and *GffAQP1* by RT-PCR **M**: 100bp DNA ladder, GenScript, **C**: Negative control **Lane 1, 2, 3**; *GffAQP1*, legs, midgut, head **Lane 4, 5, 6**; *G. f. fuscipes* GAPDH (control), legs, midgut and the head respectively. **Lane 7-10**; *GfmAQP1*, proboscis, legs, midgut, and head **Lane 11-14**; *G. f. martini* GAPDH (control), proboscis, legs, midgut, head respectively.

#### 4.6 Double strand RNA synthesis

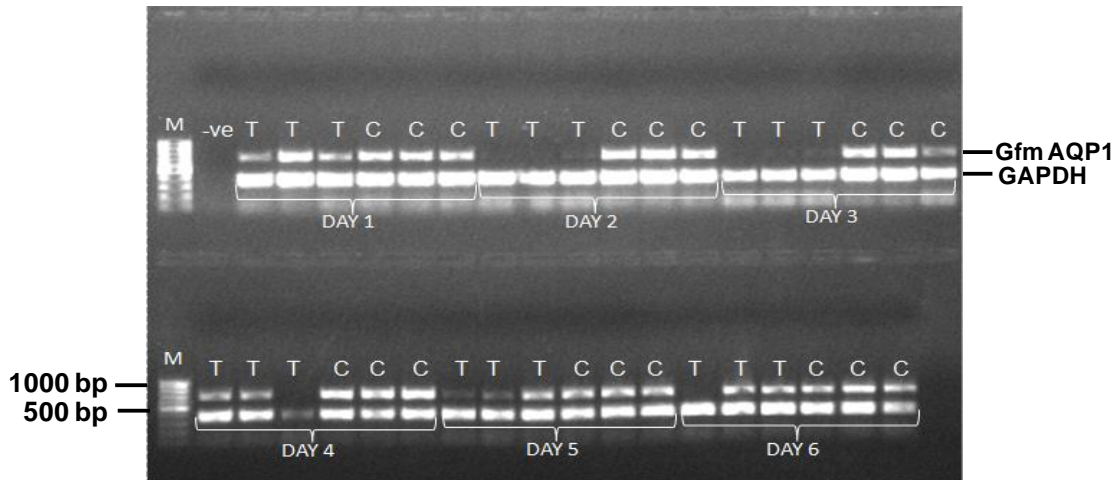
Good yield of dsRNA was obtained from the transcription reaction and 1/10 dilution of the reaction product gave a strong band confirming the success of transcription (Figure 4.12a). However, there were residual nucleotides, short oligonucleotides and salts in the RNA which prompted further purification prior to microinjections (Figure 4.12b).



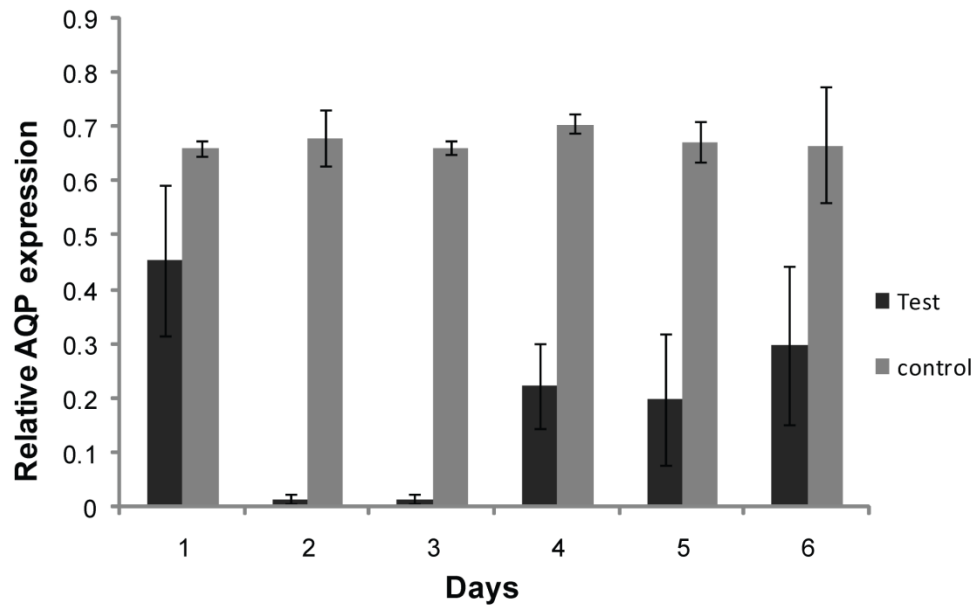
**Figure 4.12a** Gel image of dsRNA corresponding to *GfmAQP1* after in vitro transcription M: 1kb DNA marker (Fermentas), **Lane 1-3:** dsRNA corresponding to *GfmAQP1* with T7 promoter at both ends. **Figure 4.12b** Purified dsRNA.

#### 4.6.1 Verification of gene knockdown

To evaluate the success of knockdown, RT-PCR was used to detect the presence of *GfmAQP1* transcripts in the dissected midguts from flies injected with the dsRNA corresponding to *GfmAQP1* and from the NFW injected control group. Up to 97% knockdown was achieved as indicated by the reduced transcript levels from the second day post-injection in the dsRNA group. Five days post injection, AQP transcript levels increased. In the control group, the aquaporin transcript levels were present in nearly equal amounts higher than in the knockdown groups (Figure 4.13 and 4.14).



**Figure 4.13** Gel image showing GfmAQP1 transcript levels (semi-quantitative RT-PCR) after gene knockdown **M**: 100 bp DNA marker, Fermentas, **-V**: Negative control, **T**: dsRNA injected test samples, **C**: NFW injected controls.

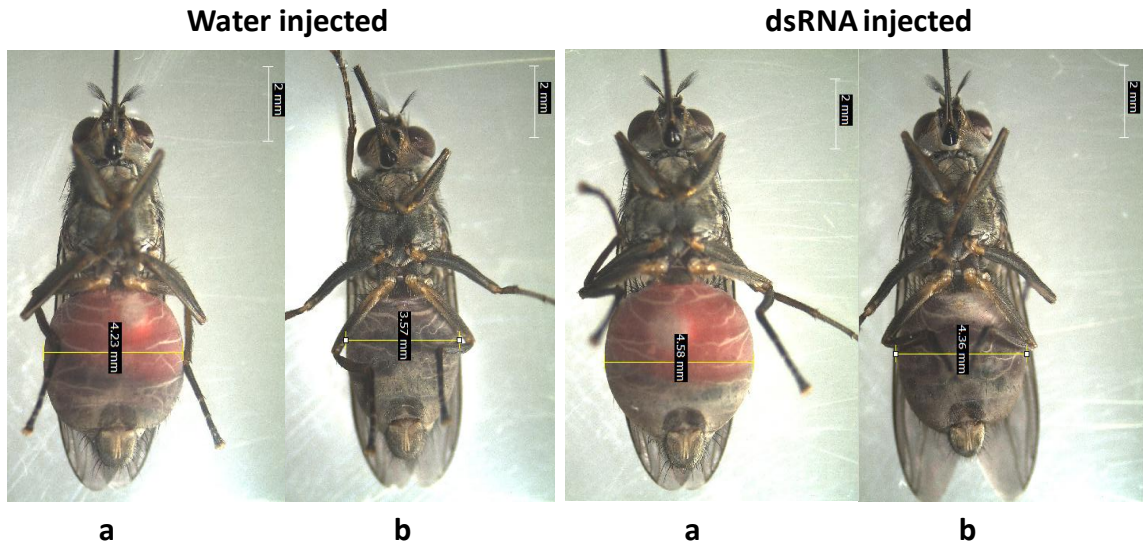


**Figure 4.14** Relative expression pattern of GfmAQP1 after gene knockdown. For each treatment  $n = 3$ . Low *GfmAQP1* expression was observed day 2 and 3 post dsRNA injection.

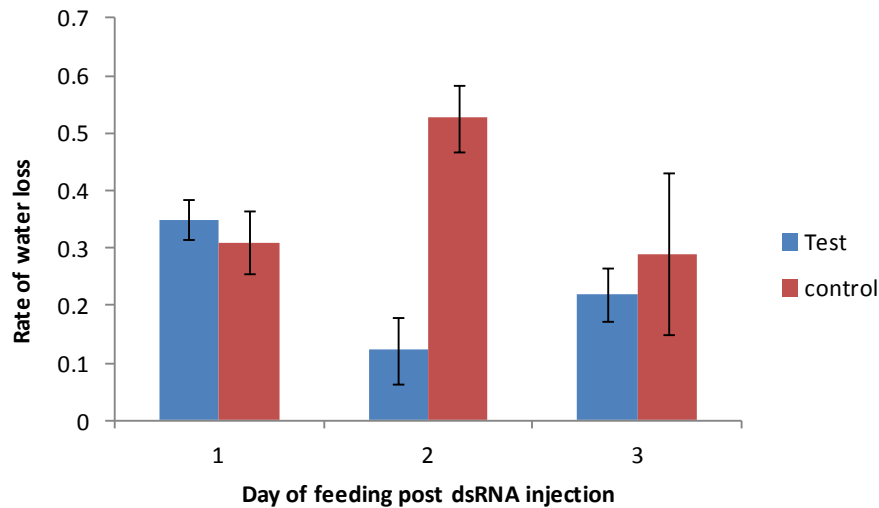
#### 4.6.2 Phenotypic trait characterization of *GfmAQP1* knockdown

Images and measurements of abdomen diameters from nine flies injected with dsRNA for gene knockdown and nine flies injected with NFW were taken and recorded after every

blood meal. Results showed that in flies with gene knockdown there was a reduction in diuresis by 30% as compared to the flies without knockdown, supported by slower reduction rates of abdomen diameter in this group (Figure 4.15 and 4.16).



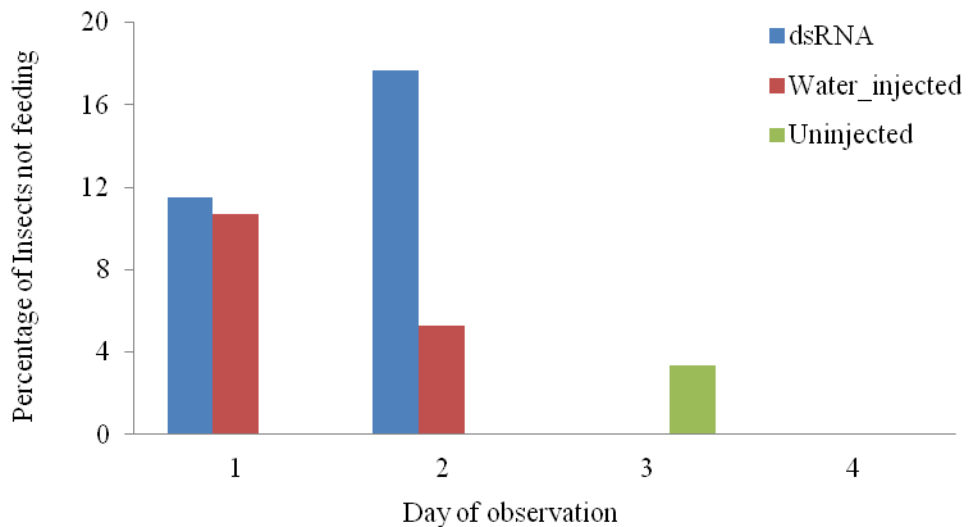
**Figure 4.15** Blood-fed tsetse fly images, taken by Leica EZ4 D stereo microscope, showing the sizes of their abdomen after (a) 20 min, and (b) 60 min post-feeding, in presence (dsRNA-injected) and absence (water-injected) of *GfmAQP1* gene silencing. *GfmAQP1* knockdown slowed down the rate of diuresis after bloodmeal evidenced by 30% difference in change of abdomen diameter between the two groups. In each treatment n = 9.



**Figure 4.16** Graphical representation of water loss rates across the abdomens of *GfmAQP1* knockdown flies and normal flies.

### 4.6.3 Feeding success of tsetse fly

The proportions of insects observed not to feed on each day of observation for each treatment group is shown in figure 4.17. On day 1 and 2, a higher proportion of dsRNA treated insects did not feed compared to the water-injected and non-injected flies. On the fourth observation, all the live insects fed. Using Chi square test, these proportions varied significantly among the three treatments (Figure 4.17) (Chi square = 8.961, df=2, p=0.0113).



**Figure 4.17** Proportion of insects not feeding on each day of observation. All flies fed on the last day of observation. For each treatment n = 32.

### 4.6.4 Mortality rates after treatment

There was a survival rate of 82%, 85% and 97% for dsRNA injected, NFW injected and the uninjected flies respectively. The results are summarized in Table 4.2. The results show that the risk of an insect dying was significantly higher in dsRNA treatment than in



uninjected controls ( $p=0.021$ ), but not significantly so for the water-injected group ( $p=0.655$ ).

**Table 4.2** Negative binomial regression model results for the number of deaths due to the three treatments.

Treatment	Incidence risk ratio	95% CI	P value
dsRNA	1	—	—
Water-injected	0.763	0.23-2.50	0.655
Uninjected	0.083	0.01-0.69	0.021

## CHAPTER FIVE

### 5. DISCUSSION, CONCLUSION AND RECOMMENDATIONS

#### 5.1 Discussion

Homologs of *Drip* (named *GfmAQP1*, *GffAQP1* and *GLAQP1*) from three tsetse fly species (*G. f. fuscipes*, *G. f. martini* and *G. longipennis*) were amplified and cloned into pGEM-T Easy cloning vector and sequenced. Nucleotide sequence comparisons with other characterized insect AQPs indicate that *GfmAQP1* were homologous to *D. melanogaster Drip* (74%), *H. irritans* BFWC1 (72.1%) and *P. regina* PregAQP1 (71.3%). Others include *A. aegypti* aquaporin and *L. longipalpis* pupative aquaporin gene with 59% and 58% homology respectively. These aquaporins have been characterized to be water-specific indicating that the studied *Glossina* AQPs could also be water-specific. Amino acid sequence comparison of *GfmAQP1*, *GffAQP1* and *GLAQP1* shows high conservation within the species, with substitution of like for like amino acids occurring where there is change. This shows that the studied AQPs are of one family and due to the high conservation their function could be similar.

Aquaporins are highly expressed in tissues with a need for high rates of water flux. The *D. melanogaster Drip* aquaporin is highly expressed in the pharynx, gut, spiracles and in the malpighian tubules (Kaufmann *et al.*, 2005). Functional analysis of *Drip* indicated that it is a water-specific channel as it only facilitated water transport but not other solutes including glycerol, ammonia, protons and urea. In this study tissue localization was done in *G. f. fuscipes* and in *G. f. martini* to determine the abundance of AQPs in

different tissues including the head, legs, proboscis and the midgut. The malpighian tubules and salivary glands (MT) were not examined because a large number of flies (which was not available at the time of the experiment) were required to pool sufficient MT for analysis. Analysis of intensity of the DNA bands (semi-quantitative RT-PCR products) on gel photo using ImageJ software (Rasband, National Institutes of Health, Bethesda, Maryland, USA) showed lower expression levels of AQP in the proboscis, legs and in the head. However, the expression level in the midgut was higher. The increased expression in the midgut could be due of the need for the insect to eliminate the excess water that comes with the blood meal and also to help the tsetse fly in osmotic regulation. Aquaporins have also been shown to be highly expressed in tsetse fly salivary glands (Bargul, unpublished data). Since the flies pass out trypanosomes into the host through saliva (Lehane, 2005), which is generated in the salivary glands during feeding, reducing expression of AQPs in this organ can greatly influence parasite transmission.

A water-specific aquaporin *RsAQP* from the brown dog tick *Rhipicephalus sanguineus* been identified in the salivary glands, gut and malpighian tubules which are tissues involved with water movement and concentration of blood meal (Andrew *et al.*, 2009). Mathew *et al.* (2011) localized an aquaporin *BtAQP1* in the gut of the whitefly *Bemisia tabaci* which is a phloem feeder. *BtAQP1* was specifically localized within the filter chamber and hind gut where it plays a key role in the movement of water across adjacent cell membrane. Six out of the ten *Glossina m. morsitans* AQPs (*gmmaqp5*, *gmmaqp4a*, *gmmaqp4b*, *gmmaqp2a*, *gmmdripa* and *gmmdripb*) are significantly highly expressed in the Malpighian tubules than in other tissues while *gmmaqp2a*, *gmmaqp2b* *gmmaqp4a* and

*gmmaqp4b* are elevated in the midgut. (Benoit *et al.*, 2014) These are tissues involved in water movement in the fly.

Developmental stage expression studies of AQP were done in the second and third instar larva, pupae, teneral and in adult flies for both *G. f. morsitans* and *G. f. fuscipes*. *G. longipennis* was not included in this analysis because it was not possible to establish a colony that would provide all the life stages during the course of the study. Results indicate that in the larval stages AQP expression levels are low compared to expression levels in the pupa and in adult flies. The highest expression level was observed in the teneral flies and this could be induced by the need of the insect to prepare adequately for their first blood meal. There was a drop in the AQP1 expression level as the flies mature. AQP Regulation of membrane water permeability can either be short-term; by phosphorylation of the AQP causing gating of the AQP pore; or long-term, by up and down-regulation of AQP gene expression in tissues involved in water movement (Andrew *et al.*, 2009). Similar studies were done in the phloem feeder *Acyrtosiphon pisum* where ApAQP1 was found to be highly expressed in the gut of mature embryos and this may be induced due to anticipation of phloem feeding after birth (Shakesby *et al.*, 2009).

Functional analysis of *GfmAQP1* was done by injection of dsRNA corresponding to *GfmAQP1* into the dorsolateral surface of the thorax of male teneral flies. Semi-quantitative RT-PCR showed reduction in the levels of *GfmAQP1* transcripts from day 2 post dsRNA injection in the midguts. Highest degree of knockdown was achieved day 3

and 4 post injection while transcript levels increased day 5 and 6 post injection. This is as a result of the transient nature of *in-vitro* transcribed dsRNA initiated gene knockdown. Use of stable DNA constructs expressing dsRNA instead of the *in-vitro* transcribed dsRNA has been showed to solve this problem (Brown *et al.*, 2003). A reduction in feeding success was observed in the first two days of feeding post dsRNA injection in the test flies compared to nuclease free water injected controls. Five days post injection, all the flies in both groups fed successfully. A similar pattern was observed in the mortality rates of both groups of flies after treatment. A high mortality rate was recorded in first days of injection and after day 5 no mortalities were recorded. This shows that *GfmAQP1* knockdown and injury stress inflicted on the flies during microinjections could be affecting tsetse fly feeding.

A reduction in the rate of diuresis by up to 30% after blood meal ingestion was observed in the knockdown flies as compared to the control flies. This shows that *GfmAQP1* in *G. f. martini* plays an important role of aiding in the elimination of excess water from the blood meal during and immediately after feeding. This effect is consistent with the observation that suppression of *gmmdripa*, *gmmdripb* and *gmmaqp5* aquaporins in *G. m. morsitans* leads to a 50% reduction in the rate of diuresis. This shows the fundamental role of AQPs in water homeostasis in tsetse flies and makes them good target candidates for development of control strategies because interfering with them reduces vector fitness. In the yellow fever mosquito *Aedes aegypti*, six AQPs (AaAQP1-6) have been identified. Three of these aquaporins AaAQP 1, 4 and 5, have been shown to play an important role in the regulation of water transport. Knockdown of either of these AQPs

reduced the rate of diuresis while simultaneous knockdown of the three AQPs exacerbated this effect (Drake *et al.*, 2010).

## **5.2 Conclusion**

This study showed that the putative AQPs (*GffAQP1*, *GfmAQP1* and *GlAQP1*) from *G. f. fuscipes*, *G. f. martini* and *G. longipennis* could be water channel proteins because they contained all the conserved features of aquaporins and they clustered together with *Drip* which has been functionally characterized to be a water specific aquaporin. AQP mRNA transcripts were present in every life stage of tsetse flies, with highest expression recorded in the teneral flies. These AQP transcripts were also present in many tissues with very high amounts present in the midguts which are tissues involved in water movement. Ablation of the aquaporins by RNAi did not result in reduced feeding and survival however it resulted in decreased diuresis after a bloodmeal. The hypothesis that *Glossina* species differentially express AQPs, which are involved in osmoregulation and water balance, in different tissues is therefore accepted.

## **5.3 Recommendations**

- i. *GfmAQP1*, *GffAQP1* and *GlAQP1* to be heterologously expressed in *Xenopus* oocytes and functional assays conducted to ascertain whether they are classical water specific aquaporins or they are aquaglyceroporins. Functional assays including water permeability, glycerol uptake, solute selectivity and mercury inhibition assays needs to be conducted.

- ii. Presence of AQPs other than *GfmAQP1*, *GffAQP1* and *GIAQP1* to be investigated in *G. f. fuscipes*, *G. f. martini* and *G. longipennis* tsetse fly species. Tissue localization and life stage expression patterns should also be carried out for these aquaporins.
  
- iii. Effect of multiple AQP knockdowns on survival and diuresis post bloodmeal should be investigated in *G. f. fuscipes*, *G. f. martini* and *G. longipennis*.

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## APPENDICES

### Appendix 1: *GfmAQP1* gene sequence with primer annealing sites for dsRNA synthesis underlined and in bold

GGATTCATGCTATTTGCTGAGCTTGCGGGGACATTCTTACTGGTTATCATTGGTAT  
AGGCAGTTGCACGAGTGGTGC GGACTGGTCCCCAGCGTGCCTCAAATAGCATTAC  
ATTTGGTTTAACTGTGGCAACGCTTGCACAGACGGTCCGGTCATATAAGTGGATGTCA  
CATAAATCCTGCTGTTACAGTCGGTTTCTTGATAGTAGGAGAGATGACTTTACTAAA  
AGCTATTTTTTACATAATAGCACAGTGCCTCGGAGCAATGGCTGGATCGGCGGTTTT  
GAGTCTAGCTATCCCTGACACACTTGGCGGTAATGGCCTGGGCGTCTCTAACTTTTCC  
TCGCTCAGTGCTGGACAGGCAGTGTCAATTGAAGCCTTCATTACAGCTATCTTAGTGC  
TGGTAGTGAAAGCAGTGTCAAGATTCAAAAACGACAAGATATAACGGGATCAGCCCCA  
TTAGCCGTAGGGCTGGCTATCGCTACGGGTCATCTTTGTGCGATTAAATTAAGTGA  
GCCAGCATGAACCCAGCACGCTCATTGCGGTCAGCCGTAGTTCACGACGTTTGGGA  
AAATCATTGGGTTTATTGGATTGGTCCCCTTGTGGCAGCGTGGTAGCTGCCGTTATT  
TATAAATTAATCTTCAAGCAATCTAAAGAAGATGATGACACGAACTCCTACGATTTT  
TTCGAA

### Appendix 2: Amino acid sequences of *GfmAQP1*, *GffAQP1* and *GIAQP1*

#### >*G. f. martini* (*GfmAQP*)

MLFAELAGTFLLVIIGIGSCTSGADWSPSPQIAFTFGLTVATLAQTVGHISGCHIN  
PAVTVGFLIVGEMTLLKAIFYIIAQCVMAGSAVLSLAIPDTLGGNGLGVSNFSS  
LSAGQAVSIEAFITAILVLVVKAVSDSKRQDITGSAPLAVGLAIATGHLCAIKLTG  
ASMNPARSFGPAVVHVDVWENHWVYWIGPLVGSVVAAVIYKLIFKQSKEDDDTN  
SYDFFE

#### >*G. f. fuscipes* (*GffAQP1*)

MLFAELAGTFLLVIIGIGSCTSGADWSPSPQIAFTFGLTVATLAQTVGHISGCHIN  
PAVTVGFLIVGEMTLLKAIFYIIAQCVMAGSAVLSLAIPDILGGNGLGVSNFSS  
LSAGQAVSIEAFITAILVLVVKAVSDSKRQDITGSAPLAVGLAIATGHLCAIKLTG  
ASMNPARSFGPAVVHVDVWENHWVYWIGPVVGSVVAAVIYKLIFKQSKEDDDTN  
SYDFFE



**>G. longipenis (GIAQP1)**

MLFAELAGTFLLVIIGIGSCTSGADWSPSPQIAFTFGLTVATLAQTVGHISGCHIN  
PAVTVGFLIVGEMTLLKAIFYIIAQCVMAGSAVLSLAIPDILGGNGLGVSNFSS  
LSAGQAVSIEAFITAILVLVVKAVSDSKRQDITGSAPLAVGLAIATGHLCAIKLTG  
ASMNPARSFGPAVVHDVWENHWVYWIGPLVGSVVAAVIYKLIFKQSKEDDDTN  
SYDFFE