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LOCALIZATION AND FUNCTIONAL ANALYSIS OF AN AQUAPORIN GENE (AQP 4886_gp) FROM TSETSE FLY, *GLOSSINA PALLIDIPES*

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A thesis submitted in partial fulfillment for the Degree of Master of Science in Biochemistry in the Jomo Kenyatta University of Agriculture and Technology

2011

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DECLARATION

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

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This thesis has been submitted for examination with our approval as University Supervisors

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DEDICATION

I dedicate this thesis to my: Dear parents; Meitewa Bargul (my father) and Samuli Bargul (my mother), Brothers; Fereiti Bargul and Loripu Bargul, and Sisters; Nuchulo Ntiliya, Athiya Mirkakona, and Dagati Harao

Your continued support, prayers and encouragement has enabled me reach this far and produce this thesis. I will not forget that I am the only one in our family who got an opportunity to go to school. This motivates me to put more effort!

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LIST OF ABBREVIATIONS

AQP	Aquaporin
bp	Base pair
°C	Degree Celsius
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
dsRNA*i*	Double-stranded RNA interference
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
EST	Expressed sequence tags
g	Gravitational force
HRP	Horse radish peroxidase
IVT	*in vitro* transcription
kb	Kilobase
M	Molar
mL	Millilitre
mM	Millimolar
Min	Minutes
mRNA	Messenger RNA
p	Significance level
PBS	Phosphate buffered saline
pmol/µl	Picomole per microlitre
RNA	Ribonucleic acid

rpm	Rounds per minute
rRNA	Ribosomal RNA
Sec	Seconds
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
T.A.E.	Tris-acetate-EDTA
T.B.E.	Tris-borate-EDTA
T.E.	Tris-EDTA
U.V. light	Ultra-violet light
V	Voltage
µL	Microlitre
µg	Microgram
%	Percentage

ABSTRACT

Tsetse flies are vectors of African trypanosomes, the protozoan agent of devastating disease, trypanosomiasis that afflicts both humans and animals. Currently, there is no promising vaccine in the horizon and treatment efforts are further constrained by the rapid increase in parasite drug resistance observed in patients. In addition, little effort is being made to develop new and effective drugs. Alternative methods to control trypanosomiasis and its transmission are therefore required. The trypanosome parasite develops into its infective metacyclic stage in the salivary glands of the tsetse fly, where the saliva provides a specific medium for its maturation and also becomes the fluid vehicle for the transfer of the parasites to the host through a blood meal. Water exchange across the salivary gland membrane occurs through aquaporin (AQP) water channels in brown dog tick, *Rhipicephalus sanguineus*. This study focused on the role(s) played by a putative water channel protein identified in the salivary glands of tsetse fly, *Glossina pallidipes*, in relation to feeding and survival. The salivary gland AQP gene (herein named AQP 4886_gp), a homolog of GMsg 4886 gene from the transcriptome of *Glossina morsitans morsitans*, was PCR-amplified and cloned from *G. pallidipes*. The AQP 4886_gp protein has a predicted molecular mass of 25.222 KDa. Topographic analysis suggested that AQP 4886_gp has the general aquaporin topology and possesses two conserved 'NPA' signature motifs (Asn-Pro-Ala) found in aquaporins. Multiple sequence alignment and protein distant tree plotted using Neighbour-Joining method shows that AQP 4886_gp is more closely related to many insect AQPs than vertebrates'. The AQP 4886_gp transcript was localized to the salivary glands, malpighian tubules, and midgut.

These tissues are involved in high rates of water exchange in insects. The gene was detected in different life-cycle stages of the fly; larva, pupa, unfed teneral fly and adult tsetse fly using semi-quantitative reverse transcription (RT)-PCR. Functional studies of AQP 4886_gp were carried out using RNA interference (RNA*i*) technique, where gene-specific double stranded RNA (dsRNA) was injected into experimental flies. The control group was injected with nuclease-free water (NFW). The effects of transient gene silencing were monitored by semi-quantitative RT-PCR, and relevant bioassays (survival, feeding success). AQP 4886_gp gene knockdown was not lethal to the flies as they continued to survive and feed. The survival rates of 83% were achieved in both injected test and control groups. Binomial test of proportions showed no significant differences in the feeding success between the test (dsRNA-injected) and control (NFW-injected) flies at $p<0.05$.

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CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Background information

The control of diseases transmitted by insect vectors such as, malaria, dengue, trypanosomiasis, and leishmaniasis is an important priority for biomedical and public health agencies, the agricultural sector and the scientific community. These complex diseases involve the interaction of the mammalian host and insect vector with the pathogenic parasite or virus. Vaccines, drugs, vector control, and public health measures are important elements to consider in reducing the negative impacts of these diseases in man (Aksoy, 2003).

Tsetse flies (Diptera: *Glossinidae*) cyclically transmit many African trypanosome species including *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*, which cause human sleeping sickness, and *Trypanosoma brucei brucei* that causes nagana (WHO, 2000). Human African trypanosomiasis (HAT) decimated the population in many parts of sub-Saharan Africa during the early part of the twentieth century (Simarro *et al.,* 2008). The colonial administrations established control programmes in the 1930s which involved systematic screening, treatment and follow-up of millions of individuals across Africa which halted the transmission by 1960s (Simarro *et al.,* 2008). However, this disease has slowly returned over time and the World Health Organization (WHO), since 1995, has expressed its concerns about the rise of HAT. The progress to break the transmission cycle is frustrated by the breakdown of surveillance related to political instability, wars, and population movements, coupled with lack of awareness and shortage of funds (WHO, 2000).

More than a third of the African continent, south of Sahara, is inhabited by the tsetse flies which expose approximately 66 million people to the risk of contracting HAT (Maudlin, 2006). It is also estimated that 30% of about 150 million cattle in tsetse-prone regions of Africa are exposed to the risk of infection (Simarro *et al.,* 2008). This leads to deaths of about three million cattle every year as a result of nagana and farmers are required to administer high doses of expensive trypanocidal drugs, many of which the parasites have developed resistance (Kennedy, 2004). *T. b. gambiense* in West and Central Africa, and *T. b. rhodesiense* in East Africa are the two causative agents of the disease in man. *T. b. gambiense* is a chronic and protracted disease that may last several years, whereas *T. b. rhodesiense* is an acute disease that can result in death in a matter of weeks or months (WHO, 2000). Both types of sleeping sickness are fatal if left untreated (Masiga *et al.,* 2002) and found uniquely in sub-Saharan Africa (Figure 1).

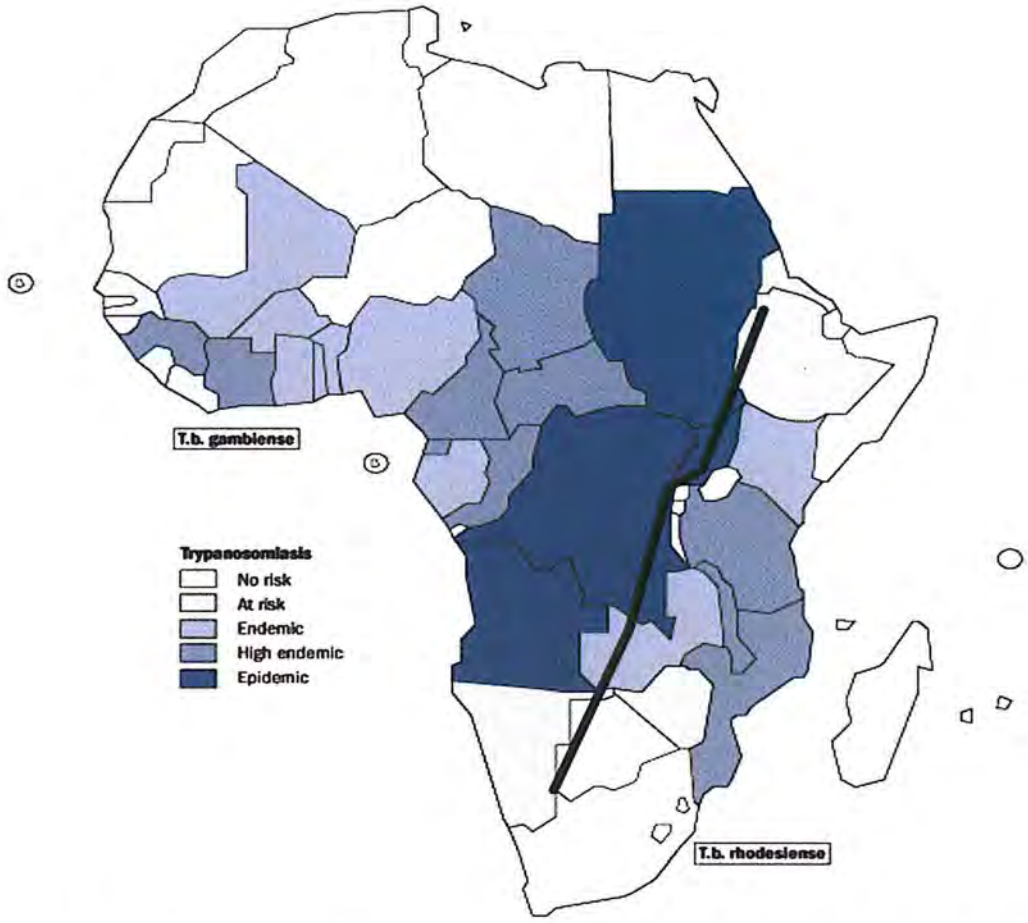

Figure 1. Distribution of *gambiense* and *rhodesiense* sleeping sickness in sub-Saharan Africa (WHO 2000).

1.2 Tsetse control

A wide variety of tsetse fly control techniques have been developed and have undergone trials. These techniques include the following;

1.2.1 Insecticides

The use of insecticides is a current vector control intervention which involves sequential aerosol spraying technique, or selective spraying application of insecticides to animals on which tsetse feed (Kabayo, 2002). The sequential aerosol technique, which entails several consecutive aerial sprayings, can effectively clear

large areas of tsetse flies in a relatively short time, but it is expensive and management intensive (Kabayo, 2002). Continuous monitoring, sustainability and vigilance are important requirements to prevent re-invasion and re-infestation of cleared areas by the vector (UNICEF/ UNDP/ World Bank/ WHO, 2004).

1.2.2 Odour-baited traps

Trapping of the tsetse flies using odour-baited traps decreases the population of flies and hence reduces man-fly contact. Vector control using traps (which are usually treated with insecticides) has the advantage of being managed by the communities for sustainability, especially when materials for the traps could be sourced locally with minimum cost (UNICEF/ UNDP/ World Bank/ WHO, 2004). Monoconical and biconical traps create a visual stimulus to which tsetse flies respond by flying into them (Schofield and Maudlin, 2001). Attractants, such as acetone, butanol, 1-acetone-3-a, p-cresol, have greatly increased the efficiency of traps over the years (Vale 1980). The cow's urine also contains some of these attractants. The findings of Belete *et al* (2004) indicated that large fly catches are possible using cow urine baited-traps for the control of *Glossina morsitans submorsitans* in Ethiopia. The likelihood that an individual fly will encounter and be killed or captured by the device depends also on the number of traps or targets relative to the local abundance of tsetse, and on the particular foraging and dispersal behaviour of the target tsetse species (UNICEF/ UNDP/ World Bank/ WHO, 2004).

1.2.3 Sterile insect technique

Sterile insect technique (SIT) involves sterilization of male flies through radiation to make them infertile. The irradiated males are then released into wild populations to compete with natural males. 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 1994 and resulted in the successful eradication of *Glossina austeni* from the island of Unguja (Vreysen *et al.,* 2000). The main advantage of the SIT method is that it slows down reproduction rate of tsetse flies; meaning eradication is a distinct possibility and is ideally suited to the final phase of local tsetse eradication (Vreysen, 2001). However, the large numbers of irradiated sterile males required and the costly implementation of the release method means that it is not viewed as a favourable method of control (Marcio *et al.,* 2003).

The impact of some of these effective tools on disease control has not been sustainable due to their local nature and extensive dependence on community participation.

1.3 Aquaporins

Understanding of interactions between the tsetse fly and trypanosomes is important in identifying ways to disrupt the transmission cycle of the parasite in their invertebrate host. The trypanosome species establish in the midgut lumen of the fly before completing maturation in the mouthparts and salivary glands (Aksoy, 2003). Both ticks and tsetse flies have been reported to salivate into the host during feeding (Valenzuela, 2004; Lehane, 2005). Ticks are blood-sucking ectoparasites of wild and domestic animals and humans. They are important vectors of pathogens such as

Anaplasma marginale which causes anaplasmosis in cattle (Kocan *et al.*, 2004). Ticks have been reported to pass approximately 70% of the water ingested in the blood meal back into the host through salivary glands by way of copious salivation (Bowman and Sauer, 2004). The findings of Andrew *et al* (2009) demonstrated that water moves through the membranes of the salivary glands of brown dog tick *Rhipicephalus sanguineus* through aquaporins. Salivation into the host by ticks forms the route by which pathogens are transmitted alongside an array of potent anti-haemostatic protein compounds described in the sialome of other blood feeding arthropods (Valenzuela, 2004; Lehane, 2005). It is conceivable that water which is the main constituent of saliva moves through the salivary glands of tsetse fly through the AQPs thus playing an important role in transporting trypanosomes to vertebrate host when the fly feeds.

The aquaporins (AQPs) are transmembrane pores that play critical roles in controlling water content of cells, therefore regulating osmotic pressure differences across cell membranes (Agre *et al.*, 1993a). This family of small, hydrophobic, integral membrane proteins, that includes both strict water channels as well as channels transporting solutes such as urea and glycerol, is widely expressed in animals, plants, insects, amphibian, yeast, and bacteria cells. To date thirteen AQPs have been identified and functionally characterized in humans (AQP0 to AQP12) (Gheorghe, 2009). The physiological importance of the AQPs in human is perhaps most profound in the kidney, where about 150-200 litres of water needs to be reabsorbed from the primary urine each day through AQP1 and AQP2 (Gheorghe,

2009). In plants, aquaporins are also critical for water absorption in the root and for maintaining the water balance throughout the plant (Agre *et al.,* 1993a).

1.3.1 Structure of aquaporin molecule

Each AQP molecule is thought to have a six-transmembrane-spanning topology as evidenced by protein conformation analysis of the amino acid sequence of the AQP family i.e. cytosolic amino and carboxy termini, extracellular loops A, C and E and intracellular loops B and D (Spring *et al.,* 2009). Aspargine-Proline-Alanine (NPA) conserved motifs appear in the B and E loops of nearly all AQPs. The intracellular loop B and extracellular loop E fold into the membrane and interact with one another, forming a 'hour-glass model' characterized by wide external openings to the channel with a narrow central constriction where the 'NPA' motifs interact, forming the functional water pore. Inhibition of the water channel to transport water by mercury (II) chloride indicates the presence of a critical sulfhydryl in the water pore, resulting in to physical blockage of the molecular flow through the pore (Gheorghe, 2009). Although each individual AQP is a functional water pore, they assemble in groups of four identical protein channels (homotetramers) in the membranes. Movement of water through each pore can be bi-directional depending on the osmotic gradient (Andrew *et al.,* 2009).

1.3.2 Insect aquaporins

Insect AQPs appear to be ubiquitous and affect cellular function in every tissue (Spring *et al.,* 2009). One of the first insect AQPs to be isolated and function determined was AQP*cic*, isolated from the filter chamber of the homopteran *Cicadella viridis*. This xylem feeder ingests large volumes of very nutrient-poor

fluid. To cope with the fluid volume, they have a filter chamber that has AQP*cic* which enables it to have higher water permeability (Beuron *et al.,* 1995). Blood-feeding insects face a similar osmotic challenge like xylem feeders. The rapid ingestion of a blood meal greatly impairs their mobility and so disposing of the excess plasma becomes paramount (Leak, 1999; Spring *et al.,* 2009).

A study conducted by Kikawada *et al* (2008) pointed to the major role played by specific AQPs in the larvae of the sleeping chironomid, *Polypedilum vanderplanki.* They isolated and expressed in *Xenopus* oocytes two cDNAs (*Pv*AQP1 and *Pv*AQP2) encoding water-selective AQPs from the chironomid. Northern blots and *in situ* hybridization studies revealed the expression of ubiquitous *Pv*AQP1 to be dehydration-inducible, whereas that of fat body-specific *Pv*AQP2 was dehydration-repressed.

The fruit fly, *Drosophila melanogaster*, has become the standard for tubule research involved in fluid transport. Several putative AQPs have been isolated from *Drosophila*, but only one, named *Drosophila* Integral Protein (DRIP, exclusively expressed in the malpighian tubule stellate cells) has been functionally expressed in proteoliposomes and found to be water permeable (O'Donnell *et al.,* 1998).

Thirty three AQP-like genes have been identified from the transcriptome of tsetse fly, *G. morsitans morsitans* and subsequently deposited in the database (*Gene*DB; http://www.genedb.org/genedb/glossina/index.jsp). Their importance in tsetse fly has not been established and they could be involved in the elimination of excess water that comes along with a bloodmeal. Functional analysis of AQP 4886_gp was investigated in the present study through RNA interference.

1.4 RNA interference

RNA interference (RNA*i*) is a novel gene regulatory mechanism whereby a gene's function can be selectively inhibited by double-stranded RNA (dsRNA) corresponding to that gene. Introduction of dsRNA into the cell leads to the sequence-specific destruction of endogenous mRNA through RNA*i* (Fire *et al.,* 1998). RNA*i*-induced gene-specific silencing was initially described in *Caenorhabditis elegans* and plants (e.g. *Arabidopsis thaliana*) (Fire *et al.,* 1998), and subsequently in a variety of other organisms such as trypanosomes (Djikeng *et al.,* 2000), ticks (Sukanya *et al.,* 2004), and tsetse fly (Lehane *et al.,* 2008). RNA*i* protects the genome against invasion by transposons and viruses, which produce aberrant RNA in the host cell when they become active (Seokyoung *et al.,* 2008).

1.4.1 RNA*i* mechanism

The RNA*i* mechanism is highly specific and the introduced dsRNA is processed through a number of sequential steps (Figure 2). First, there is cleavage of dsRNA by the RNase III enzyme dicer into shorter 21-23 nucleotide long dsRNA pieces termed short interfering RNAs (siRNA). These siRNAs have a characteristic phosphorylated 5' end and a two nucleotide overhang at the 3'OH end. The siRNAs then enter an RNA-induced silencing complex (RISC). A helicase, belonging to the argonaute (AGO1) family or slicer, unwinds the two strands of the siRNA to form single stranded RNAs (ssRNAs), and RISC scans the mRNAs in the cytoplasm and cleaves the molecules that are found complementary to the RISC-contained siRNA. This leads to down regulation of mRNA transcript and hence gene silencing ensues.

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RNA*i* has been used to silence target genes and analyze gene functions in many insect species. Lehane *et al* (2008) applied this technique to study the role of transferrin *2A192* transcript in tsetse fly, *G. m. morsitans*. Transferrin was found to play a critical role in protection of tsetse fly from trypanosome infection.

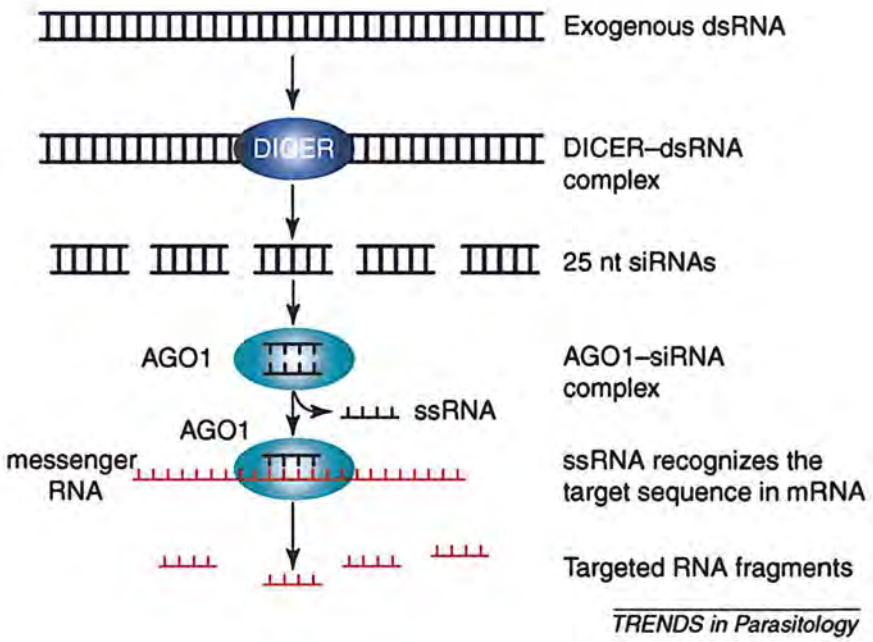

Figure 2. General RNA*i* mechanism (taken from Balana-Fouce and Reguera, 2007).

1.5 Statement of the problem

Trypanosomiasis is one of the most severe medical and veterinary problems in Africa that prevents the development of sustainable and productive agricultural systems. Currently, the most efficient way to contain the disease is through an integrated pest management approach. The available methods to control the tsetse fly are limited and thus tsetse control has not been sustainable. Therefore, there is need for developing alternative techniques that may supplement the traditional methods. This can be achieved through targeted basic research. Molecular biology techniques could provide an avenue for scaling up control and management efforts of this important

vector of African trypanosomes. Since aquaporins (AQPs) allow transmembrane water movements in cells, it is conceivable that tsetse fly saliva passes through these water channels hence allowing transfer of trypanosomes to the mammalian host when the infected fly feeds. Therefore, the AQPs of tsetse fly could be targeted as a possible avenue for breaking the transmission of trypanosomes to the vertebrate host. The aim of our study was to validate an aquaporin of tsetse fly as a possible gene target for designing a control strategy.

1.6 Justification of the study

African trypanosomiasis is one of the world's most neglected tropical diseases. As such, there is need to establish concerted efforts to control this important disease. Most control efforts have targeted the parasite; however, no vaccine is yet available due to the variant surface glycoproteins (VSGs) of the trypanosome. However, control that targets the vector seems promising because of the sequencing of *G. m. morsitans* genome which is currently in progress. It is hoped that this genome sequence will provide opportunities to study vector-parasite interactions and identify potential genes that can be exploited to design vaccines and new drugs. Our target genes, in this study, are the aquaporins which are involved in water homeostasis. Functional Studies of AQPs in *G. pallidipes* (the main vector of *Trypanosoma congolense*, *T. vivax* and *T. brucei*) will help determine their roles in this important vector of African animal trypanosomiasis. Comparative analysis of the identified AQPs with other insect homologues will shed some light on the evolutionary history of AQPs, which may be conserved within and across different insect orders. This physiological machinery may form a target for enhancing vector control, for

example, through studies of potential water-transport inhibitors (e.g. mercury (II) chloride; Preston *et al.,* 1992) hence bringing genomics to applied biology thus improving the prospects of tsetse fly control and management.

1.7 Hypothesis

Abrogation of AQP 4886_gp gene activity through RNA*i* disrupts water transport across salivary gland membranes thus inhibiting tsetse fly feeding.

1.8 Objectives

1.8.1 Main objective

To investigate expression profiles and the effect(s) of AQP 4886_gp gene knockdown on the feeding behaviour and survival in tsetse fly, *G. pallidipes*.

1.8.2 Specific objectives

i) To investigate tissue localization of AQP 4886_gp and other selected putative AQPs in tsetse fly.

ii) To investigate expression patterns of AQP 4886_gp in relation to developmental stages of the tsetse fly.

iii) To determine the role of RNA*i*-silenced AQP 4886_gp on mortality and feeding success.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Analytical pipeline

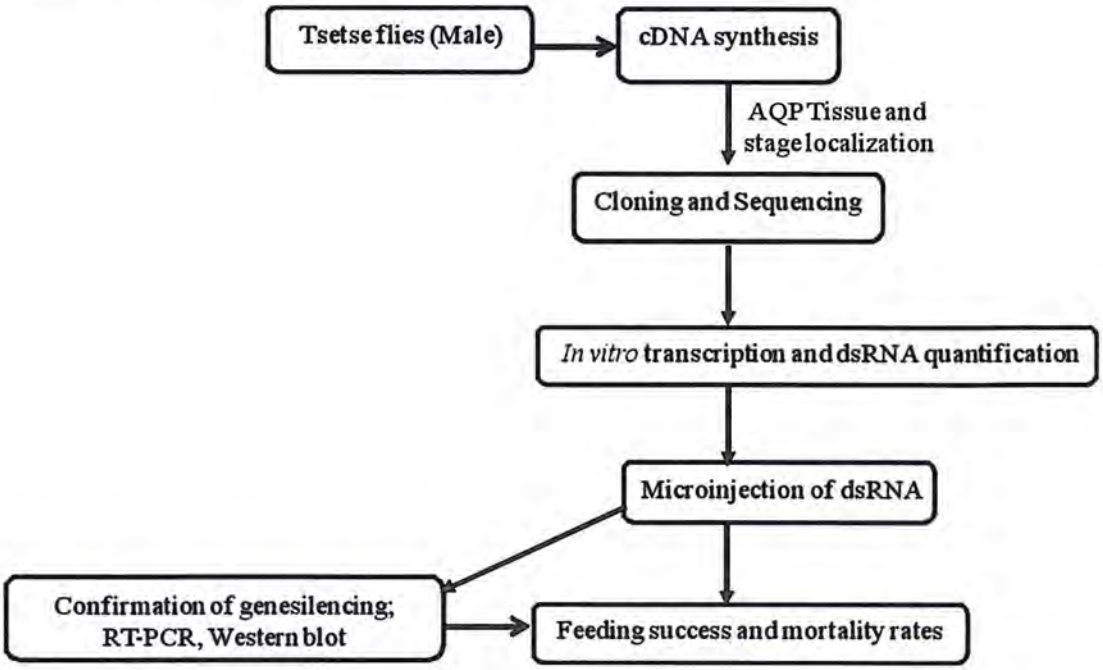

Figure 3. Experimental design flow chart. This chart describes a summary of the work involved in this study.

2.2 Insects

A laboratory colony of *G. pallidipes* maintained at ICIPE was used in all experiments. The flies were maintained on porcine blood (fed *in vitro* via a silicone membrane; Bauer and Wetzel, 1976) and reared under controlled conditions of temperature (26±1 °C) and humidity (60-70%).

2.3 RNA extraction

The tsetse flies were anaesthetized by chilling on ice for 30 min. Each fly was dissected, on a microscope glass slide placed on ice, under a dissection microscope

(Wild Heerbrugg M5, Switzerland). Total RNA was isolated using RNAgents® Total RNA isolation system (Promega, Madison, WI, USA) from fresh tissue homogenate following the manufacturer's instructions. The microfuge tubes, pipette tips and pestles required for extraction of RNA were treated in 0.1% diethyl pyrocarbonate (DEPC) for one hour at room temperature and then autoclaved for 30 min to destroy any residual DEPC. Four main steps involved in the extraction included; effective disruption of tissue, inactivation of endogenous ribonuclease, denaturation of nucleoprotein complexes, and purification of RNA away from contaminating DNA and protein. Immediate inactivation of endogenous RNase activity released from membrane bound organelles upon cell disruption was accomplished using two of the most potent known inhibitors of RNase supplied in the denaturing solution, i.e. guanidine thiocyanate and ß-mercaptoethanol. All procedures were performed on ice.

The salivary glands were pooled from twenty flies and placed in 1.5 mL pre-chilled Eppendorf tubes. Tissues were weighed so as to determine the appropriate volume of reagents to add for different amounts of starting material. Sixty microlitres of denaturing solution (26 mM sodium citrate, pH 4.0, 0.5% N-lauryl sarcosine, 0.125 M ß-mercaptoethanol, and 4 M guanidine thiocyanate) was added to the salivary gland tissue and homogenized. Then, 6 µL of 2 M sodium acetate (pH 4.0) was added and mixed thoroughly by inversion. Sixty microlitres of Phenol: Chloroform: Isoamyl alcohol mixture (125:24:1, pH 4.7) was added to the mixture, vortexed for 10 sec and then incubated on ice for 15 min. The mixture was centrifuged in a BIOFUGE fresco centrifuge (DJB Labcare Ltd, UK) at (10,000 x *g*, 20 min, 4°C) and the top aqueous phase which contained the total RNA was carefully pipette into

a fresh DEPC-treated tube. Most DNA and proteins remained in the organic phase and at the interface. An equal volume of chilled isopropanol was added to the aqueous phase containing RNA and incubated overnight at -20 °C to precipitate the RNA. The RNA was pelleted the following day by centrifugation in a BIOFUGE fresco centrifuge, DJB Labcare Ltd, UK (10,000 x *g*, 15 min, 4 °C). The RNA pellet was centrifuged again under same conditions in 1 mL of ice-cold 75% ethanol. The pellet was air-dried for 5 min and resuspended in 25 µL of nuclease free water. Total RNA was stored at -80 °C.

The samples contaminated with proteins were re-extracted in Phenol: Chloroform: Isoamyl alcohol (125:24:1, pH 4.7), while the DNA contaminated samples were treated with RNase-free DNase I to digest the DNA (section 2.3.1).

2.3.1 DNase treatment of total RNA samples

The DNA-contaminated RNA samples were treated with RQ1 RNase-Free DNase by setting up the following reaction as per the manufacturer's protocol (RNAgents® Total RNA isolation system, Promega, Madison, WI, USA). The components were added in order as follows; 50 µL of total RNA (2 µg), 20 µL of nuclease-free water, 10 µL of 10X RQ1 DNase buffer, 100 mM DTT, 2 units of RQ1 RNase-free DNase, 100 units of recombinant RNasin® ribonuclease inhibitor. A final volume of 100 µL prepared was incubated at 37 °C for 15 min. The RNA was extracted with an equal volume of the Phenol: Chloroform: Isoamyl alcohol (99:24:1, pH 4.7), vortexed for 1 min and centrifuged in a BIOFUGE fresco centrifuge (DJB Labcare Ltd, UK) at 12000 *x g* for 2 min. The upper aqueous phase was transferred to a new tube using pipette and a second extraction performed using Chloroform: Isoamyl alcohol (24:1)

following the same steps. The RNA in the upper aqueous phase was ethanol precipitated by addition of 0.3M sodium acetate and 2.5 volumes of 100% ethanol. The tube contents were mixed, incubated at -20 °C for 30 min and then centrifuged at 12,000 *x g* for 5 min. The RNA pellet was washed with 1 mL of 70% ethanol and air-dried before dissolving it in 10 µL of DEPC-treated water. The RNA yield, purity and integrity were determined as described in section 2.3.2 below.

2.3.2 Determination of RNA yield and quality

a) Yield and purity

The concentration of RNA was determined by diluting an aliquot of the preparation (1:100) in T. E. buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and reading the absorbance (Biospec-mini DNA/RNA/Protein analyzer, Shimadzu Corporation, Tokyo) at 260nm. The yield of total RNA was determined as follows;

$$1\ A_{260nm} = 40\mu g\ RNA/mL,\ Therefore,\ A_{260nm} \times Dilution\ factor \times 40 = \mu g/mL\ RNA$$

The purity of the isolated RNA was determined by reading absorbance at 260 nm and 280 nm. The ratio of A_{260nm}/A_{280nm} indicated the purity of the sample. Pure RNA samples exhibit A_{260nm}/A_{280nm} ratios of 2.0. A lower ratio than 1.7 confirms contamination of the sample, particularly with proteins, thus prompting for further purification steps.

b) RNA integrity

The RNA was analyzed on non-denaturing agarose gel. An aliquot of the RNA solution (1 µg RNA) was heated at 75°C for 5 min and placed on ice before loading on a 1% ethidium bromide-stained agarose gel to determine its

integrity. When resolved by gel electrophoresis, the 28S and 18S eukaryotic ribosomal RNAs were expected to exhibit a near 2:1 ratio of ethidium bromide staining.

2.4 Reverse transcription-polymerase chain reaction

2.4.1 First strand synthesis

The first strand cDNA reaction was performed using oligo(dT)$_{18}$ primer. To a sterile tube, 2 µg of total RNA was added followed by 5 µM oligo(dT)$_{18}$ primer. Water was added to bring the volume to 12 µL. The tube contents were mixed gently, centrifuged briefly in a BIOFUGE fresco centrifuge (DJB Labcare Ltd, UK) before incubating it at 65 °C for 5 min. The tube was placed back on ice, spun down briefly and the following components were added to bring the total volume to 20 µL according to the manufacturer's protocol (RevertAid^{TM} H Minus First Strand cDNA synthesis kit, Fermentas, Lithuania); 1X Reaction buffer (250 mM Tris-HCl, pH 8.3, 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT), RiboLock^{TM} RNase inhibitor (20 U/µL), 1 mM dNTP mix and RevertAid^{TM} H Minus M-MuLV reverse transcriptase (200 U/µL). The tube contents were mixed gently, centrifuged briefly and incubated at 42 °C for 60 min. The reaction was terminated by heating at 70 °C for 5 min. Gene-specific primers were designed (section 2.4.2 below) and used in amplification of target genes from the cDNA.

2.4.2 Primer design

Thirty-three putative AQP gene sequences from *G. m. morsitans* transcriptome were retrieved from *Gene*DB (http://www.genedb.org/genedb/glossina/index.jsp). The multiple alignment of 33 AQP genes exhibited low sequence identity to allow for the

design of degenerate primers to target all of them for this study. The AQP coding regions (i.e. the open reading frames) clustered into multiple clades that produce significant sequence similarity as shown below (Figure 4). Therefore AQP 4886_gp and AQP 0306_gp were chosen for the present study to represent two of the groups. However, most work was done using AQP 4886_gp. AQP 0306_gp was only used for comparative purposes to study AQP expression in different tissues of the tsetse fly.

Primers were designed manually to target the longest open reading frames (ORF) of the selected AQPs (Table 1), after considering the protein expression study required later during antibody production for use in Western-blots.

AQP 4886_gp and AQP 0306_gp gene sequences were retrieved from the *Gene*DB. In order to select the longest ORFs (including the start and stop codons), the DNA sequences were translated into amino acids using ExPASy translate tool (www.expasy.ch/tools/dna.html). Primers were then designed, based on the DNA sequence, to target the identified frames. The best primer parameters were selected using DNA calculator (www.sigma-genosys.com).

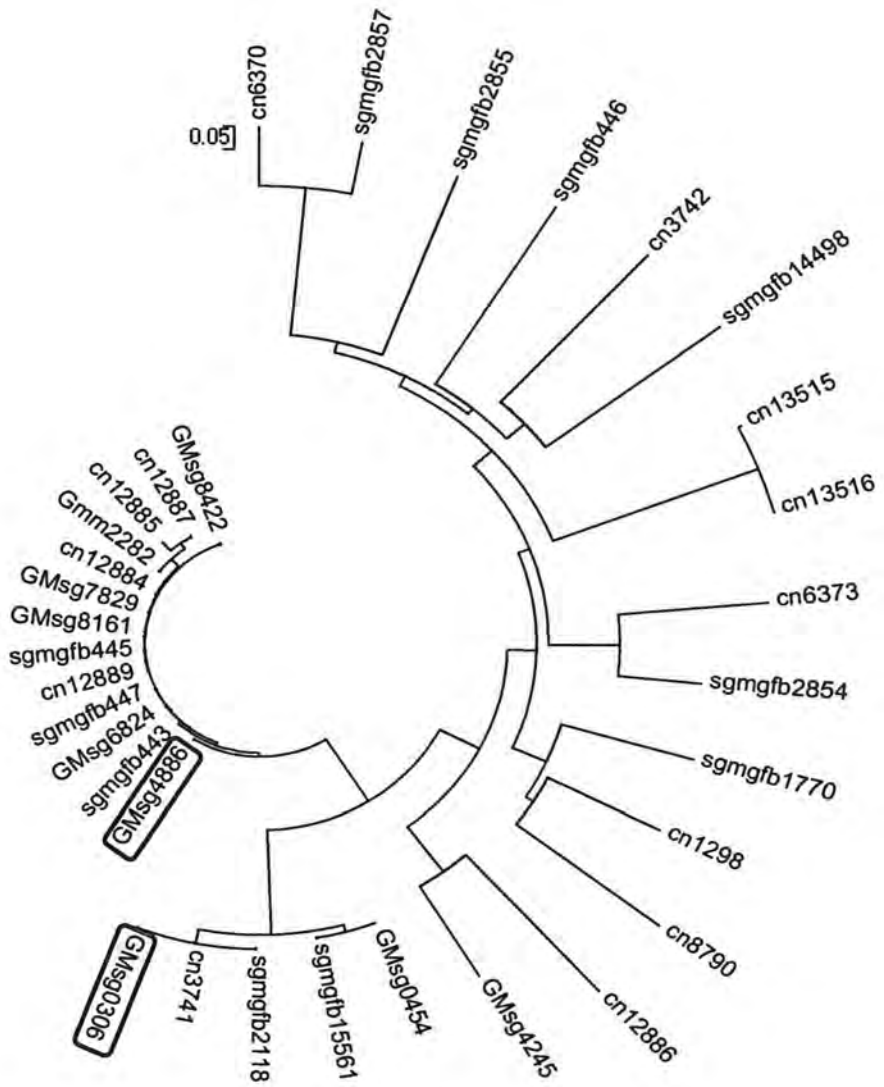

Figure 4. The circular phylogenetic tree view of the coding regions of 33 putative AQPs identified from transcriptome of *G. m. morsitans*. Tree constructed using amino acid sequences with Neighbour-Joining method using MEGA (4.0). Scale bar represents an estimate of the number of amino acid substitutions per site. The two rounded rectangles with black outline represent the two AQPs considered in this study.

Table 1. Primer sequences for construction of cloning and expression vectors.

PRIMER NAME	SEQUENCE (5' to 3')	ENZYME
Fw4886	TATA ***GAA TTC*** ACC ATG CTA TTT GCT GAG CTT GCG	*Eco*RI
Fw4886t	TATA ***GAA TTC*** ACC ATG GCG GTT TTG AGT CTA GCT	*Eco*RI
Rv4886	TATA ***AAG CTT*** TTA AAA ATC GTA GGA GTT CGT	*Hind*III
Fw0306	TATA ***GAA TTC*** GT ACC ATG GTA GAG AAA TTA GAT ATG	*Eco*RI
Rv0306	TATA ***AAG CTT*** TTA AAT GCT TTC TGC ACT GCT	*Hind*III
AQP4886F	***GGA TCC*** ATG CTA TTT GCT GAG CTT GCG	*Bam*HI
AQP4886R	***TTC GAA*** AAA ATC GTA GGA GTT CGT GTC	*Bst*BI
GAPDH Fw	TAA AAT GGG TGG ATG GTG AGA GTC	-
GAPDH Rv	CTA CGA TGA AAT TAA GGC AAA AGT	-
RNAi-Fw4886	AGC GTG CCT CAA ATA GCA TTC	-
RNAi-Rv4886	TTA AAA ATC GTA GGA GTT CGT	-
dsRNA-Fw4886	TATA ***GAA TTC*** ATG CTA TTT GCT GAG CTT GCG	*Eco*RI
dsRNA-Rv4886	TATA ***AAG CTT*** CCG AAT GAG CGT GCT GGG TTC	*Hind*III

Fw (or F) and Rv (or R) represent the forward and reverse primers, respectively. The restriction enzyme sites introduced in the primer sequences are in bold and italicized. The letter ***t*** *(in Fw4886t)*; represents the primer that produces *truncated* gene product. GAPDH Fw and GAPDH Rv are primers designed from *G. pallidipes* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene and were used in internal control reactions during expression studies. RNA*i*-Fw/Rv4886 and dsRNA-Fw/Rv4886 represent primers used in RNA*i* experiments.

2.4.3 PCR amplification of first strand cDNA

The product of first strand cDNA synthesis was used directly in PCR (1 µL in 25 µL of PCR reaction). Gene specific primers (Table 1) designed according to the ORF of AQP 4886_gp (Fw4886/Rv4886; 681 bp) and AQP 0306_gp (Fw0306/Rv0306; 720 bp) were used. The amplification of the cDNA product was performed with Phusion® High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) using the following cycling parameters; 98 °C for 30 sec, 35 cycles of 98 °C for 15 sec, 56 °C for 30 sec,

and 72 °C for 45 sec, followed by elongation for 8 min at 72 °C. The reaction was done in 9800 Fast Thermal Cycler (Applied Biosystems, Carlsbad, California). Dimethyl sulfoxide (DMSO) was added to the PCR master-mix to a final concentration of 6%. Reverse transcriptase minus (RT-) negative control without the enzyme and No template negative control (NTC) which lacked only the cDNA template were included. The RT- negative control tested for any DNA contamination in the cDNA sample, while the NTC control tested for contamination of the PCR reagents.

The RT-PCR products were electrophoresed through ethidium bromide-stained 1% agarose gel for 1 hr at 70 V (Bio-Rad model 200/2.0 power supply and wide mini-sub cell GT horizontal electrophoresis system, Bio-Rad laboratories, Inc., USA).

2.4.4 Gel extraction

The PCR products (total volume of 25 µL) were loaded on 1% ethidium-bromide stained agarose gel and subjected to electrophoresis (70 V for 1 hr). When the run was completed, the products were visualized under UV illumination to check the size of the bands by comparing with known DNA molecular weight standard (DNA marker, Fermentas, USA). The DNA bands were gel purified using QuickClean 5M Gel Extraction Kit protocol (GenScript Corporation, Piscataway, NJ) as follows; using a clean sharp scalpel, the DNA band was excised from the agarose gel. Extra agarose gel was removed to reduce the size of gel slice. The gel slice was then placed in a colourless pre-weighed 1.5 mL Eppendorf tube and its weight recorded. Three volumes of binding solution II (100 mg ≈ 100 µL) was added and incubated at 50 °C for 10 min with occasional vortexing to dissolve the gel slice. One volume of

isopropanol was added to the tube contents and mixed. The mixture was transferred to QuickClean column for centrifugation at 14,824 ×*g* for 30 sec (BIOFUGE fresco centrifuge, DJB Labcare, UK). The flow-through in the collection tube was discarded. Five hundred microlitres of wash solution was added to the column with bound DNA and centrifuged at 14,824 ×*g* for 1 min. This wash procedure was repeated once. Finally, the column was transferred into a clean 1.5 mL microcentrifuge tube and then followed by addition of 30 µL of elution buffer to the center of the column membrane prior to 1 min incubation at room temperature. The salivary gland AQP 4886_gp and AQP 0306_gp DNA was finally eluted by centrifugation at 14,824 ×*g* for 1 min. Six microlitres of the clean products were loaded on 1% ethidium bromide-stained agarose gel to confirm recovery of the DNA before sequencing.

2.5 Tissue and life stage distribution of AQP 4886_gp mRNA

Adult male *G. pallidipes* were dissected on ice under dissection microscope (Wild Heerbrugg, Switzerland) to isolate main tissues involved in high water-exchange rates in insects. These included; salivary glands, malpighian tubules, midgut and testes. The tissues were pooled from twenty tsetse flies. The following tsetse fly (*G. pallidipes*) life stages were also obtained from the insectary: first-instar larva (a day old), one-day old pupae, 15-days old pupae, unfed teneral fly (1 hr old since emergence), and an adult fly (30 days old).

Total RNA was isolated (Section 2.3) from the above tissues and used in semi-quantitative RT-PCR analysis (Section 2.4.1 and 2.4.3) to amplify AQP 4886_gp. During localization studies, AQP 0306_gp gene, which had been PCR-amplified and

cloned from the salivary glands of *G. pallidipes,* was included for comparative expression study. RT-PCR was normalized using GAPDH primers (Table 1) generating a fragment of 400 bp. The products were visualized on 1% ethidium bromide-stained agarose gel under the UV illumination. The PCR products were purified (section 2.4.4) and sequenced.

2.6 Gene cloning and dsRNA synthesis for microinjection

The RNA interference (RNA*i*) method adopted for the selective silencing of AQP 4886_gp expression was based on the injection of AQP 4886_gp-specific double stranded RNA (dsRNA) molecules into the tsetse fly. AQP 4886_gp was amplified using dsRNA-Fw4886 and dsRNA-Rv4886 primers (Table 1), cloned into pGEM®-T Easy vector (Promega Corporation, Madison, WI), and then subcloned into an RNA*i* plasmid, pLL10, which has two opposing T7 promoters (Appendix 1).

2.6.1 Preparation of LB-agar plates

LB-agar media was prepared by adding 15 g of agar to 1 litre of Lauria Bertani medium (LB; 10 g Bacto® tryptone, 5 g Bacto® yeast extract, 5 g NaCl, pH adjusted to 7.0 with NaOH). The LB-agar media was autoclaved (Laboratory Thermal equipment, England) and allowed to cool to 50 °C before adding ampicillin (100 µg/mL), Isopropyl-β-D-thiogalactopyranoside (IPTG; supplemented with 0.5 mM IPTG), and chromogenic dye, 5-bromo-4-chloro-indolyl-D-galactoside (X-Gal; 40 µg/mL) (Promega Corporation, Madison, WI) for phenotypic screening. The white colonies on the plates are ideally expected to carry recombinant plasmids, while the blue ones indicate the clones transformed with recircularized plasmids.

About 30 mL of the LB-agar/ampicillin/IPTG/X-Gal media was poured into 85 mm petri dishes and allowed to harden in the hood (Bellco Glass, Inc, Vineland, USA). The plates were stored at 4 °C for a maximum period of one month.

2.6.2 Ligation and transformation

2.6.2.1 Preparation of competent cells

The *E. coli* DH5α competent cells (Promega Corporation, Madison, WI) were streaked on LB plate (without ampicillin) and grown overnight at 37 °C (Inoue's method, 1990). A discrete colony was picked for seeding the start up culture (2 mL of LB media) that was incubated overnight at 37 °C with shaking at 150 rpm. The starter culture was used, the following day, to inoculate 250 mL of SOB media and grown (18 °C, 150 rpm) to an OD_{600nm} of 0.6. The culture was transferred into an ice water bath for 10 min prior to harvesting the cells by centrifugation at 12,096 ×*g* (Beckman J-25I centrifuge, USA) for 20 min at 4 °C. The supernatant was poured off and the cells gently resuspended in 80 mL of ice cold transformation buffer (TB; 10 mM PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$, and 250 mM KCl). The centrifugation step was repeated once and the cells were resuspended in 20 mL of ice cold TB. Then, 1.5 mL of DMSO was added to the cell suspension and mixed gently by swirling. The cells were incubated on ice for 10 min and finally 100 µL volumes were aliquoted into pre-chilled 1.5 mL tubes. The competent cells were frozen immediately in dry ice and stored at -70 °C ready for use.

2.6.2.2 Cloning of AQP 4886_gp into pGEM®-T Easy vector

A ligation reaction was set up as follows (pGEM®-T and pGEM®-T Easy vector systems, Promega Corporation, Madison, WI); To a 0.5 mL microcentrifuge tube, 5

μL of 2× Rapid ligation buffer, 50 ng of the pGEM®-T Easy Vector, 1 μL of PCR product, and 1 μL of T4 DNA ligase (3 Weiss units/μL) was added and topped up to 10 μL with nuclease-free water. A positive control DNA (supplied with the kit) and background control (that lacked the insert DNA) were included. The reactions were mixed by pipetting up and down, followed by a brief centrifugation step and finally incubated overnight at 4 °C to facilitate ligation. The freshly prepared *E. coli* DH5α competent cells (section 2.6.2.1) were transformed with ligation reaction. Five microlitres of ligation mix was added to clean 1.5 mL Eppendorf tube followed by 50 μL of competent cells. The tube contents were mixed gently and incubated on ice for 20 min. The competent cells were heat shocked at 42 °C for 1 min in order to take up the foreign DNA. The cells were supplied with 1 mL of rich media, Super Optimal broth with Catabolite repression (SOC), followed by an incubation at 37 °C for 1 hr with shaking (150 rpm) on a rotary shaker (Shellab Mini Shaker, US, 150 rpm). Meanwhile, the LB-agar plates (containing ampicillin, IPTG and X-Gal) were placed at room temperature inside a hood (Bellco Glass, Inc, Vineland, USA) so as to warm up. After an hour of incubation in SOC media, the cells were pelleted by centrifugation (BIOFUGE fresco centrifuge, DJB Labcare, UK) at 5000 ×*g* for 5 min. The excess media was discarded using a pipette and 100 μL of the cells were plated on LB-agar plates followed by an overnight incubation at 37 °C. Colony PCR on selected white colonies on plates was done to screen for the positive-insert colonies using the following conditions; 1 cycle of 5 min at 95 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 56 °C and 2 min at 72 °C. A final extension cycle of 8 min at 72 °C was provided. PCR was done using PTC-100 Programmable Thermal Controller (96-well) (MJ Research, Gaithersburg). Gene-specific primers

for AQP 4886_gp were used in PCR, i.e. dsRNA-Fw4886 and dsRNA-Rv4886 primers (Table 1). The positive-insert clones on the LB-agar plates were cultured in LB broth (section 2.6.1) supplemented with 100 μg/mL of ampicillin. Recombinant plasmids were purified using PureYield™ plasmid miniprep system (Promega Corporation, Madison, WI) according to the instructions of the manufacturer as described below (section 2.6.2.3).

2.6.2.3 Plasmid isolation

Six hundred microlitres of an overnight culture was added to a sterile 1.5 mL microcentrifuge tube followed by 100 μL of cell lysis buffer (Promega Corporation, Madison, WI). The mixture was mixed gently by inverting the tube six times. The solution changed from opaque to clear blue indicating complete lysis. Then, 350 μL of ice-cold neutralization solution was added and mixed thoroughly by inverting the tube six times. The lysate was centrifuged in a BIOFUGE fresco centrifuge (DJB Labcare Ltd, UK) at 16,060 ×*g* for 3 min at room temperature. The centrifugation step pelleted the bacterial chromosomal DNA and other cellular components, while the plasmids remained in the supernatant. The supernatant was transferred using a pipette onto a PureYield™ minicolumn that was placed into a PureYield™ collection tube. The tube contents were subjected to centrifugation (BIOFUGE fresco centrifuge, DJB Labcare Ltd, UK) at 16,060 ×*g* for 15 sec (room temperature). The flow-through was discarded and 200 μL of endotoxin removal wash was added to the minicolumn and centrifuged under the same conditions described. The centrifugation step was repeated using 400 μL of column wash solution. The minicolumn was transferred into a clean 1.5 mL microcentrifuge tube followed by

addition of 30 µL of elution buffer to the column matrix. The column was allowed to stand at room temperature for a minute before elution of DNA by centrifugation. The purified plasmids were quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and sequenced.

2.6.2.4 Sequencing of plasmid constructs

The purified recombinant plasmids (section 2.6.2.3) were sequenced using M13 forward and M13 reverse universal primers at the International Livestock Research Institute (ILRI), SegoliP Unit, Kenya. The sequences were edited using ChromasPro version 1.33 software program (Copy© 2003-2005 Technelysium Pty Ltd) and analyzed through BLAST of National Center for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov/). Sequence alignment with the *G. m. morsitans* homolog (GMsg 4886) retrieved from *Gene*DB was done (Appendix 1).

2.6.3 Sub-cloning of AQP 4886_gp into pLL10 RNA*i* vector

2.6.3.1 Plasmid construction and digest

The sequenced AQP 4886_gp initially cloned into pGEM®-T Easy vector was digested using *Eco*RI and *Hind*III (Fermentas, USA). The sites had been incorporated into the primers Fw4886 and Rv4886 (Table 1). The insert was ligated into pLL10 RNA*i* plasmid that had been digested with the same enzymes (*Eco*RI and *Hind*III). The ligation reaction was used to transform competent *E. coli* DH5-α cells (Promega Corporation, Madison, WI). After screening by colony PCR (as described in section 2.6.2.2) using universal and insert-specific primers, recombinant clones were grown in LB-broth and plasmids were purified as described before (section 2.6.2.3). The constructs were sequenced and used in dsRNA preparation. The

recombinant pLL10 vector (i.e. contains AQP 4886_gp insert) was digested singly, in two separate tubes, with *Xho*I and *Xba*I enzymes (Fermentas, USA) for transcription of both sense and antisense RNA strands.

2.6.3.2 Purification of the digested pLL10 construct

The restriction digests were treated with 0.05 mg/mL of proteinase K (Fermentas, USA) to digest proteins. Then, 3.75 µL of 10% SDS was added to 50 µL of the digest, followed by incubation in water bath (RB-200 Life Technologies, Van Allen Way, USA) at 50 °C for 30 min. The linearized plasmid DNA was extracted using 80 µL of buffer saturated Phenol: Chloroform. The tube contents were vortexed and centrifuged at room temperature (centrifuge 5415 C, Eppendorf, Hinz GmbH Company, Germany) for 5 min at 16,060 ×*g* to collect the upper aqueous phase that contained the DNA. Then, 80 µL of chloroform was added to aqueous phase; vortexed again, and centrifuged under same conditions as above. The DNA was precipitated using 56 µL of room temperature isopropanol for 15 min. The mixture was centrifuged (centrifuge 5415 C, Eppendorf, Hinz GmbH Company, Germany) at 16,060 ×*g* for 15 min, followed by a wash step using 80 µL of 70% (v/v) ethanol. The pellet was air-dried and dissolved in 20 µL of DEPC-treated water. The purified linear DNA became the template for *in vitro* transcription reactions using the Megascript RNA*i* kit (Ambion, 2130 Woodward St. Austin TX 78744).

2.6.3.3 *In vitro* transcription

The transcription reactions were assembled at room temperature. To a sterile microfuge tube, the following reagents were added; nuclease-free water (to a final volume of 20 µL), 2 µg of linearized plasmid, 2 µL of 10× T7 reaction buffer, 2 µL

each of dNTPs (ATP, CTP, GTP, UTP) and 2 μL of T7 enzyme mix. The mixture was mixed by pipetting up and down before a brief centrifugation step to collect the reaction mixture at the bottom of the tube. The reaction was incubated at 37 °C for 16 hrs to allow transcription of the template to occur. A positive transcription control template (pTRI-Xef) supplied with the kit was included in the experimental set up. The next step involved annealing of the synthesized sense and anti-sense strands of RNA to form dsRNA. About 0.5 μL of both sense and anti-sense RNA was reserved for gel analysis.

Equal volume of the sense and antisense RNAs were mixed in a 1.5 mL sterile tube. The tube containing the mixture was capped properly and incubated in boiling water in a 1L beaker for 5 min. The heat source was removed and the samples were left in the beaker to cool down slowly to room temperature. This leads to annealing of the complementary RNA strands. The dsRNA was further purified from any remaining single-stranded RNA (ssRNA) and initial template DNA by enzymatic digestion (37 °C for 1 hr) using RNase and DNase I provided in the kit. This nuclease treatment step does not digest the dsRNA in the solution because it is not a substrate of either enzyme. The next step involved column purification of the dsRNA to remove proteins, free nucleotides, and nucleic acid degradation products. This was achieved by using MEGAclear™ Purification Kit (Ambion, 2130 Woodward St. Austin TX 78744).

The dsRNA binding mix was applied onto the filter in the filter cartridge and centrifuged (Bench centrifuge 5415 C, Eppendorf, Hinz GmbH Company, Germany) at 16,060 ×*g* for 2 min at room temperature, followed two wash steps with 2X wash solution. The dsRNA was recovered through addition of 100 μL of hot elution

solution to the filter in the filter cartridge then followed by centrifugation for 2 min at maximum speed. Finally, the dsRNA was quantified by spectrophotometry (section 2.3.2). The resultant dsRNA ($1/400^{th}$ dilution) was run on a 1% ethidium bromide-stained agarose gel to check its integrity and efficiency of duplex formation.

2.6.3.4 Concentration of dsRNA

The dsRNA (from section 2.6.3.3) was further concentrated using ammonium acetate. Five molar NH_4Ac was added to the purified dsRNA in a ratio of 1:10. Then 2.5 volumes of 100% ethanol was added to the sample, mixed well, and incubated at -20 °C for 30 min. The sample was centrifuged (BIOFUGE fresco centrifuge, DJB Labcare Ltd, UK) at 4 °C for 15 min at 16,060 ×*g* to pellet the RNA. The supernatant was carefully discarded and the remaining pellet was washed using 500 µL of 70% (v/v) ice-cold ethanol. The RNA pellet was briefly air-dried and resuspended in nuclease-free water at a final concentration of 5 µg/µL. The dsRNA samples were stored at -80°C.

2.7 Microinjection

Teneral flies (12-24 hr old) were used in microinjection experiments. The flies were provided with a first bloodmeal on day 0 and injected on day 1. The unfed flies were removed and excluded from this study. Tsetse flies were anaesthetized by chilling to +4°C in their cages and then put into plastic petri dishes on ice in small batches (10 - 12 flies). Ten micrograms (i.e. 2 µL of 5 µg/µL RNA) of the dsRNA corresponding to AQP 4886_gp was injected using a fine glass needle (2.00 mm outside diameter, formed into a fine point using needle puller; PC10; Narishige, Japan) into the dorsolateral surface of the thorax of male flies under the dissection microscope

(Figure 5). The optimal quantity of dsRNA for RNA*i* of a series of genes in *G. m. morsitans* has been determined in Lehane's laboratory to be 6-10 μg/fly (Lehane *et al.,* 2008). In order to identify the correct delivery medium for RNA*i* experiments, Walshe *et al.* (2009) determined the effect of different solutions on tsetse fly mortality rates and immune responses to trypanosomes. The best medium for delivery which gave consistent results was nuclease-free water (NFW). A volume of 2 μL NFW was previously adopted in RNA*i* by injection to knockdown the attacin gene in *G. m. morsitans* (Hu and Aksoy, 2006).

Two control groups were included; the first one was simply chilled for half an hour and not injected at all. The second control group was chilled and injected with 2 μL of NFW. At least 35 flies were used in each group. After injections, the flies were put into cages (20 flies per cage) at room temperature and a piece of damp paper placed over the cage during recovery. The flies were thereafter kept in well ventilated cages under controlled conditions of temperature (26±1 °C) and humidity (60-70%) and fed on porcine blood 24 hrs post-injection (day 2). The analysis of gene knockdown was done by RT-PCR starting at day 3 and followed after every 48 hrs.

Figure 5. Microinjection of tsetse fly. The arrow points to the injection position on intrathoracic cavity. Image source: Peggy Greb, USDA.

2.7.1 Verification of gene silencing

The degree of knockdown achieved was measured by semi-quantitative RT-PCR. Total RNA was extracted from a fresh tissue after chilling the flies on ice to immobilize them. Dissections under dissection microscope were done on a glass slide placed on ice to maintain cold conditions during the process. Insect saline (Appendix 2) was used during dissections. Tissue samples, including salivary glands and midguts, were pooled from five flies in each group. The intimate association of the fat body with the midgut made it impossible to remove all traces of fat body from dissected midguts. Isolated total RNA was reverse transcribed (RT) using oligo$(dT)_{18}$ primer to synthesize the first strand that produces the template for PCR using gene-specific aquaporin RNA*i* primers (RNA*i*-Fw4886 and RNA*i*-Rv4886, Table 1). PCR controls were included using the same procedures but without reverse transcriptase (test for DNA contamination in the RNA preparation) and also a no template (cDNA) control (detect contamination of the PCR reagents). PCR products were electrophoresed through 1% ethidium bromide-stained agarose gels to check the size of amplified fragments by comparison to a known DNA molecular weight marker (1 kb DNA Ladder, Promega Corporation, Madison, WI). Gel visualization, editing and documentation were accomplished using KODAK Gel Logic 200 Imaging System (Raytest GmbH, Straubenhardt). Reduction in AQP 4886_gp gene expression was determined by densitometric measurement of bands using the software KODAK Gel Logic 200 Imaging System (Raytest GmbH, Straubenhardt). Change in gene expression in dsRNA-injected and nuclease-free water (NFW)-injected flies was determined by the ratio between band intensity of the AQP 4886_gp gene and its

correspondent loading control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.7.2 Tsetse fly survival

Mortality of the flies in the three groups (test, injected controls and uninjected controls) was regularly assessed in each cage to evaluate tsetse fly survival after the treatments. The dead flies were counted and removed from the cages. Feeding success was also recorded. Tsetse fly mortality and feeding success was compared between dsRNA-, mock-injected and uninjected flies.

2.8 Heterologous protein expression for antibody production

2.8.1 Gene cloning

Gene-specific primers (AQP4886Fw and AQP4886Rv; Table 1) were designed and *Bam*HI and *Bst*BI sites were included in forward and reverse primer respectively. AQP 4886_gp DNA was amplified from cDNA as described in section 2.4.3. The gene was ligated into pGEM®-T Easy vector, and cloning was done as described before (Section 2.6.2.2). The plasmid construct was sequenced using M13 forward and M13 reverse primers. Then, AQP 4886_gp gene was sub-cloned into pRSET-A (Section 2.8.2 below) after considering the restriction sites available in the vector.

2.8.2 Sub-cloning into pRSET-A

The pRSET vectors are pUC-derived expression vectors designed for high-level protein expression and purification from cloned genes in *E. coli*. The expression of DNA sequences cloned into the pRSET vectors are made possible by the presence of the T7 promoter. To facilitate cloning, the pRSET vector is provided in three

different reading frames; pREST-A, pRSET-B and pRSET-C. They differ only in the spacing between the sequences that code for the N-terminal peptide and the multiple cloning site. pREST-A was chosen for cloning AQP 4886_gp gene.

The AQP 4886_gp insert in pGEM®-T Easy vector (Appendix 1) was sub-cloned into pRSET-A through double digestions of both pGEM®-T-AQP 4886_gp construct and pRSET-A vector using *Bam*HI and *Bst*BI restriction endonucleases (FastDigest®, Fermentas, USA). The following reagents were added to a sterile microfuge tube; 10 µL of nuclease-free water, 2 µL of 10× FastDigest® buffer, 6 µL of the plasmid (concentration; 113 ng/µL), and 1 µL of *Bam*HI and *Bst*BI. The tube contents were mixed by gently flicking the bottom of the tube and then briefly spun in a BIOFUGE fresco centrifuge, DJB Labcare Ltd, UK (16,060 ×*g*, 15 sec, at room temperature). The reaction was incubated at 37 °C for 2 hrs in a water bath (RB-200 Life Technologies, Van Allen Way, USA). The digestion products were run on a 1% ethidium bromide-stained agarose gel and both the digested-out insert (AQP 4886_gp) and the linearized pRSET-A vector were gel extracted as described before in section 2.4.4. Five microlitres of the gel-purified products were visualized in 1% ethidium bromide-stained agarose gel to confirm recovery. AQP 4886_gp was then ligated into linearized pRSET-A vector (since both were digested with same restriction endonucleases; *Bam*HI and *Bst*BI, they should carry sticky overhangs). Ligation was performed using T4 DNA ligase (Promega Corporation, Madison, WI) and cloning proceeded as described in section 2.6.2.2.

2.8.3 Sequencing of pRSET-A constructs

The pRSET-A/AQP4886 constructs were sequenced using T7 promoter forward primer (5'-TAA TAC GAC TCA CTA TAG GG-3') and T7 Reverse primer (5'-TAT GCT AGT TAT TGC TCA G-3'). The nucleotide sequences were translated into amino acids using ExPASy translate tool (www.expasy.ch/tools/dna.html) to confirm that the gene of interest is in frame with the N-terminal histidine tag of pRSET-A vector and in the proper orientation before proceeding with protein expression studies. Glycerol stocks were prepared as described in section 2.8.3.1 below.

2.8.3.1 Glycerol stock

Two milliliters of BL21(DE3)pLysS carrying the recombinant pRSET plasmid was grown (overnight at 37 °C) in LB broth containing 100 µg/mL ampicillin. Then, 0.85 mL of the overnight culture was combined with 0.15 mL of sterile 100% glycerol in a sterile freezing vial. The tube contents were vortexed and stored at -80 °C.

2.8.4 Induction of protein expression

A pilot expression study was performed in order to establish optimal expression conditions for AQP 4886_gp protein. A starter culture was prepared by inoculating 2 mL of LB broth with a single recombinant *E. coli* clone of BL21(DE3)pLysS. The culture was incubated overnight at 37°C with shaking (150 rpm) in presence of 50 µg/mL ampicillin and 35 µg/mL chloramphenicol. The following day, 25 mL of sterile LB broth was inoculated with overnight culture to an OD_{600nm} of 0.1 and further grown to an OD_{600nm} of 0.4-0.6under the same conditions as described above. Prior to induction with IPTG, 1 mL of the culture was aliquoted into 1.5 mL Eppendorf tube, centrifuged and resultant pellet stored at -20°C. This represented

time zero sample. IPTG was then added to a final concentration of 1 mM and growth continued. After 1 hr of incubation, 1 mL of the sample was pelleted and the pellet stored at -20 °C. The samples were similarly collected at 1 hr interval for 6 hrs and the last collection was taken after an overnight incubation. When all the time points were collected, the pellets were prepared as described below (section 2.8.4.1).

2.8.4.1 Quick outer membrane preparation

Each pellet was resuspended in 100 µL of the lysis buffer (10 mM Tris-HCl pH 9.0, 2% SDS [w/v], 10 mM EDTA) and 1 µL of 20 mg/mL DNase/RNase. The pellet was passed through 26-gauge needle several times to break it up. The needle was rinsed with PBS-Tween (1× PBS pH 7.4, 0.05% [v/v] Tween 20) between samples. Two microlitres of 0.1 M HCl was added to each tube and incubated at 37 °C for 1 hr. Thereafter, 100 µL of 2× SDS-PAGE sample buffer (Appendix 3), containing 10% ß-mercaptoethanol, was added to each sample and boiled in a steamer for 5 min. Twenty microlitres of each sample was separated on a 12% polyacrylamide gel. Initially, the electrophoresis was set at 80 V until samples entered the resolving gel, and then it was raised to 120 V for 1 hr. To visualize the separated proteins in the gels, Coomassie Brilliant Blue staining (20% methanol, 1% acetic acid, and 0.15% R-250 dye) method was employed. The gel was stained overnight with gentle shaking. The following day, the gel was transferred into a destain solution (Appendix 3) to wash off excess dye. Kimwipes were placed in the solution in order to absorb the excess dye released from the gel. A band of increasing intensity at the expected size range for the recombinant protein (6×His-tagged protein) was anticipated, and Western blot was performed to confirm its reactivity.

2.8.4.2 Western blot

The identity of recombinant AQP 4886_gp was confirmed by Western blotting (Burnette, 1981). Because the recombinant protein was expected to carry a 6×His-tag at its N-terminal region, detection can be done using anti-His antibodies or probes which are commercially available. The protein samples (From section 2.8.4.1) were separated on 15% Tris-SDS-PAGE and then electrophoretically blotted onto a nitrocellulose membrane (Bio-Rad, Canada). This was achieved following an overnight incubation at +4 °C in transfer buffer (0.2 M zwitterionic glycine, 25 mM Tris base, 20% [v/v] methanol) at 30 V. The membrane was blocked in 5% [w/v] non-fat dry milk in 1× PBS (137 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) for 3 hrs followed by incubation in 1% w/v non-fat dry milk in 1× PBS containing HisProbe^{TM}-HRP (1:5000) for 1 hr with shaking. HisProbe^{TM}-HRP (Pierce Biotechnology, USA) is a nickel (Ni^{2+}) activated derivative of horseradish peroxidase (HRP, a high activity enzyme label) used for direct detection of recombinant poly-histidine-tagged fusion proteins and other histidine-rich proteins.

The membrane was washed three times for 5 min in PBS-Tween (1× PBS pH 7.4, 0.05% [v/v] Tween 20) followed by three more washes each for 1 min in 1× PBS. HRP developer (Bio-Rad, Canada) was prepared by combining 100 mL of 1× PBS, 50 mg of diaminobenzidine (DAB), and 0.5 mL of 6% cobalt chloride. Sixty microlitres of hydrogen peroxide was added to the developer, mixed quickly and added to the washed blot. The blot was left shaking for few minutes to develop the dark brownish colour for the reactive protein. The developed blot was rinsed in distilled water (to stop the reaction) and left for 2 min to air dry before scanning for documentation.

2.8.5 Re-cloning in pBADHisA

It was clear from the culture growth profile that the recombinant protein exhibited toxicity to the cells. This was evidenced by slow or no increase at all of the absorbance readings at OD_{600nm} over time. Therefore, it was necessary to try different expression vectors and hosts. Re-clonings were done into pBADHisA vector (provided by Dr. Brad Clarke, University of Guelph, Canada) which has more tightly regulated arabinose induced araBAD promoter. The vector has a 6×His-tag for detection of expressed protein. The entire coding-region of AQP 4886_gp was cloned into the expression vector pRSET-A (Invitrogen Corporation, Carlsbad, CA) but recombinant protein was not produced at detectable levels. As a result of the difficulty in expressing hydrophobic transmembrane proteins in bacteria (Loukas *et al.,* 1999), the last three transmembrane domains of the aquaporin protein including the C-terminal hydrophilic domain of AQP 4886_gp (Figure 8) was also cloned into pBADHisA for expression studies.

The primers, Fw 4886, Fw 4886t and Rv 4886 (Table 1; synthesized by Sigma-Genosys, Oakville, CA, USA) were designed manually and appropriate restriction sites introduced based on the multiple cloning site of pBADHisA. Both full length (ORF, 681bp) and a truncated AQP 4886_gp gene (431 bp) sequences were PCR-amplified following protocols described before (Section 2.4.3).

The PCR products and pBADHisA vector were purified and double digested in FastDigest® *Eco*RI and *Hind*III (FastDigest®, Fermentas, USA) as described earlier in section 2.8.2. The digestion products were purified by gel extraction procedure (Section 2.4.4) and ligated using T4 DNA ligase (Promega Corporation, Madison, WI) in 2× rapid ligation buffer as described in section 2.6.2.2. The ligation product

(2 μL) was used to transform fresh competent *E. coli* DH5α cells as described earlier in section 2.6.2.2. The recombinant plasmid constructs were purified and sequenced.

2.8.5.1 Transformation of expression hosts

The AQP 4886_gp inserts in pBADHisA were confirmed to be in frame from the translation results using ExPASy translate tool (www.expasy.ch/tools/dna.html) and in proper orientation based on the N- and C-terminal ends of the AQP 4886_gp sequence in relation to the 6×His tag in pBADHisA vector (Appendix 1). The recombinant plasmids were transformed by heat shocking (described in section 2.6.2.2) into the following expression hosts; Rosetta(DE3)pLysS (Novagen, Darmstadt) and BL21(DE3)pLysS (Invitrogen Corporation, Carlsbad, CA). The transformed cells were plated on LB-plates containing 50 μg/mL ampicillin and 35 μg/mL chloramphenicol for selection.

2.8.5.2 Induction of protein expression

A starter culture was prepared by inoculating 2 mL of LB media with a single recombinant *E. coli* colony of Rosetta(DE3)pLysS /pBADHisA-AQP 4886t (t: truncated gene) and grown overnight at 37 °C with shaking in presence of 50 μg/mL ampicillin and 35 μg/mL chloramphenicol. The culture was diluted 100-fold into 500 mL of fresh LB media supplemented with 0.5% w/v glucose in 2 L flask, and propagated (37 °C, 200 rpm) to an OD_{600nm} of 1.0. Expression was induced by 0.2% w/v arabinose, and then followed by additional 3 hrs of incubation under the same conditions. The cells were subsequently harvested by centrifugation at 5,000 *x g* for 30 min, followed by resuspension of the pellet in 5 mL of lysis buffer (1× PBS, 1 mM Phenylmethylsulfonyl fluoride (PMSF), 1 mM $MgSO_4$ and 1 μg/mL DNase I),

and then lysed by sonication. Short pulses of 15 sec were supplied to the sample on ice for six times with 10 sec rests in between the pulses to avoid overheating. The lysate was centrifuged (centrifuge 5415 C, Eppendorf, Hinz GmbH Company, Germany) at 12,000 *x g* for 30 min at +4 °C to obtain the supernatant. The pellet was stored at -20 °C and later used for gel analysis. The 6×His-tagged protein was column purified using Ni-NTA agarose (Qiagen Inc., Ontario, Canada), and then resolved by 15% Tris-SDS-PAGE. The separated proteins were transferred into nitrocellulose transfer membrane as detailed in section 2.8.4.2. The membrane was subsequently blotted with HRP-HisProbe followed by incubation in HRP developer for visualization of the reactive proteins.

2.9 Data analysis

2.9.1 Bioinformatics and phylogeny

AQP 4886_gp sequences were edited to remove ambiguous base calls and primer sequences using the ChromasPro version 1.33 software program (Copy© 2003-2005 Technelysium Pty Ltd). A search to identify protein sequences similar to AQP 4886_gp was performed using tBLASTx algorithm of NCBI GenBank and also *Gene*DB (tsetse fly EST database). The retrieved sequences were aligned, in order to identify conserved regions, using CLUSTALW (Thompson *et al.,* 1994) and, where necessary, re-alignments adjusted manually. Amino acid sequences were used to estimate phylogeny with the neighbour-joining, minimum evolution or maximum parsimony methods. The dendrogram was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (Tamura *et al.,* 2007) with 10,000 bootstrap replicates. All methods for estimating phylogeny gave trees with similar

topology and approximate bootstrap values; therefore only the neighbor-joining tree is presented. Percentage homology/genetic distance among similar AQPs to AQP 4886_gp was computed using MEGA software. Topographical analysis of AQP 4886_gp to determine the transmembrane regions was performed using SOSUI software (http://bp.nuap.nagoya-u.ac.jp/sosui/), while its 3D structure was predicted using the Swiss-Pdb Viewer (http://www.expasy.org/spdbv/). Phosphorylation sites were determined using NetPhos 2.0 (Blom *et al.,* 1999) on ExPASy proteomic server (http://expasy.org/tools/#ptm).

2.9.2 Statistical analysis

R-software (R Development Core Team, 2010) was used to analyze the data. Normality of the data was tested using Shapiro-Wilk's test and when the data was non-normal, arcsine transformation was applied. When the data did not normalize after the transformation, Kruskal-Wallis rank sum test (non-parametric test) was used to verify the differences between groups. Binomial test for proportion was used whenever differences between two groups were tested. In all cases, $p<0.05$ was considered statistically significant.

CHAPTER THREE

3.0 RESULTS

3.1 RT-PCR results

The total RNA was analyzed by non-denaturing agarose gel electrophoresis to determine the quality of the isolated RNA (Figure 6a). Intact rRNA bands (28S and 18S subunits) were observed indicating good integrity of the isolated total RNA. The 28S and 18S eukaryotic rRNAs exhibited an expected near 2:1 ratio of ethidium bromide staining, indicating that no gross degradation of RNA occurred. An average concentration of 150 µg/mL of RNA was obtained through spectrophotometry.

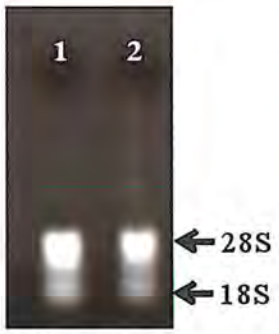

Figure6a

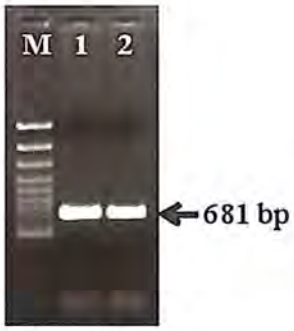

Figure6b

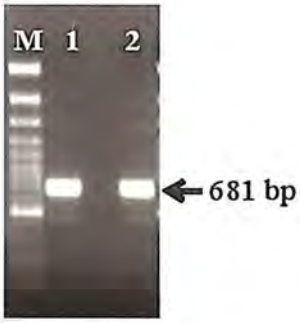

Figure6c

Figure 6a. Total RNA integrity. RNA Electrophoresed through 0.3% agarose gel to check its integrity. The 18S and 28S ribosomal bands are present indicating good integrity of the RNA samples (Lanes **1 and 2**).

Figure 6b. RT-PCR products electrophoresed through 1% ethidium-bromide stained agarose gel. **M:** 100 bp plus DNA ladder (Fermentas). Lanes **1 and 2:** AQP 4886_gp. **Figure 6c.** Gel purified PCR products (Lanes **1 and 2:** AQP 4886_gp). **M:** 100 bp plus DNA ladder (Fermentas).

The mRNA was reverse transcribed to synthesize the first strand in a reaction driven by a reverse transcriptase. Gene specific primers were employed in a PCR-amplification of AQP 4886_gp gene using cDNA as template. The RT-PCR products were visualized on a 1% ethidium bromide-stained agarose gel under UV illumination (Figure 6b). The PCR products were gel purified and loaded on 1% ethidium bromide-stained agarose gel to check the recovery of the product (Figure 6c).

3.2 Tissue and developmental stage expression of AQP

3.2.1 Tissue expression

AQP 4886_gp and AQP 0306_gp genes were PCR-amplified and cloned from *G. pallidipes* salivary gland cDNA. Therefore, both of these AQPs were localized in the salivary glands of tsetse fly, *G. pallidipes.* Water channel proteins were also expected to be expressed in other tsetse fly tissues, especially the regions known to be involved in high water-flux in most insects. Semi-quantitative RT-PCR demonstrated the presence of AQP 4886_gp mRNA in the three tissues studied in *G. pallidipes*; malpighian tubules, midgut, and testes. However, AQP 0306_gp, included for comparative expression studies, was absent in the studied tissues (Figure 7a). The tissue specificity, functions and some pathological implications of thirteen human AQPs have been reported (Gheorghe, 2009). The absence of AQP 0306_gp in the malpighian tubules and midgut may point to its role in the salivary glands where it is expressed. Because of its expression in all considered tissues, AQP 4886_gp attracted our attention to study it further.

Figure 7a. The tissue expression of AQP 4886_gp and AQP 0306_gp in *G. pallidipes*. Semi-quantitative RT-PCR products were electrophoresed through 1% ethidium bromide-stained agarose gel. The loading order of the samples in the above lanes was as follows; **M:** 1Kb Smart DNA ladder (Eurogentec Inc., North America). Lane **1:** Negative control (lacked only the cDNA template), Lanes **2, 5, and 8:** Internal control (GAPDH) for malpighian tubules (MT), midgut (MG), and testes (TE), respectively. Lanes **3, 6, and 9:** AQP 0306_gp in MT, MG, and TE, respectively. Lanes **4, 7, and 10:** AQP 4886_gp in MT, MG, and TE, respectively.

3.2.2 Life stage expression of AQP 4886_gp

Semi-quantitative RT-PCR analysis of life cycle stages revealed that AQP 4886_gp mRNA was present in whole body extracts of 1st instar larva, a day old pupa, 15-days old pupa, unfed teneral fly, and 30-days old adult tsetse fly (Figure 7b). The AQP 4886_gp transcript was detected at lower level in larval stage, while the transcript was particularly abundant in teneral stage of the fly. These results were consistent for the three independent semi-quantitative RT-PCR experiments performed.

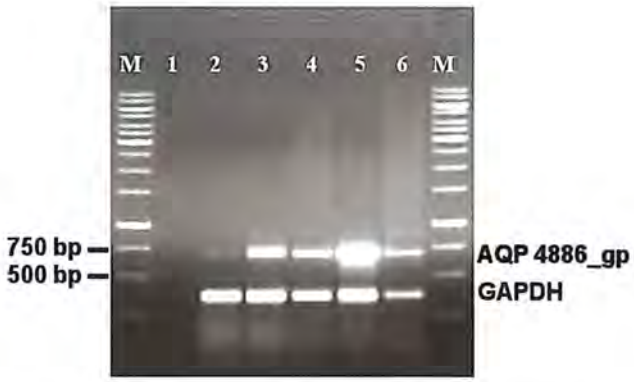

Figure 7b. The life stage expression of AQP 4886_gp in *G. pallidipes*. RT-PCR products were electrophoresed through 1% ethidium bromide-stained agarose gel. The loading order from left was as follows; Lane **1:** Negative control (no cDNA template), Lane **2:** 1st instar larva, Lane **3:** One-day old pupa, Lane **4:** 15-days old pupa, Lane **5:** Teneral fly, and Lane **6:** Adult tsetse fly. **M:** 1Kb DNA ladder, Fermentas, USA. Gel loading was normalized to GAPDH (400 bp product).

3.3 AQP 4886_gp sequence analysis

3.3.1 Topology and homology of AQP 4886_gp

The AQP 4886_gp encodes a protein containing 227 amino acids (An equivalent of 681 bp) with a predicted molecular weight of 25.222 KDa and an isoelectric point of 5.36. Topology and hydrophobicity predictions showed that AQP 4886_gp has 6 transmembrane-spanning regions and cytosolic N- and C- termini (Figure 8) as is standard for AQP family members (Gheorghe, 2009). AQP 4886_gp possesses two characteristic NPA (asparagine, proline, alanine: Asn-Pro-Ala) motifs in transmembrane loops B and E at positions 57-59 and 173-175, respectively. Loop B (intracellular) and loop E (extracellular) folds into the membrane and interact with one another to form an 'hour-glass model'. Consequently, NPA motifs interact, generating a functional water pore (Spring *et al.,* 2009). There are a total of 6

predicted phosphorylation sites in AQP 4886_gp; however, three of these sites are within transmembrane regions (highlighted in red and underlined in the following Table 2). Two intracellular residues potentially involved in regulation of water transport were identified (Ser140, and Ser215 with prediction scores of 0.977, and 0.995, respectively). Phosphorylation of RsAQP1 of *Rhipicephalus sanguineus*, with four predicted phosphorylation sites, was studied using a protein kinase C activator (phorbol 12, 13-dibutyrate) and resulted into reduced water flux (Andrew *et al.*, 2009). Therefore, phosphorylation status of AQP indicates role in water transport regulation.

Table 2. The six transmembrane helices of AQP 4886_gp. The underlined residues, which are highlighted in red, are the predicted transmembrane phosphorylation sites (NetPhos 2.0).

No.	N terminal	Transmembrane region	C terminal	Type
1	1	MLFAELAGTFLLVIIGIGSCTSG	23	PRIMARY
2	27	SPSVPQIAFTFGLTVATLAQTIG	49	SECONDARY
3	68	GEMTLLKAIFYIIVQCVGAMAGS	90	PRIMARY
4	117	GQAVSIEAFITAILVLVVKAVSD	139	PRIMARY
5	148	SAPLAVGLAIATGHLCAIKLTGA	170	SECONDARY
6	192	VYWIGPLVGSVVAAVIYKLIFKQ	214	PRIMARY

Prediction of transmembrane helices was done using SOSUI software:

http://bp.nuap.nagoya-u.ac.jp/sosui/.

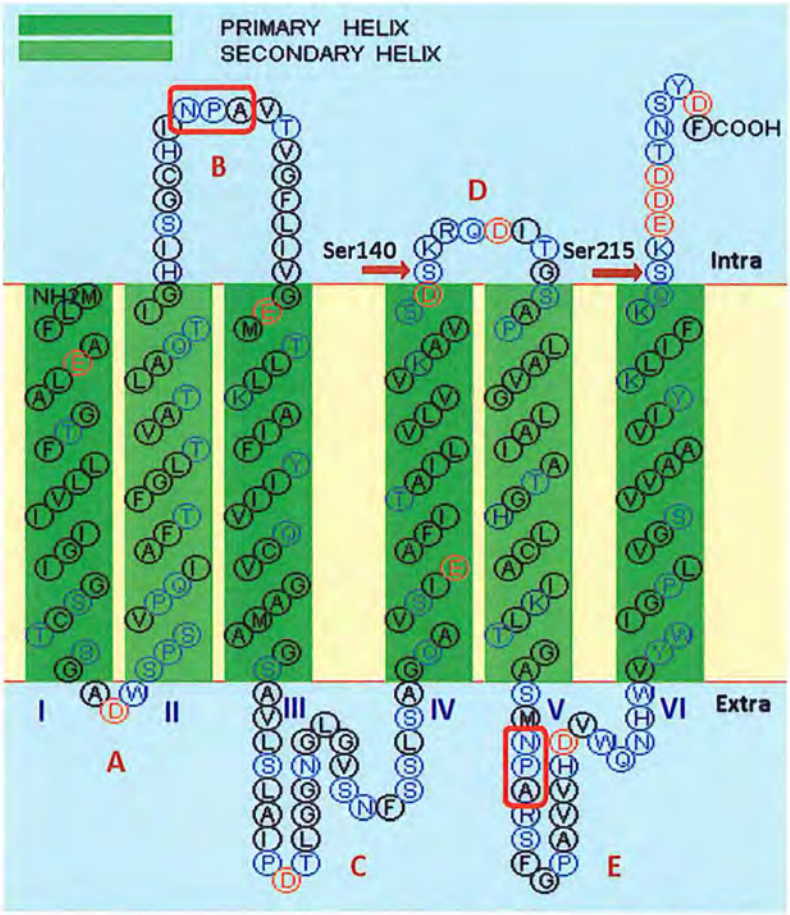

Figure 8. The membrane topology of AQP 4886_gp. Using predictive residue software, the amino acid sequence for AQP 4886_gp was plotted using SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/) as it would appear in the plasma membrane of cells prior to complete folding of pore hemispheres into the hourglass shape. Loops are labeled A-E and transmembrane regions I-VI. The two rounded rectangles with red outline represent the two NPA motifs. Hydrophobic residues: *black*, Positively charged: *blue*, Negatively Charged: *red*. The two dark-red *arrows* indicate predicted intracellular phosphorylation sites (Ser140 and Ser215). The average of hydrophobicity of AQP 4886_gp is 0.797778.

The Swiss-Pdb Viewer (http://www.expasy.org/spdbv/) server was used to predict the 3D structure of AQP 4886_gp. The two 'NPA' conserved motifs interacted at the centre of the pore as expected (Figure 9).

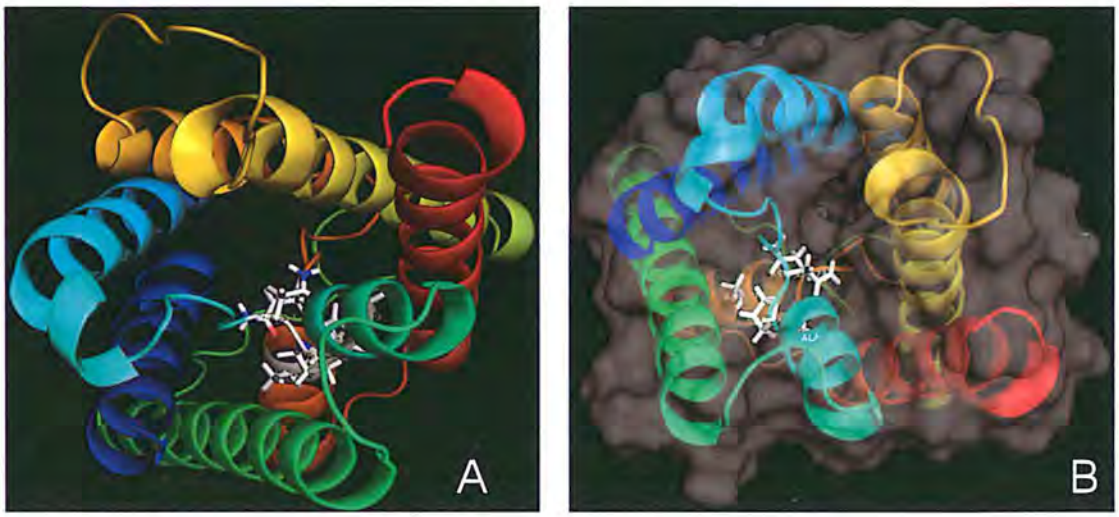

Figure 9. The 3D structure of AQP 4886_gp. (A) Shows the ribbon diagram of AQP 4886_gp monomer, while (B) shows its space-filling model. The projecting residues, in stick representation and coloured white, shown in the middle of the pore (surrounded by the helices) indicates the interacting NPA motifs.

3.3.2 Sequence comparison and evolutionary relationships of AQP 4886_gp

The two salivary gland homologues from *G. m. morsitans* (GMsg 4886) and *G. pallidipes* (AQP 4886_gp), differ in their gene sequences as demonstrated by nucleotide sequence alignment (Appendix 1). These changes (mutations) in the aquaporin gene sequence in these two different species of tsetse fly, belonging to morsitans group that occupy mainly savanna areas, could affect the rates of water exchange across the water channels.

A BLAST analysis using AQP 4886_gp nucleotide and protein sequence on *G. morsitans morsitans* transcriptome (*Gene*DB) yielded 98.2% identity to GMsg 4886 (http://old.genedb.org/genedb/Search?organism=glossina&name=GMsg-4886&isid=true). A comparison of the coding sequence of AQP 4886_gp with the NCBI GenBank database, using tBLASTx algorithm, revealed the highest amino acid identities to *G. m. morsitans* GM-509 (EZ422025) and GM-513 (EZ423775) putative AQPs with 99.3% and 99.1% sequence identities, respectively. Other best hits revealed by the search included; the polypeptide encoded by the adult buffalo fly, *Haematobia irritans exigua*, water channel (BFWC1) mRNA (with 71.7% identity), followed by 70.0% identity to *Drosophila melanogaster* integral protein (DRIP; aquaporin) (Table 3).

A multiple sequence alignment (CLUSTALW) of the AQP 4886_gp coding sequence with other aquaporin sequences is shown in Figure 10. This alignment illustrates the highly significant amino acid sequence homologies between the *G. pallidipes* AQP 4886_gp sequence and several insect aquaporin sequences deposited in GenBank of NCBI. In particular, the two 'NPA' amino acid sequence motifs are a characteristic feature of all aquaporin channel proteins (Agre *et al.*, 1993b; Knepper, 1994) and AQP 4886_gp sequence contains these motifs as well as surrounding regions of identical and conserved amino acids. Inspection of the multiple alignment also shows closer similarity between AQP 4886_gp sequence and the eight insect AQPs, as opposed to the five vertebrate AQPs, with respect to the location of the gaps introduced into the sequences. The protein distance tree, as described below (section 3.3.3), illustrates these relationships.

Table 3. Similarity of AQP 4886_gp deduced protein sequence to other related AQP proteins. NCBI GenBank database was scanned for homology.

Acc. No	Description	% Identity	E value	Reference
(EZ422025)	Transcriptome Shotgun Assembly (TSA): *G. morsitans morsitans* GM-509 mRNA	99.3%	7e-86	Alves-Silva *et al.*, 2010
(EZ423775)	TSA: *G. morsitans morsitans* GM-513 mRNA sequence	99.1%	4e-145	Alves-Silva *et al.*, 2010
(U51638.1)	*Haematobia irritans exigua* water channel (BFWC1) mRNA, complete cds	71.7%	1e-103	Elvin *et al.*, 1999
(NM_165833.2)	*Drosophila melanogaster* DRIP, transcript variant A, mRNA	70.0%	1e-90	Hoskins *et al.*, 2007
(AF218314.1)	*Aedes aegypti* aquaporin mRNA, complete cds	59.2%	4e-41	Pietrantonio *et al*, 2000
(X97159.1)	*Cicadella viridis* mRNA for aquaporin	53.3%	2e-64	Le Caherec *et al.*, 1996
(NM_001043454.1)	*Bombyx mori* aquaporin (AQP-Bom1), mRNA, complete cds	52.9%	3e-58	Kataoka *et al.*, 2009
(U48397.1)	*Mus musculus* mercurial-insensitive water-channel 1 (mMIWC1) mRNA, complete cds	51.4%	2e-52	Ma *et al.*, 1996
(NM_001085862.1)	*Xenopus laevis* aquaporin 2 (collecting duct) (AQP2), mRNA, complete cds	49.1%	4e-59	Kubota *et al.*, 2006
(NM_001105719.1)	*Rattus norvegicus* major intrinsic protein of lens fiber (MIP), mRNA	45.8%	6e-54	Grey *et al.*, 2009
(U34846.1)	Human mercurial-insensitive water channel mRNA, form 2, complete cds	45.3%	2e-52	Yang *et al.*, 1995
(NM_001101199.1)	*Bos taurus* aquaporin 2 (collecting duct) (AQP2), mRNA	44.8%	7e-53	Zimin *et al.*, 2009
(NM_008600.4)	*Mus musculus* major intrinsic protein of eye lens fiber (MIP), mRNA	43.8%	2e-53	Rivera *et. al.*, 2009

The table shows the best thirteen database matches on the basis of their percentage identity (to AQP 4886_gp) and *E* values. Abbreviations used are as listed from the NCBI GenBank search. The accession numbers (Acc) are shown in the first column.

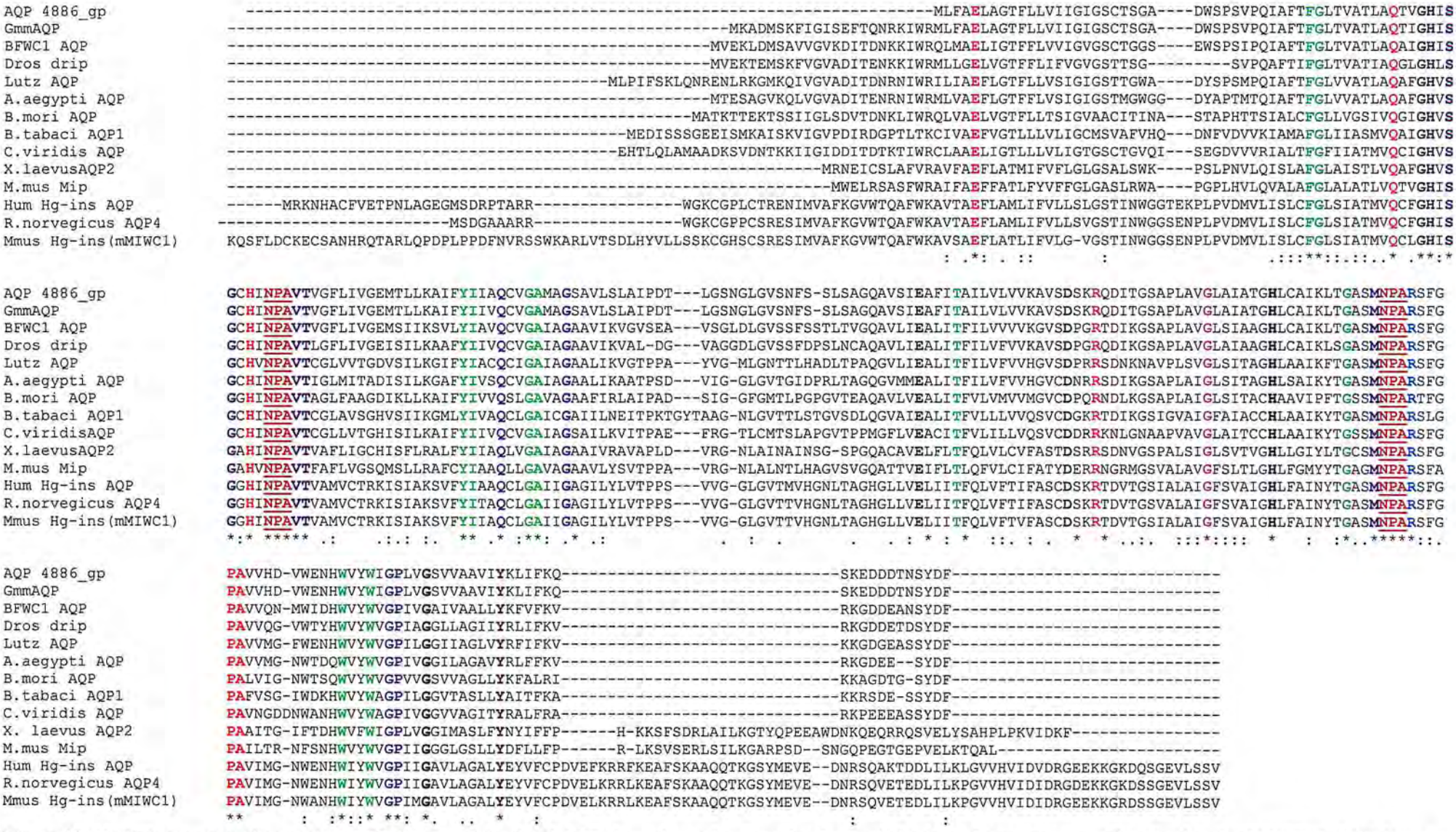

Figure 10. Multiple sequence alignment of AQP4886_gp with related AQP channel proteins from the GenBank database. Sequences were aligned using the CLUSTAL W (1.8) programme with identical residues indicated by (*) and conserved residues shown by (:). Sequence abbreviations are described in section 3.3.2 and 3.3.3. The 'NPA' motif signatures are underlined.

3.3.3 Phylogeny

The evolutionary history of AQP 4886_gp (Figure 11) was inferred using the Neighbor-Joining method (Saitou and Ni, 1987) and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) are shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the dendrogram. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.,* 2007).

Three major clusters of sequences resulted from this analysis. The first group (i) comprises the insect aquaporins; *G. m. morsitans* putative AQPs (GMsg 4886, GM-509 and GM-513), *D. melanogaster* DRIP gene, *Haematobia irritans exigua* water channel (BFWC1), *Aedes aegypti* aquaporin, *Bombyx mori* AQP, and *Cicadella viridis* AQP. The second group (ii) comprises MIP-homologues from a frog (*Xenopus laevus*), house mouse (*Mus musculus*), and cattle (*Bos taurus*), while the third group (iii) contains members of the mercury-insensitive AQPs isolated from brain tissue of a house mouse, and human. The protein distance tree shows that the database-retrieved vertebrate MIPs are less related to AQP 4886_gp as compared to insect AQPs, a result which is consistent with multiple sequence alignment presented in Figure 10.

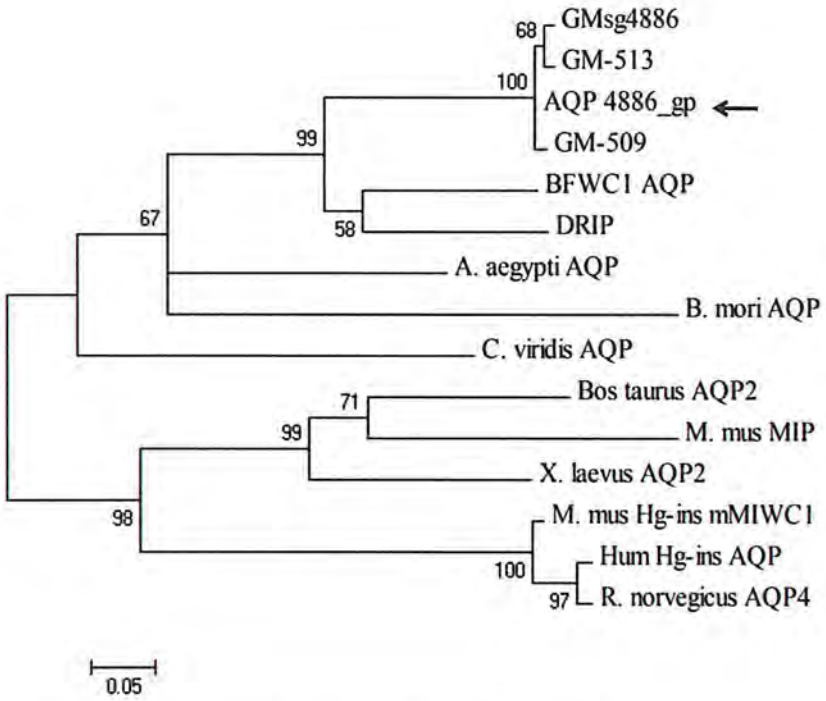

Figure 11. A dendrogram of *G. pallidipes* AQP 4886_gp deduced protein sequence to other related MIP-family sequences listed in Table 3.

3.3.4 Prediction of putative AQP 4886_gp function

A number of AQPs have been studied and functionally characterized as either pure water channels or aquaglyceroporins that can transport water and other small molecules such as glycerol or urea. The atomic resolution structures of human AQP1 have been solved using electron microscopy at a medium resolution (3.8 Å; Murata *et al.,* 2000). Since then, more structures came up including; the *E. coli* glycerol facilitator, GlpF (2.2 Å; Fu *et al.,* 2000) and bovine AQP1 (2.2 Å; Sui *et al.,* 2001). Water permeation in AQPs has been extensively studied by classical molecular dynamics simulations (Zhu *et al.,* 2001). The constriction region of GlpF was found to be almost 1 Å wider than in AQP1 which gives water favourable steric accessibility through both the AQP1 and GlpF channels but not glycerol in AQP1

(Sui *et al.,* 2001). The residues at the selectivity filter (water pore) between classical water channels and aquaglyceroporins also differ. Sui *et al* (2001) identified three residues, in the water pore, responsible for water specificity of bovine AQP1 (i.e. R197, H182 and F58) and further illustrated conservation of these three residues across the studied water-specific AQPs, unlike in aquaglyceroporins, through amino acid sequence alignments. By the same approach, the three conserved residues in the AQP1 constriction region were identified in AQP 4886_gp sequence (Figure 12, conserved residues at positions; R176, H161 and F37). Therefore, AQP 4886_gp may only transport water and not any other small uncharged solutes such as glycerol and urea (as is the case for aquaglyceroporins).

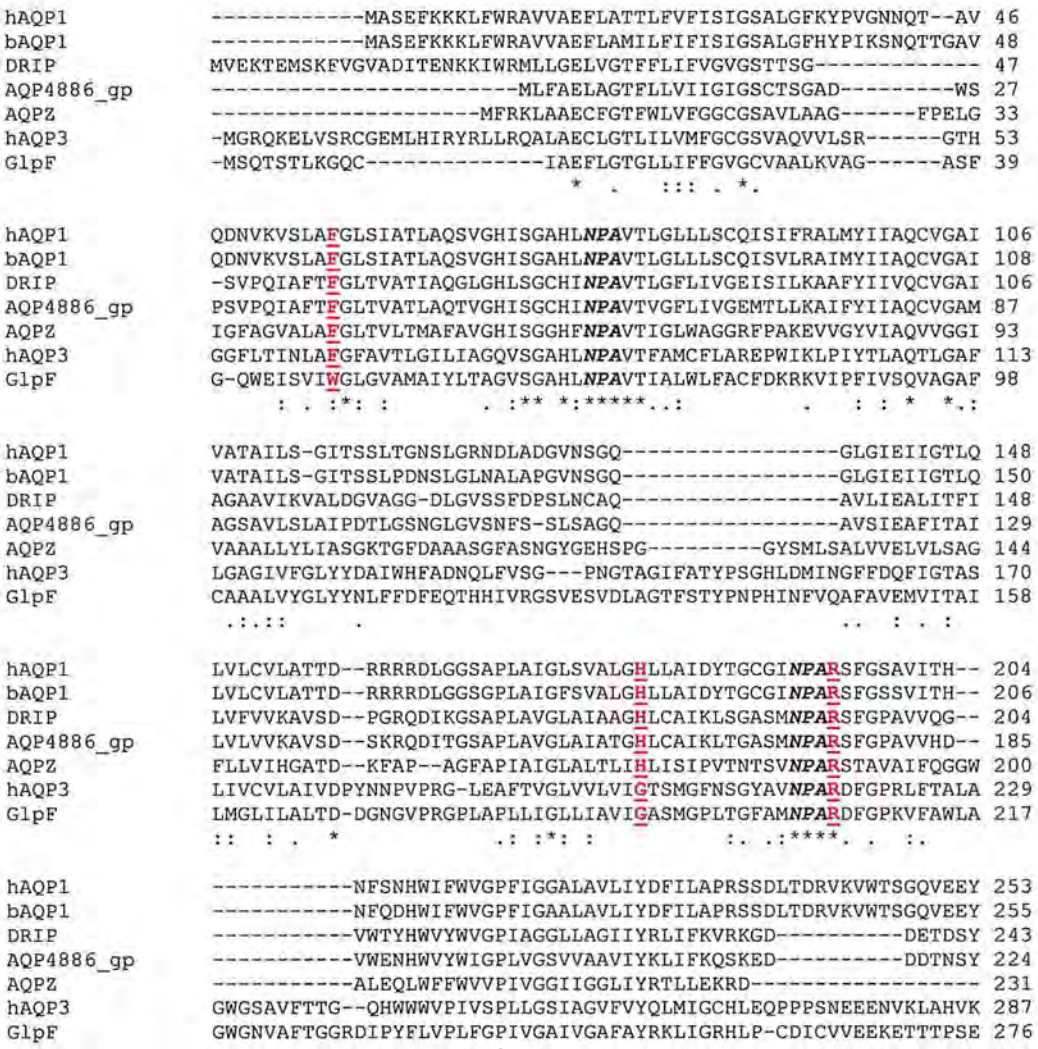

Figure 12. Amino-acid sequence alignment of bovine AQP1 (bAQP1), human AQP1 (hAQP1), human AQP3 (hAQP3), *E. coli* AQPZ, Drosophila integral membrane protein (DRIP), AQP 4886_gp, and *E. coli* GlpF produced using CLUSTALW. Residues lining the extended narrow pore (selectivity filter) of bovine AQP1 and human AQP1 are indicated by underlined red bolden letters. The 'NPA' motifs are italicized. The known aquaglyceroporins (GlpF and hAQP3) were included in the above alignment for comparison of the conserved residues in water-specific AQPs. The identical residues are indicated by (*) and conserved residues shown by (:).

3.4 RNA*i* studies

3.4.1 Gene cloning and sequencing

AQP 4886_gp gene was successfully amplified from the tsetse fly *G. pallidipes* salivary gland cDNA, ligated into pGEM®-T Easy vector and subsequently subcloned into pLL10 RNA*i* plasmid (Appendix 1). The presence of T7 promoter sites on both ends of the insert (in pLL10 plasmid) was confirmed before proceeding with *in vitro* transcription.

3.4.2 *In vitro* transcription

It was important to ensure that the plasmid DNA is relatively free of contaminating proteins and RNA in order to have greatest yields from *in vitro* transcription reactions. The circular pLL10 plasmid containing AQP 4886_gp gene was digested to linearize the plasmid downstream of the insert. This is because RNA polymerases are very processive hence can generate extremely long, heterogeneous RNA transcripts from the circular plasmid template. Both sense and antisense RNA strands were prepared from the AQP 4886_gp gene that was cloned into pLL10 vector. The resultant two complementary RNA strands were annealed together to form a duplex that was purified and then quantified. The dsRNA initial concentration of 159.79 μg/mL was further concentrated to 5 μg/μL through ammonium acetate precipitation procedure. The dsRNA was loaded on 1% ethidium bromide-stained agarose gel to visualize the expected band size (Figure 13). The gel results indicated two bands after the nuclease digestion and purification steps; one at the expected size and the other at double the expected size. The dsRNA that contains double-sized band has

been reported before and does not affect RNA*i* (Megascript RNA*i* kit, Ambion, 2130 Woodward St. Austin TX 78744). The RNA was stored at -20 °C.

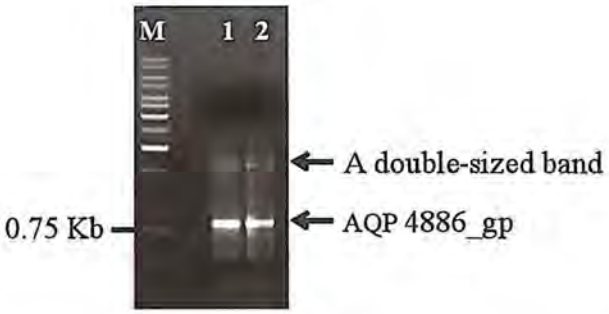

Figure 13. Purified dsRNA for microinjection studies (Lanes 1 and 2). **M:** 1 Kb DNA ladder, Fermentas. The arrow indicates the position of desired product.

3.4.3 Verification of gene silencing

The degree of knockdown achieved was monitored separately in the salivary glands, midgut and remaining carcass of the fly by semi-quantitative RT-PCR using RNA*i*-Fw4886 and RNA*i*-Rv4886 primers (Table 1) to amplify 597 bp product. A whole fly homogenate was also included for analysis. RT-PCR demonstrated that day 10 post-injection, AQP 4886_gp transcripts were absent in experimental flies (Figure 14). The disappearance of salivary gland transcripts was also observed on day 8. On day 12 post-injection, the transcripts in different tissues was detected again. This indicated transient gene knockdown.

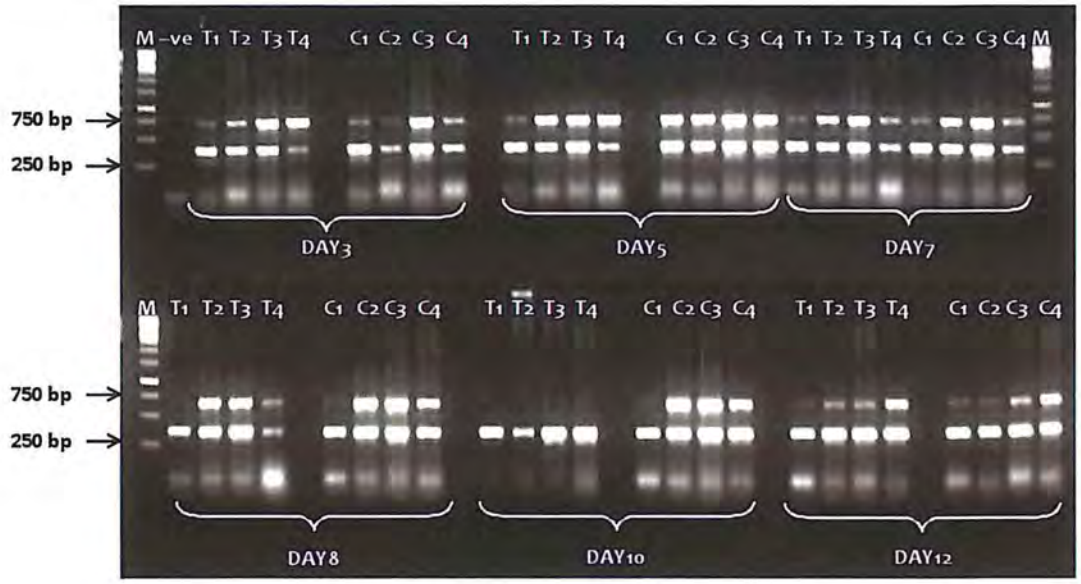

Figure 14. Verification of gene-knockdown. The PCR products were separated on a 1% agarose gel electrophoresis. **M:** 1Kb DNA ladder, Fermentas.

Test samples (dsRNA-injected; **T1-T4): T1**- Test salivary gland (SG), **T2**- Test midgut (MG), **T3**- Test carcass (CAR), and **T4**-Test Whole fly (WF). Control samples (NFW-injected; **C1-C4): C1**- Control SG, **C2**- Control MG, **C3**- Control CAR, and **C4**- Control WF. The upper band in each lane represents AQP 4886_gp (681 bp product), while the lower one is the internal control (GAPDH, 400 bp product). **–ve:** represents the reverse transcriptase negative control comprising the reaction without reverse transcriptase enzyme.

3.4.4 Effects of AQP 4886_gp knockdown on feeding success and survival rates

Trends of reduced feeding were observed in the dsRNA-injected group (Table 4), particularly on day 1 post-injection, where 12 flies, out of 34, did not feed after two attempts to supply them with blood source. In the nuclease-free water (NFW)-injected control group, a total of only three flies did not feed on first two days after injection. On the other hand, all flies in the uninjected control group fed. The reduction in feeding trends for the injected groups may have been contributed by the injuries inflicted on flies by the injection needle. Chilling of flies at 4 °C for 30 min did not seem to affect feeding because all flies that were only chilled and not injected fed until the last day of analysis.

Survival rates of 83% (6 flies died, total number of flies in each group was 35; Table 4) were observed for both dsRNA- and NFW-injected flies. All flies in the uninjected control group survived to the last day of analysis indicating that chilling of the flies at 4°C for 30 min simply makes them inactive, but it is not lethal. The mortality cases in NFW-injected control group was recorded for only two days after injection, while in the dsRNA-injected flies, the mortality (even though low) was noted almost daily (Table 4). The higher mortality of the NFW-control group during the first two days after injection could have been caused by the injury inflicted on the flies by the injection needle and handling stresses during injection.

Table 4. Microinjection experiment on male tsetse fly, *G. pallidipes*. The teneral flies were followed for 12 days after treatment. Each group consisted of 35 tsetse flies.

TREATMENT	DATE	Total No. Alive	Total No. Dead	No. of Flies that Fed	No. of flies that did not feed
TEST FLIES	31.03.2010 **Injected (dsRNA)**	-	-	-	-
(dsRNA-AQP4886_gp)	1.04.2010	34	1	22	12
	2.04.2010	33	1	32	1
N = 35	4.04.2010	27	1	26	1
	6.04.2010	21	1	20	1
	8.04.2010	15	1	15	0
	10.04.2010	9	1	9	0
	12.04.2010	3	0	-	0
Totals	-	-	**6**	-	**15**
INJECTED CONTROLS	31.03.2010 **Injected (NFW)**	-	-	-	-
(Nuclease-Free Water)	1.04.2010	33	2	31	2
	2.04.2010	31	3	30	1
N = 35	4.04.2010	23	0	23	0
	6.04.2010	18	0	18	0
	8.04.2010	13	0	13	0
	10.04.2010	8	0	8	0
	12.04.2010	3	0	-	0
Totals	-	-	**6**	-	**3**
UNINJECTED CONTROLS	**31.03.2010 Chilled on ice for 30 min**	-	-	-	-
(Chilled only)	1.04.2010	35	0	35	0
	2.04.2010	35	0	35	0
N = 35	4.04.2010	35	0	35	0
	6.04.2010	35	0	35	0
	8.04.2010	35	0	35	0
	10.04.2010	35	0	35	0
	12.04.2010	35	0	35	0
Totals	-	-	**0**	-	**0**

3.5 Statistical analysis and data presentation

The average proportion of the fed flies was determined in each group. The data was analyzed using Kruskal-Wallis rank sum test (which is a non-parametric test that does not require the normality of the data). The following result was obtained; Kruskal-Wallis chi-squared = 6.597, df = 2, p-value = 0.03. The $p<0.05$ indicated that the treatment groups (dsRNA-injected, nuclease free water (NFW)-injected and uninjected controls) were significantly different. To further study the differences

between different groups, the average proportions were subjected to a binomial test for proportion (Table 5).

Table 5. The average proportion of fed flies in different groups. All flies in the uninjected control group fed throughout the analysis period.

TREATMENT	Proportion fed	Standard error
Injected dsRNA	0.79[a]	0.139
Injected NFW	0.84[a]	0.140
Uninjected controls	1.00[b]	0

Average proportion followed by the same lower case letter are not significantly different at $p<0.05$ (binomial test for proportions).

The means and the standard errors of the three groups are graphically presented in the figure below (Figure 15). There was no significant difference, in terms of feeding success, between the dsRNA- and NFW-injected groups at $p<0.05$

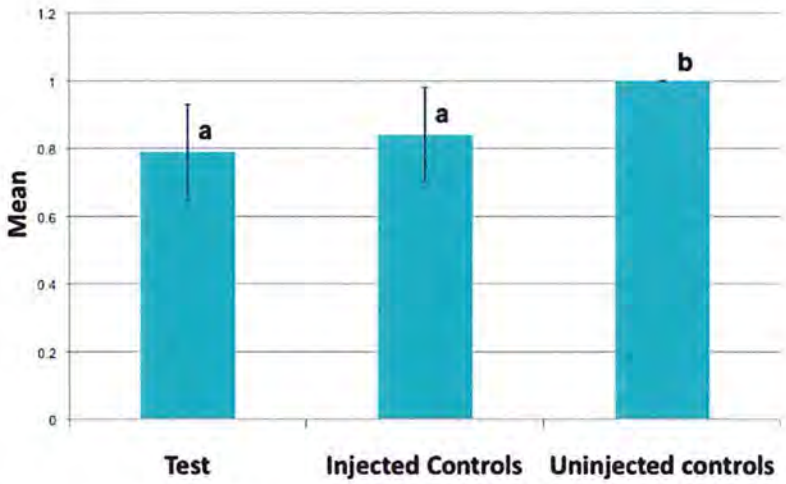

Figure 15. A graph that compares means and the standard errors of the three groups. The data is provided in Table 5 above.

3.6 Protein expression

Verification of gene knockdown is very important in RNA*i* studies. Semi-quantitative RT-PCR and Western blotting techniques are used widely for confirmation of gene silencing. Currently, there are no commercially available antibodies for AQP 4886_gp. Therefore, it was important to raise the antibody first before conducting the Western blot experiments to study the protein profiles before and after the gene knockdown. This was important because some proteins have long half-lives such that they can still provide the gene function in the absence of the mRNA (due to gene silencing). This is important when studying gene functions.

The protein expression was done in bacteria (described in section 3.6.1 below) with an anticipation to produce enough protein for raising the antibody in rabbits. However our results indicate that the aquaporin protein was highly toxic to *E. coli,* thus could not be produced in detectable amounts.

3.6.1 Expression of tsetse fly protein (AQP 4886_gp) in *E. coli*

The expression of AQP 4886_gp (cloned into pRSET-A) was induced in *E. coli* BL21(DE3)pLysS culture at an optical density (OD_{600nm}) of 0.6. The samples were collected at different time points before and after induction with 1 mM IPTG. A positive control, TcrX gene from *Mycobacterium avium* subspecies *paratuberculosis* which had been cloned into pRSET-A, was included. The samples were resolved on 12% SDS-PAGE. The positive control, TcrX, of about 31 KDa effectively expressed and was detectable through Coomassie staining (Figure 16a) and Western blot (results not shown). Staining of AQP 4886_gp protein (expected size of 28.74 KDa) did not reveal any difference in terms of banding pattern on SDS-PAGE (Figure

16b). However, a unique band of approximately 45 KDa was present in the overnight incubated sample. The 45 KDa protein was absent in IPTG-induced BL21(DE3)pLysS transformed with pRSETA vector. Western blot using HRP-HisProbe did not with the 45 KDa band.

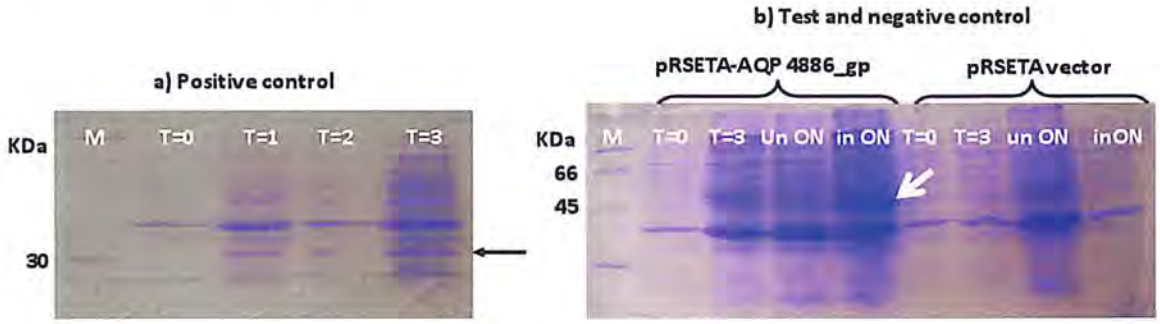

Figure 16. Protein expression in *E. coli* BL21(DE3)pLysS and resolution on 12% SDS-PAGE. **a)** Positive expression control (TcrX protein) was detectable by both Coomassie staining. **b)** Analysis of recombinant AQP 4886_gp 6× His-tagged protein. A negative control of bacteria transformed with pRSET-A vector was included. **T;** Time (h), **ON;** overnight incubated culture (UnON- uninduced, inON-induced overnight culture). The arrows indicate unique bands. Numbers on the left indicate position of protein molecular markers.

3.6.2 Observation on growth profile of cells

The behaviour of recombinant *E. coli* BL21(DE3)pLysS/pRSET-A/AQP 4886_gp was different in the absence or presence of the inducer. Immediately after IPTG induction, the growth of recombinant cells was arrested (Figure 17). There was no inhibition of growth in the absence of inducer and increase in OD_{600nm} was comparable in both control (carried pRSET-A vector) and test cells (recombinant pRSET-A/AQP 4886_gp). This indicated the toxicity of the channel protein

expression in *E. coli* which exerted negative effect on the cell growth. Lian *et al* (2008) found out that fusion proteins such as maltose binding proteins, glutathione-s-transferase and thioredoxin greatly improve the expression of an *E. coli* aquaporin Z in *E. coli* and imparts least toxicity to the cells. These relatively larger fusion proteins (as compared to common histidine tags) aid in preventing the insertion of recombinant protein into the membranes of *E. coli*, with hope that soluble proteins are produced. However, these fusion proteins tend to form mostly inclusion bodies in *E. coli* (Lian *et al.,* 2009) which poses another difficulty to refold the protein.

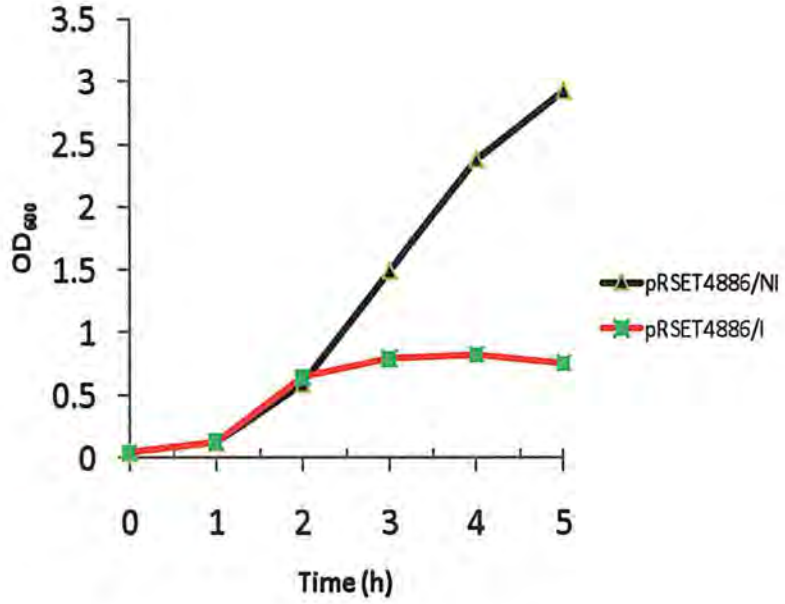

Figure 17. The growth curve of transformed BL21(DE3)pLysS in LB medium. The cells were transformed with recombinant pRSET-A/AQP4886_gp and growth observed with (I; filled green squares) or without (NI; filled dark triangles) induction by 1 mM IPTG. The arrow indicates the point at induction.

Different conditions of IPTG and temperature were employed in order to optimize the expression conditions. The growth media, LB broth, was supplemented with

0.5% w/v glucose to repress induction of the *lac* promoter by lactose. The cultures were induced at mid-log phase of growth (OD_{600nm} ~ 0.6) and also at an OD_{600nm} of 1.0 to study the effects of induction timing. The results were recorded in the following Table 6. There was no significant improvement in terms of growth of cells at different concentrations of the inducer and incubation temperatures. However, increased growth rate was noted from the increase in OD_{600nm} readings before induction when glucose was supplemented in the media.

Table 6. Expression of AQP 4886_gp in *E. coli*.

TIME	pRSETA vector in BL21(DE3)pLysS (Control 1)	BL21(DE3)pLysS Cells (Control2)	pRSET/AQP4886 in BL21(DE3)pLysS (Test)				
		OD_{600nm} at 37°C					
2.5hr	0.663	0.678	0.700	-	-	0.645	0.657
3hr	1.057	1.105	-	0.939	0.983	-	-
		Induction with IPTG					
	37°C	37°C	18°C	25°C	25°C	37°C	37°C
	1mM	1mM	1mM	1mM	2mM	0.5mM	1mM
3hrs	2.413	2.336	0.862	1.076	1.060	0.767	0.701
5hrs	-	-	0.880	1.096	1.066	0.712	0.695
O/N			0.908	-	-	-	-

Induction was done under different conditions of temperature, IPTG concentration, and post-induction timing. Induction controls (1 and 2) were included to monitor bacterial growth.

3.6.3 Detection of AQP 4886_gp by SDS-PAGE and Western-blot analysis

The expected band size of AQP 4886_gp monomer is 28.74 KDa. This protein was not detected by Coomassie staining. However, Western-blot using HRP-HisProbe revealed a band of 25 KDa in cells (induced at OD_{600nm} of 0.9 and incubated at 25 °C) carrying recombinant expression plasmid (Figure 18). However, the control cells transformed with the expression vector also showed reactivity at the same position (data not shown). The detected cross-reacting protein could have been an *E. coli* histidine rich protein.

Figure 18. Western-blot detection of purified His-AQP 4886_gp from *E. coli* BL21(DE3)pLysS. **M;** protein marker, **P;** pellet, **FT;** flow-through (supernatant after binding), **10-200mM;** increasing concentrations of imidazole for elution of the fusion protein, **+ve:** His-tagged protein used as a control in Western-blot procedure. The arrow indicates unique bands. Numbers on the left indicate position of protein molecular markers.

3.6.4 Similar work on aquaporin expression in *E. coli*

Elvin *et al* (1999) expressed an aquaporin gene (AqpBF1) from adult buffalo fly in *E. coli*, XL1-Blue using pQE10 expression vector. AqpBF1 has 71.7% identity to AQP 4886_gp according to NCBI GenBank search. The cells were grown at 37 °C on double-strength LB medium to an OD_{600nm} of 1.0 and induced with 1.0 mM IPTG and grown further for 2 hrs. The yield of AqpBF1 recombinant protein was 8 mg from 40 litres of IPTG-induced culture which implies low expression due to the toxicity of the protein.

Another challenge of expressing toxic proteins in *E. coli* arises when basal expression of the recombinant protein occurs before induction. That exerts a negative effect in growth of bacteria which translates into minimal yields of the protein. That brings importance to the choice of the vector and also the expression host to be employed. The aquaporin gene was re-cloned and expressed under arabinose-inducible arapBAD promoter of pBADHisA which is a more tightly regulated system that curtails the problem of basal expression levels in *E. coli.* Both full length (ORF) and truncated AQP gene sequence were re-cloned.

3.7 Expression in pBADHisA vector

3.7.1 Cloning into pBADHisA and sequencing

The AQP 4886_gp gene was PCR-amplified (Figure 19) using gene-specific primers (*Fw 4886/Rv 4886:* Full length, *Fw 4886t/Rv 4886:* Truncated gene. Table 1). Both full length and truncated AQP 4886_gp genes were cloned into pBADHisA. The sequences were confirmed to be in frame and in proper orientation (Appendix 1).

Figure 19. PCR amplification results of full length and truncated AQP 4886_gp gene Full length (1 and 2) and truncated (3 and 4) AQP 4886_gp gene as visualized in ethidium bromide-stained 1% agarose gel. **M:** 1 Kb DNA ladder, **C:** Negative control.

3.7.2 Expression of truncated AQP4886_gp and detection by Western-blot

The truncated AQP 4886_gp sequence cloned into pBADHisA was transformed into Rosetta(DE3)pLysS (Novagen, Inc. Canada), which is a strain of *E. coli* that carries rare tRNAs in *E. coli* thus could overcome the codon bias of *E. coli* for enhanced protein expression.

A culture of 500 mL supplemented with 0.5% w/v glucose was prepared and induced at an OD_{600} of 1.0 with 0.2% arabinose at 37 °C with shaking at 200 rpm for 3 hrs. The protein was purified and resolved by 15% Tris-SDS-PAGE, and transferred into nitrocellulose transfer membrane for Western blot analysis to detect 6×His-tagged proteins.

Figure 20. Expression and Western-blot detection of purified 6×His-AQP 4886t in *E. coli* Rosetta(DE3)pLysS. **M;** protein marker, **P;** pellet, **FT;** flow-through (supernatant after binding), **10-200mM;** increasing concentrations of imidazole, **+ve:** His-tagged protein used as a control in Western-blot procedure. The arrow indicates the position of unique bands. Numbers on the left indicate position of protein molecular markers.

The expected monomer size of the truncated gene (431 bp) was 15.96 KDa (1 KDa ≈ 27 bp). The 6×His fusion tag added 3 KDa, thus 18.96 KDa band was expected. A band of 25 KDa showed reactivity (Figure 20) at imidazole concentrations of 60 mM and 200 mM and the resin also indicated bound protein. A control sample of Rosetta(DE3)pLysS cells transformed with pBADHisA vector was included in this experiment and treated the same way. A weak signal was also present at almost the same position of 25 KDa. Thus the detected signal could be a cross-reacting histidine-rich protein from *E. coli* origin.

From the above results, it was clear that induction of AQP 4886_gp expression leads to death of *E. coli* as evidenced by the changes in OD_{600nm}. The integral membrane proteins have been reported to be toxic to the bacterial expression host when

overexpressed (Lian *et al.,* 2008). Some are highly toxic even at low concentration in the cells, thus tightly regulated systems that minimizes basal expression levels before induction are required. The growth of cells improved when pBADHisA vector was used for AQP 4886_gp protein expression study. However, *E. coli* may not be the best expression host for eukaryotic membrane proteins (Junge *et al.,* 2008). This is because posttranslational modifications (e.g. glycosylation and phosphorylation) of recombinant eukaryotic membrane proteins are unlikely to occur in prokaryotic expression hosts, which sometimes may hamper stability, targeting, and functional folding of these proteins (Junge *et al.,* 2008).

CHAPTER FOUR

4.0 DISCUSSION

The tsetse saliva is the fluid vehicle for maturation and transfer of the infective metacyclic trypanosomes to the mammalian host during the blood-feeding process (Vickerman, 1985). Water movement across cell membranes occurs via AQPs. In the brown dog tick, *Rhipicephalus sanguineus*, an AQP (named *Rs*AQP1), was identified to be involved in movement of water through the cell membranes of salivary glands thus important in tick osmoregulation (Andrew *et al.,* 2009).

In the present study, an AQP (named AQP 4886_gp) from *G. pallidipes* salivary glands cDNA was identified and cloned using primers based on a *G. m. morsitans* putative aquaporin, GMsg4886 (http://www.genedb.org/genedb/glossina/index.jsp). AQP 4886_gp has all the major hallmarks of aquaporin gene family. Amino acid sequence analysis of AQP 4886_gp reveals residues that have been identified, in classical AQPs, to be involved in rapid water-selective transport (Sui *et al.,* 2001). AQP 4886_gp showed high homology to insect AQPs such as *D. melanogaster* DRIP (70.0% identity), *Aedes aegypti* aquaporin (59.2%), and *Cicadella viridis* AQP (53.3%). These three insect AQPs belong to the sub-family of pure water channels. This information predicts AQP 4886_gp to be a water-specific protein channel, although water and solute permeability assays needs to be performed to confirm the prediction. This can be achieved through expression studies of AQP 4886_gp protein in *Xenopus laevus* oocytes or proteoliposomes followed by physiological assays involving incubation of the recombinant oocytes in solutions of different

physiological strengths (isotonic, hypotonic and hypertonic medium) to monitor water movement across the cell membrane.

The AQP 4886_gp gene was localized in different tissues of the tsetse fly, *G. pallidipes*. Semi-quantitative RT-PCR results indicated the presence of AQP 4886_gp transcript in the malpighian tubules, midgut and the testes of tsetse fly (Figure 7a). Aquaporins associated with malpighian tubules have been found to mediate the flux of water into the tubule lumen to form the primary urine (Kaufmann *et al.,* 2005). In ticks, water is absorbed from the blood meal across the gut wall and expelled into the host via the salivary glands (Sauer and Hair, 1972). The *Rs*AQP1 from brown dog tick, *Rhipicephalus sanguineus*, was localized in the salivary glands, malpighian tubules and gut where it is believed to be involved in mass movement of water, hence concentrating the blood meal (Andrew *et al.,* 2009). Expression of AQP 4886_gp transcript in tissues of tsetse fly e.g. malpighian tissues that have been reported to be involved in high rates of water flux (Leak, 1999) supports its potential role in mediating transmembrane water movements thus may play a role in eliminating excess water (plasma) in their diet. It will be of interest to determine whether there are specific regions of the gut and associated diverticuli with higher levels of AQP 4886_gp that could indicate particular regions of the alimentary tract associated with water uptake. Shakesby *et al* (2009) localized an aquaporin, ApAQP1, to the apposed stomach and distal intestine of adult pea aphids, *Acyrthosiphon pisum,* which was found to play an important role in water cycling in the aphid (a phloem-feeder) gut contributing to the dilution of the concentrated ingesta in the stomach.

The developmental stage expression of AQP 4886_gp mRNA transcripts was also studied. Semi-quantitative RT-PCR analysis of life stages of *G. pallidipes* revealed that AQP 4886_gp mRNA was present in whole body homogenates of larva, pupae, unfed teneral fly, and adult fly. The results indicate a higher relative presence of AQP 4886_gp mRNA in teneral stage as compared to the larval stage. The AQP 4886_gp may also be present in the egg stage of tsetse fly, although this was not investigated here. The expression of AQP 4886_gp at many stages during development suggests that this protein plays important roles throughout the organism's life cycle.

In this study, dsRNA*i* knockdown of AQP 4886_gp was carried out in teneral male tsetse flies, *G. pallidipes*. The gene knockdown effect was validated by semi-quantitative RT-PCR. On day 10 post-injection of dsRNA, AQP 4886_gp transcript was undetected in the test samples as compared to controls (injected with nuclease-free water). The absence of salivary glands AQP 4886_gp transcripts was also realized on day 8 post-injection in the dsRNA-injected samples. This indicated transient AQP 4886_gp gene-knockdown.

The dsRNA-injected group had reduced trends of feeding when provided with rabbit blood for up to 4 days after injections (and two days post-injection for the injected controls). However, improved feeding success was noted after four days post-injection. The mortality rates in the injected groups also decreased with time. Relatively high mortality rates observed in the first few days after injection could be attributed to stresses associated with handling and injection. Therefore, knock-down of AQP 4886_gp gene was by no means lethal to the flies. This may be due to the

transient nature of the knock-down. Secondly, survival could be due to compensatory expression of other AQPs in the tsetse fly gut and salivary glands. It is also possible that the AQP 4886_gp protein has a long half-life that enables it to persist during the entire knockdown period, thus providing the function of the protein in the absence of protein synthesis. A similar study was done in pea aphid in which dsRNA*i*-knockdown of an AQP, ApAQP1, in pea aphid did not affect mortality or body weight gain (Shakesby *et al.*, 2009).

Determining protein expression profile of AQP 4886_gp gene is paramount to making valid conclusions. However, this hydrophobic membrane protein was toxic when overexpressed in *E. coli*. Therefore, it is important to induce the expression in another expression system such as yeast or insect cell lines. These eukaryotic expression systems have post-translational modification capacities, lacking in *E. coli*, such as phosphorylation which was predicted to occur in AQP 4886_gp (NetPhos 2.0). Thirteen codons that are rare in *E. coli* are also present in AQP 4886_gp, which could inhibit protein expression in bacteria (www.justbio.com; batch codon usage tool).

4.1 Conclusion

AQP 4886_gp mRNA transcript was present in most life cycle stages of tsetse fly, *G. pallidipes*, as well as tissues involved in mass water movement associated with concentrating the blood meal. This may point to its importance in tsetse fly osmoregulation. Maintenance of fluid homeostasis throughout the life cycle of these insects presents multiple targets for insect population control. Thus aquaporins could be targeted for control efforts, for example, by production of recombinant vaccines

(immunological approach) or through the study of potential water-transport inhibitors where eco-friendly insecticides could be developed.

The *ds*RNA-knock down of AQP 4886_gp in male tsetse flies, *G. pallidipes*, led to reduction in feeding trends. The transient knock down was validated by semi-quantitative RT-PCR. However, in this study, AQP 4886_gp protein levels were not measured before and after injection of dsRNA, but only the mRNA. The AQP 4886_gp heterologous protein proved to be toxic when expressed in bacteria. Thus, future possibilities to improve this study would include Western blots using AQP 4886_gp-specific antibodies.

Despite the reduction in feeding trends for the dsRNA-injected flies, the tsetse flies did survive and feed. Therefore, transient deletion of AQP 4886_gp mRNA seems not critical to the survival of the tsetse fly. This study on AQP 4886_gp gene silencing highlights the utility of dsRNA*i* to assess the viability of candidate drug targets.

4.2 Recommendations

1. It is important to confirm the transport function of putative AQP 4886_gp and other AQPs in tsetse fly through expression in *Xenopus laevus* oocytes and performing studies such as sensitivity to Hg^{2+}.
2. The *Glossina* species are known to occupy different niches. Osmoregulatory mechanisms must therefore be different in the diverse environmental conditions of their respective life histories. It is therefore conceivable that specific adaptation have occurred in their AQPs. A study to compare AQPs in different tsetse fly species occupying different ecological niches is called for.
3. Although feeding success has been studied in the present study, the volume of blood ingested by a fly in each group (dsRNA-injected, nuclease-free water injected and uninjected controls) was not performed (i.e. by haemoglobin assay). This bioassay is important to study other effects of gene knockdown.

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APPENDICES

Appendix 1: Sequence results after cloning AQP 4886_gp gene into various vectors

CLUSTAL 2.0.12 multiple sequence alignment

```
GMsg4886        ATGCTATTTGCTGAGCTTGCGGGGACATTCTTACTGGTTATCATTGGTATAGGCAGTTGC 60
AQP 4886_gp     ATGCTATTTGCTGAGCTTGCGGGAACATTCTTACTGGTTATCATTGGTATAGGCAGTTGC 60
                ************************ ************************************

GMsg4886        ACGAGTGGTGCGGACTGGTCCCCCAGCGTGCCTCAAATAGCATTCACATTTGGTTTAACT 120
AQP 4886_gp     ACGAGTGGTGCGGACTGGTCCCCCAGCGTGCCTCAAATAGCATTCACATTTGGTTTAACT 120
                ************************************************************

GMsg4886        GTGGCAACGCTTGCACAGACGGTCGGTCATATAAGTGGATGTCACATAAATCCTGCTGTT 180
AQP 4886_gp     GTGGCAACGCTTGCACAGACTATTGGTCATATAAGTGGATGTCACATAAATCCTGCTGTT 180
                ********************  * ************************************

GMsg4886        ACAGTCGGTTTCTTGATAGTAGGAGAGATGACTTTACTAAAAGCTATTTTTTACATAATA 240
AQP 4886_gp     ACAGTCGGTTTCTTGATAGTAGGAGAGATGACTTTATTAAAAGCTATTTTTTACATAATA 240
                ************************************ ************************

GMsg4886        GCACAGTGCGTCGGAGCAATGGCTGGATCGGCGGTTTTGAGTCTAGCTATCCCTGACACA 300
AQP 4886_gp     GTACAGTGCGTCGGAGCAATGGCTGGATCGGCGGTTTTGAGTCTAGCTATCCCTGACACA 300
                * ************************************************************

GMsg4886        CTTGGCAGTAATGGCCTGGGCGTCTCTAACTTTTCCTCGCTCAGTGCTGGACAGGCAGTG 360
AQP 4886_gp     CTTGGCGGTAATGGCTTGGGCGTGTCTAACTTTTCCTCGCTCAGTGCCGGACAAGCGGTG 360
                ****** ******** ******* ********************** ***** ** ***

GMsg4886        TCAATTGAAGCCTTCATTACAGCTATCTTAGTGCTGGTAGTGAAAGCAGTGTCAGATTCA 420
AQP 4886_gp     TCAATTGAAGCCTTCATTACAGCTATCTTAGTGCTGGTAGTGAAAGCAGTGTCAGATTCA 420
                ************************************************************

GMsg4886        AAACGGCAAGATATAACGGGATCAGCCCCATTGGCCGTAGGGCTGGCTATCGCTACGGGT 480
AQP 4886_gp     AAACGACAAGATATAACGGGATCAGCCCCATTGGCCGTAGGCCTGGCTATCGCTACGGGT 480
                ***** ************************************ ******************

GMsg4886        CATCTTTGTGCGATTAAATTAACTGGAGCCAGCATGAACCCAGCACGCTCATTCGGTCCA 540
AQP 4886_gp     CATCTTTGTGCGATTAAATTAACTGGAGCCAGCATGAACCCTGCGCGCTCATTCGGACCG 540
                ****************************************** ** *********** **

GMsg4886        GCCGTAGTTCACGACGTTTGGGAAAATCATTGGGTTTATTGGATTGGTCCCCTTGTTGGC 600
AQP 4886_gp     GCCGTAGTTCACGATGTTTGGCAAAATCATTGGGTTTATTGGATTGGTCCCCTTGTTGGC 600
                ************** ****** ****************************************

GMsg4886        AGCGTGGTAGCTGCCGTTATTTATAAATTAATCTTCAAGCAATCTAAAGAAGATGATGAC 660
AQP 4886_gp     AGTGTGGTAGCTGCCGTTATTTATAAATTAATCTTCAAGCAATCTAAAGAAGATGATGAC 660
                ** ************************************************************

GMsg4886        ACGAACTCCTACGATTTTTAA 681
AQP 4886_gp     ACGAACTCCTACGATTTTTAA 681
                **********************
```

Figure: Sequence alignment of putative AQP homologues from two different species of tsetse fly. Both GMsg 4886 (from *G. morsitans morsitans*) and AQP 4886_gp (*G. pallidipes*) were amplified from salivary gland cDNA. Primers used for amplification of AQP 4886_gp were designed from (the above) GMsg 4886 gene sequence retrieved from *Gene*DB, because the same gene sequence was not available for *G. pallidipes*. The bases highlighted in red are different in both homologues, indicating mutations. The identical residues indicated by (*) and conserved residues shown by (:).

>AQP 4886_gp (in pGEM®-T Easy vector)

ATGCTATTTGCTGAGCTTGCGGGGACATTCTTACTGGTTATCATTGGTATAGGCAGTTGCACGAGTGGTGCGGA
CTGGTCCCCCAGCGTGCCTCAAATAGCATTCACATTTGGTTTAACTGTGGCAACGCTTGCACAGACGGTCGGTCA
TATAAGTGGATGTCACATAAATCCTGCTGTTACAGTCGGTTTCTTGATAGTAGGAGAGATGACTTTACTAAAAGC
TATTTTTTACATAATAGCACAGTGCGTCGGAGCAATGGCTGGATCGGCGGTTTTGAGTCTAGCTATCCCTGACAC
ACTTGGCAGTAATGGCCTGGGCGTCTCTAACTTTTCCTCGCTCAGTGCTGGACAGGCAGTGTCAATTGAAGCCTTC
ATTACAGCTATCTTAGTGCTGGTAGTGAAAGCAGTGTCAGATTCAAAACGGCAAGATATAACGGGATCAGCCCC
ATTGGCCGTAGGGCTGGCTATCGCTACGGGTCATCTTTGTGCGATTAAATTAACTGGAGCCAGCATGAACCCAGC
ACGCTCATTCGGTCCAGCCGTAGTTCACGACGTTTGGGAAAATCATTGGGTTTATTGGATTGGTCCCCTTGTTGGC
AGCGTGGTAGCTGCCGTTATTTATAAATTAATCTTCAAGCAATCTAAAGAA**GATGATGACACGAACTCCTACGA
TTTTTAA**

NB: *The primer sequences are in bold and underlined.*

>AQP 4886_gp (in pLL10 RNA*i* plasmid)

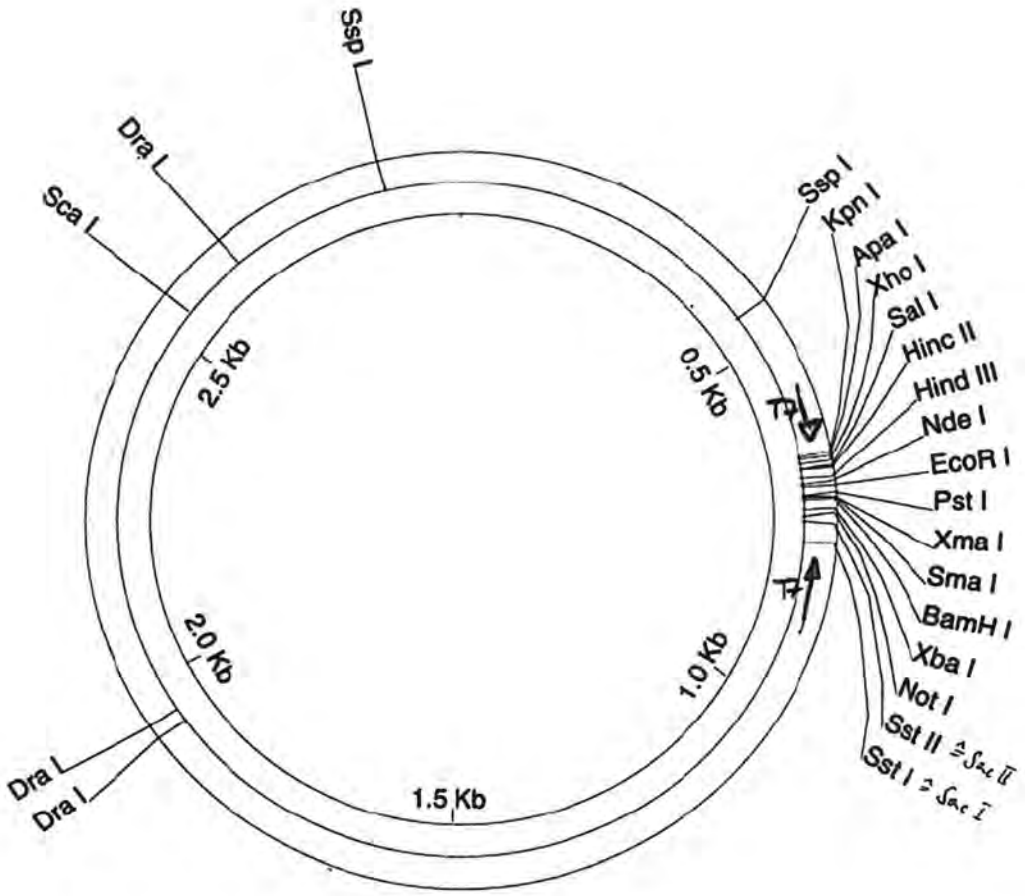
ACGGGCCCAGTTGATTGTATACGACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTAT
CGATAAGCTTCATATGGAATTCACTAGTGATT**GGATCCATGCTATTTGCTGAGCTTGCG**GGAACATTCTTACTG
GTTATCATTGGTATAGGCAGTTGCACGAGTGGTGCGGACTGGTCCCCCAGCGTGCCTCAAATAGCATTCACATTT
GGTTTAACTGTGGCAACGCTTGCACAGACTATTGGTCATATAAGTGGATGTCACATAAATCCTGCTGTTACAGTC
GGTTTCTTGATAGTAGGAGAGATGACCTTATTAAAAGCTATTTTTTACATAATAGTACAGTGCGTCGGAGCAATG
GCTGGATCGGCGGTTTTGAGTCTAGCTATCCCTGACACACTTGGTGGTAATGGCTTGGGCGTGTCTAACTTTTCCT
CGCTCAGTGCCGGACAAGCGGTGTCAATTGAAGCCTTCATTACAGCTATCTTAGTGCTGGTAGTGAAAGCAGTGT
CAGATTCAAAACGACAAGATATAACGGGATCAGCCCCATTGGCCGTAGGCCTGGCTATCGCTACGGGTCATCTTT
GTGCGATTAAATTAACTGGAGCCAGCATGAACCCTGCGCGCTCATTCGGACCGGCCGTAGTTCACGATGTTTGGC
AAAATCATTGGGTTTATTGGATTGGTCCCCTTGTTGGCAGTGTGGTAGCTGCCGTTATTTATAAATTAATCTTCAA
GCAATCTAAAGAA**GATGATGACACGAACTCCTACGATTTTTAA**TTCGAAAATCGAATTCCTGCAGCCCGGGGG
ATCCACTAGTTCTAGAGCGGCCGCCACCGCGGGCCCTATAGTGAGTCGTATTAGAGCTCCAGCTTTTGTTCCCTTT
AGTGAGGGTTAATTTCGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTTGTTATCCGCTCACAA
TTCCACACAACATACGAGCCGGAGCATAAAGTGTAAAGCCTGGGTGCTATGAGTGAGCTAACTCACATTTATTGC
GTGCGCTCACTGCCGCTTCAGTAGAAACTGTCTGCAGCTGCATTAATGAATCGCCAACGCGCGGGAAGGCGTGCT
ATTGGGCTCTTCCGGC

NB: *The primer sequences are in bold and underlined.*

>AQP 4886_gp (in pRSET-A expression vector)

IHMRGS**HHHHHH**GMASMTGGQQMGRDLYDDDDKDRWGSMLFAELAGTFLLVIIGIGSCTSGADWSPSVPQIAFTFG
LTVATLAQTIGHISGCHINPAVTVGFLIVGEMTLLKAIFYIIVQCVGAMAGSAVLSLAIPDTLGGNGLGVSNFSSLSAGQ
AVSIEAFITAILVLVVKAVSDSKRQDITSAPLAVGLAIATGHLCAIKLTGASMNPARSFGPAVVHDVWQNHWVYWIGP
LVGSVVAAVIYKLIFKQSKEDXDTNSYDFFEASGCQSPKGSXGCCHRXITSITPWGLTGLX

NB: *The underlined shows the 6× His tag for detection of the expressed protein.*



The pLL10 vector map.

>AQP 4886_gp, full length (in pBADHisA expression vector)

CCTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCATACCCGTTTTTTGGGCTAACAGGAGGAATTAACCATGG
GGGGTTCT***CATCATCATCATCATCAT***GGTATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGGGATCTGTACG
ACGATGACGATAAGGATCGATGGGGATCCGAGCTCGAGATCTGCAGCTGGTACCATATGG**GAATTCGTACCAT**
GCTATTTGCTGAGCTTGCGGGAACATTCTTACTGGTTATCATTGGTATAGGCAGTTGCACGAGTGGTGCGGACT
GGTCCCCCAGCGTGCCTCAAATAGCATTCACATTTGGTTTAACTGTGGCAACGCTTGCACAGACTATTGGTCATAT
AAGTGGATGTCACATAAATCCTGCTGTTACAGTCGGTTTCTTGATAGTAGGAGAGATGACCTTATTAAAAGCTAT
TTTTTACATAATAGTACAGTGCGTCGGAGCAATGGCTGGATCGGCGGTTTTGAGTCTAGCTATCCCTGACACACTT
GGTGGTAATGGCTTGGGCGTGTCTAACTTTTCCTCGCTCAGTGCCGGACAAGCGGTGTCAATTGAAGCCTTCATT
ACAGCTATCTTAGTGCTGGTAGTGAAAGCAGTGTCAGATTCAAAACGACAAGATATAACGGGATCAGCCCCATT
GGCCGTAGGCCTGGCTATCGCTACGGGTCATCTTTGTGCGATTAAATTAACTGGAGCCAGCATGAACCCTGCGCG
CTCATTCGGACCGGCCGTAGTTCACGATGTTTGGCAAAATCATTGGGTTTATTGGATTGGTCCCCTTGTTGGCAGT
GTGGTAGCTGCCGTTATTTATAAATTAATCTTCAAGCAATCTAAAGAAGATGATGAC**ACGAACTCCTACGATTT**
TTAAAAGCTTGGCTGTTTTGGCGGATGAGAGAAGATTTTCAGCCTGATACAGATTAAAT

NB: *The 6X His (6xCAT) tag is italicized in the above sequence. The primer sequences are in bold and underlined.*

>AQP 4886_gp, truncated sequence (in pBADHisA expression vector)

GCTTTTTATCGCAACTCTCTACTGTTTCTCCATACCCGTTTTTTGGGCTAACAGGAGGAATTAACCATGGGGGGTT
CT***CATCATCATCATCATCAT***GGTATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGGGATCTGTACGACGATG
ACGATAAGGATCGATGGGGATCCGAGCTCGAGATCTGCAGCTGGTACCATATGG**GAATTCGTACCATGGCGGT**
TTTGAGTCTAGCTATCCCTGACACACTTGGTGGTAATGGCTTGGGCGTGTCTAACTTTTCCTCGCTCAGTGCCGG
ACAAGCGGTGTCAATTGAAGCCTTCATTACAGCTATCTTAGTGCTGGTAGTGAAAGCAGTGTCAGATTCAAAACG
ACAAGATATAACGGGATCAGCCCCATTGGCCGTAGGCCTGGCTATCGCTACGGGTCATCTTTGTGCGATTAAATT
AACTGGAGCCAGCATGAACCCTGCGCGCTCATTCGGACCGGCCGTAGTTCACGATGTTTGGCAAAATCATTGGGT
TTATTGGATTGGTCCCCTTGTTGGCAGTGTGGTAGCTGCCGTTATTTATAAATTAATCTTCAAGCAATCTAAAGAA
GATGATGAC**ACGAACTCCTACGATTTTTAAAAGCTT**GGCTGTTTTGGCGGATGAGAGAAGATTTTCAGCCTGAT
ACAGATTAAATCAGAACGCAGAAGCGGTCTGATAAAACAGAATTTGCCTGGCGGCAGTAGCGCGGTGGTCCCAC
CTGACCCCATGCCGAACTCAGAAGTGAAACGCCGTAGCGCCGATGGTAGTGTGGGGTCTCCCCATGCGAGAGTA
GGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTG

NB: *The 6XHis (6xCAT) tag is italicized in the above sequence. The primer sequences are in bold and underlined.*

Appendix 2: Common laboratory reagents

Insect saline for dissection

NaCl (9.1 g/L), KCl (0.52 g/L), $CaCl_2.2H_2O$ (1.2 g/L), and $MgCl_2.6H_2O$ (8 g/L)

T.E. buffer

10 mM Tris-HCl pH 8.0, 0.25 mM EDTA.

1X T.A.E. electrophoresis buffer

Prepare 50X using: 242 g Tris base, 57.1 mL glacial acetic acid, and 100 mL of 0.5 M EDTA (pH 8.0). Then dilute to 1X T.A.E. working solution with distilled water

1X T.B.E. electrophoresis buffer

Tris base (108 g), boric acid (55 g), 0.5 M EDTA (20 mL). Add distilled water to 1.0 L to make 10X T.B.E.

Appendix 3: Reagents preparation for Laemmli SDS-PAGE

Stock reagent preparation;

Acrylamide/Bis (30%)

Weigh 146.0 g of acrylamide and 4.0 g of N'N'-bismethylene-acrylamide. Add distilled water to 500 mL, filter and store at 4 °C in the dark. Maximum shelf life under these conditions is 30 days.

1.5 M Tris-HCl, pH 8.8

Weigh 54.45 g of Tris base and add 150 mL of distilled water. Adjust pH to 8.8 with 1 N HCl. Top up to 300 mL with distilled water and store at 4 °C.

0.5 M Tris-HCl, pH 6.8

Take 6 g of Tris base and add 60 mL of distilled water. Adjust pH to 6.8 with 1 N HCl. Top up to 100 mL with distilled water and store at 4 °C.

10% (w/v) SDS

Dissolve 10 g of SDS in 100 mL of distilled water and stir gently to dissolve.

10% (w/v) Ammonium persulfate

Dissolve 100 mg of ammonium persulfate in 1 mL of distilled water. APS (10%) has to be prepared afresh every time when polyacrylamide gels are casted.

Sample buffer

Add the following to a clean falcon tube; 4 mL distilled water, 1 mL of 0.5 M Tris-HCl (pH 6.8), 0.8 mL of glycerol, 1.6 mL of 10% SDS, 0.4 mL of ß-

mercaptoethanol, and 0.2 mL of 0.05% (w/v) bromophenol blue (in water). Dilute the sample at least 1:4 with sample buffer and boil at 95 °C for 4 min.

5X Electrode (running) buffer (25 mM Tris, 192 mM glycine and 1% SDS, pH 8.3)

Add 45 g Tris base, 216 g Glycine, and 15 g SDS together and add 3 L of distilled water. Store at 4 °C. Warm the buffer to 37 °C before use if precipitation occurs. Dilute 300 mL of 5X stock with 1.2 L of distilled water for one electrophoretic run.

Destain for Coomassie (20% methanol and 10% glacial acetic acid)

To make 1 L, add 100 mL of glacial acetic acid to 700 mL of deionized distilled water (ddH_2O) followed by 200 mL of methanol and mix. Store the destain solution at room temperature in a sealable container.

1X transfer buffer (For Western blot procedure)

To make a litre; dissolve 3 g of Tris-base and 14 g of glycine in 1000 mL of ddH_2O.

10X Phosphate buffered saline (PBS)

Dissolve the following reagents in 800 mL ultrapure water; 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4. Adjust pH of PBS Buffer Solution to 7.4 with HCl and then bring the volume to 1 liter with distilled water. Autoclave or sterilize by filtration.

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