EXPRESSION OF THE *GLOSSINA* PROTEOLYTIC LECTIN (*Gpl*) GENE IN TSETSE FLIES AND OTHER HAEMATOPHAGOUS ARTHROPODS

BY

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DECLARATION

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

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DEDICATION

This work is dedicated to my mother, Jane Burugu, brothers, Peter, James, and Elias, and sisters, Mary, Rachel and Naomy.

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ABSTRACT

African animal trypanosomosis, transmitted by the tsetse fly (*Glossina* spp.) remains a major constraint to food security in areas of Africa that hold the continent's greatest potential for expanded agriculture production. The flies also transmit the parasite responsible for human sleeping sickness. Other haematophagous arthropods like stable flies also transmit trypanosomes mechanically. Mechanical transmission only accounts for a small percentage (~16 %) of the trypanosomes transmission since the haematophagous arthropods do not provide an environment for trypanosome development within their tissues unlike in tsetse flies. The life cycle of the parasitic African trypanosome in its invertebrate vector begins when the tsetse fly feeds from an infected mammalian host. Within the fly, the parasites are transformed from the bloodstream forms to procyclic forms and an infection is established. Many midgut enzymes have been shown to play a role in the establishment phase of the infection. Lectin-trypsin complex is one of the molecules that have been shown to play a role in the transformation of trypanosomes from bloodstream forms to procyclic forms *in vitro*, the first crucial step in the establishment of primary midgut infections in the fly. *Glossina* proteolytic lectin (*Gpl*) gene encodes the α subunit of the lectin-trypsin protein. A study was undertaken to evaluate the expression profile of *Gpl* in *Glossina fuscipes fuscipes* using RT- PCR based techniques. In teneral flies, very low levels of transcript could be detected, but the expression was rapidly induced as early as 1 h post bloodmeal acquisition. Transcript levels decreased slightly after 6 h and stabilized, then increased after 24 h and peaked between 48 and 72 h of starvation. A further decrease of transcript levels was observed. The transcript abundance after 120 h of starvation was higher than in teneral guts. This non-constitutive mode of expression suggests that the gene is regulated at the transcription level. Analysis for the expression of *Gpl* was also carried out in *Anopheles gambiae, Phlebotomus dubosci, Rhipicephallus appendiculatus* and *Stomoxys calcirans* besides the known cyclic vectors of trypanosomes using RT-PCR technique. The evaluation of *Glossina* proteolytic lectin gene in these haematophagous arthropods by PCR revealed that this gene is only expressed in *Glossina* species. The conservation of this gene in *Glossina* species only may be the reason for the cyclic mode of transmission of trypanosomes exhibited by tsetse flies.

CHAPTER ONE

INTRODUCTION

1.1 Background to the study

Tsetse flies are the true vectors of the African trypanosomes that cause nagana in livestock and sleeping sickness in man (Gooding, 1997). Tsetse flies are haematophagous insects of the genus *Glossina* (Diptera: Glossinidae). They infest 36 countries in an area of between 9 to 10 million Km^2 in tropical Africa (Mira and Ralph, 1989). Thirty-one species and subspecies have been described in sub-Sahara Africa (WHO, 2001; Mira and Ralph, 1989), where about 50 million cattle and 10 million small ruminants are at risk of trypanosomosis (FAO, 1994). According to WHO (2002), more than 60 million people, living mainly in rural areas of sub-Sahara Africa are at risk of Human African Trypanosomosis (HAT). Effects on human and livestock health lead to serious economic loss and significantly constrain socio-economic development in Africa (WHO, 2002).

Tsetse flies are the only haematophagous insects reported to transmit trypanosomes cyclically. Other haematophagous arthropods have been implicated in mechanical transmission of trypanosomes (Mira and Ralph, 1989). These include flies of the genus *Tabanus, Stomoxys, Chrysops, Haematopota,*, and blood-sucking bugs of the family *Reduviidae* transmit trypanosomes mechanically (Le Ray and Van Meirvenne, 1985). In addition mechanical transmission

occasionally occurs through placental or blood transfusion (Le Ray and Van Meirvenne, 1985). Since mechanical transmission also contributes to trypanosomes transmission, the presence of *Glossina* proteolytic lectin gene was also evaluated in *Anopheles gambiae, Phlebotomus dubosci, Rhipicephallus appendiculatus* and *Stomoxys calcirans* besides the known cyclic vectors of trypanosomes. . Within the tsetse fly's midgut, trypanosomes differentiate from their mammalian bloodstream forms into procyclic (midgut) forms after undergoing dramatic changes in surface- antigen expression, metabolic development, cellular morphogenesis and cell cycle events (Mathews and Gull, 1998). This differentiation is a crucial first step in the establishment of an infection within the vector (Imbuga *et al.*, 1992a; Maudlin, 1991).

Several factors including the source of food (Nguu *et al.*, 1996; Cross and Manning, 1973); cis-aconitate levels (Brun and Schonenberger, 1981), a shift in temperature from 37- 27° C (Bienen *et al.*, 1981), mild acidic conditions (Rolin *et al.*, 1998) and trypsin concentration (Hunt *et al.*, 1994; Imbuga *et al.*, 1992a) have been shown to induce the differentiation *in vitro.* Following differentiation, there is a massive attrition in the number of procyclic cells in the tsetse fly's gut. Lectins have been suggested to play a role in this attrition process since feeding lectin inhibitory sugars to blood fed flies resulted in increased parasite establishment (Welburn *et al.*, 1989). Attempts at purification of the tsetse fly

lectin yielded a 67 KDa agglutinin (Stiles *et al.*, 1990) and a complex with trypsin- lectin activity of 61000 ± 3000 Da (Osir *et al.*, 1995).

In other blood-sucking insects, similar observations have been made. For instance, in *Aedes aegypti*, midgut trypsin has been shown to activate the *Plasmodium*- encoded enzyme chitinase, which the parasite uses to traverse the peritrophic matrix barrier (Shahabuddin *et al.*, 1996). Since the midgut factors are bloodmeal-induced, the type of host blood influences the infection prevalence at the time of an infective meal (Mihok *et al.*, 1993; Moloo, 1981). Host blood also influences the types and quantities of midgut factors released (Nguu *et al.*, 1996). Therefore, in this study, all experimental arthropods were fed artificially on whole pig blood (domestic) since domestic host blood has been shown to support high infection prevalence of the parasites (Nguu *et al.*, 1996).

Gene expression is a process that begins with the transcription of DNA to an RNA messenger (mRNA), which is then translated to a protein (Harford and Morris, 1997; Watson *et al.*, 1983). Post- transcriptional regulation also occurs by either mRNA metabolism or by post- transcriptional gene regulation. Many methods have been employed for the study of gene expression. Traditional assays for mRNA abundance, such as RNA gel blots or RNase protection assays, require relatively large amounts of RNA, thus ruling them out as the method of choice for low abundance transcript or limited tissue availability. In recent years, a number

of methods have been developed that combine reverse transcription (RT) of mRNA with polymerase chain reaction (PCR) to determine transcript concentrations. These methods avoid the need for large amounts of RNA and offer sensitive non-radioactive detection in small tissue samples (Siebert and Larrick, 1992). Quantitative (or competitive) reverse transcriptase polymerase chain reaction (Q-RT-PCR), while offering the highest accuracy of these methods, requires the construction of an appropriate competitor template that should be included in the RT- reaction at several concentrations (Riedy *et al.*, 1995). Construction of such a competitor is time consuming and the need for several RT-reactions makes the procedure expensive.

In contrast, a semi-quantitative RT-PCR measures transcript level based on the band intensity of amplification products relative to an internal control and does not require the synthesis of a competitor. However, to obtain accurate readings the amplification reaction must be terminated before it reaches a plateau, thus requiring that several control reactions be run before the experiment (Kinoshita *et al.*, 1993). Another semi-quantitative RT-PCR approach is to perform amplification on a dilution series of the cDNA template. The dilution at which the template becomes limiting for PCR can then be used as an indicator of transcript abundance (McDowell *et al.*, 1996). In the absence of an internal control to measure the efficiency of reverse transcription and amplification, it is

necessary to quantify cDNA concentrations of the samples to allow comparative statements to be made.

Recently, the *Glossina* proteolytic lectin (*Gpl*)) which is implicated in the induction of both lysis and transformation of bloodstream trypanosomes to procyclic forms has been cloned and sequenced in *Glossina fuscipes fuscipes* (Abubakar, 2003). Results from this study also showed that the native protein induced transformation of *T. b. brucei in vitro,* which was strongly inhibited by D-glucosamine (Abubakar, 2003).

1.2 Justification

Trypanosomosis is a disease caused by protozoan parasites of the genus *Trypanosoma*, which are mainly transmitted by tsetse fly (*Glossina spp*). Other blood sucking arthropods also transmits the parasites mechanically. The disease is a major impediment to livestock production and economic development in many countries in sub-Saharan Africa, where it is endemic. While the tsetse fly is not the only vector of African trypanosomes, cyclical transmission of infection represents the most important problem because a tsetse fly, once infected, remains infective for a long period, in contrast to the ephemeral nature of non-cyclical transmission by other biting flies (d'leteren and Kimani, 2003). At the same time, trypanosomes infect a wide range of hosts, including wild and domestic animals, which represent reservoirs for the parasite. Apart from this, the enormous

geographical area affected by trypanosomes, the variety of ecosystems, and the limitations of methods currently available for extensive control contribute to the difficulty of containing the disease (Hide *et al.*, 1998). While eradication of trypanosomes remains an unrealistic goal for most of Africa, considerable effort has been invested in control of this disease by the use of trypanocidal drugs, management of the vector and exploitation of the genetic resistance exhibited by indigenous breeds such as N'Dama cattle and Djallonké sheep. An additionally environmentally acceptable method for eradicating tsetse consists of an integrated campaign using insecticide-treated screens followed by massive release of sterile males. Sterile insect technique (STI) involves breeding up of thousands of male Glosina, which are sterilized using radiation and then released at regular intervals, thus swamping the population with males that are unable to fertilize females successfully. *Glossina austeni* has been successfully eradicated from Zanzibar using this technique (Vreysen *et al.*, 2000)

Currently, the use of trypanocidal drugs is well established and represents the most widely adopted approach to control trypanosomosis. Reports show increasing cases of trypanosome resistance to current drugs, both in individual cases and regionally, especially in East and West Africa due to under-dosing (Clausen *et al.*, 1992; d'Ieteren *et al.*, 1997; Rowlands *et al.*, 1993). There appears to be little hope for developing new trypanocidal drugs to benefit

smallholder's farmers in the short term. Given the actual or potential problem of drug resistance in many areas, drug

usage clearly cannot be relied upon continuously as the sole method of trypanosome control. A potential vaccine against trypanosomosis is an attractive option. However, there is little hope that a conventional, anti-infection vaccine will be produced in the near future. Thus further knowledge of the biochemistry of insects midgut genes that have been shown to play a role in transmission and eventually the genetic engineering of these genes may provide the means to improve disease control, for example by blocking transmission through genetic manipulation of the vector. Analysis of insect midgut genes in other biting flies that are implicated in mechanical transmission of trypanosomes will enhance our understanding of the transmission process of trypanosomes by these vectors.

1.3 Null Hypotheses

1) *Glossina* proteolytic lectin gene abundance does not vary with time post-bloodmeal in tsetse fly's midgut.
2) Other haematophagous arthropods do not express the *Glossina* proteolytic lectin gene in their midguts.

1.4 Objectives

1.4.1 General objective

To study the expression of the *Glossina* proteolytic lectin (*Gpl*) gene in tsetse flies and other haematophagous arthropods

1.4.2. Specific objectives

1. To determine the expression levels of the *Gpl* gene in tsetse flies at different intervals post-bloodmeal.
2. To identify the *Gpl* gene in other haematophagous arthropods post-bloodmeal.

CHAPTER TWO

LITERATURE REVIEW

2.1 Trypanosome biology

Trypanosomes are microscopic, elongated unicellular parasites which move with the help of a single flagellum. They are obligatory parasites, which multiply in the body tissues (especially blood) of the vertebrate host (Mira and Ralph, 1989). They evade elimination by the host immune system by undergoing antigenic variation. This process is accomplished by the expression of genes encoding antigenically variable surface glycoprotein (VSGs) (Schopf and Mansfield, 1998; Mansfield, 1995; Kamper and Barbet, 1992).

Taxonomically, trypanosomes species infecting animals belong to the order trypanosomatida (kinetoplastida), family trypanosomatidae and genus *Trypanosoma* (Service, 2001; Mira and Ralph, 1989; Hoare, 1972). Genus *Trypanosoma* consist of dixenous parasites and is divided into 2 major sections: stercorarian and salivarian. The stercorarian trypanosomes develop in the midgut and hindgut of the vector (posterior station development), and the metacyclic trypanosomes are usually passed in the vector through faecal contamination (Service, 2001; Mira and Ralph, 1989). Stercoraria consists of 3 subgenera- *Megatrypanum, Herpetosoma* and *Schizotrypanum.* None of this is of particular importance as an animal pathogen apart from *Schizotrypanum,* which contains the

zoonotic *Trypanosoma cruzi*, the cause of chaggas disease in central and South America (Service, 2001; Mira and Ralph, 1989).

The salivarian trypanosomes are transmitted via the mouthparts of the vector (anterior station development), involving either proboscis (subgenera *Dutonella* and *Nannomonas*) or salivary glands (subgenera *Trypanozoon* and *Pycnomonas*) (Service, 2001). These subgenera include species causing severe disease in animals. Haematophagous insects transmit them by both cyclical and non-cyclical means. Those that undergo cyclical development are transmitted by tsetse flies while mechanical transmission is by a range of flies, including tabanid and stable flies (Hide *et al.*, 1997). Tsetse transmitted trypanosomes usually infest a range of animal species, but cattle and horses are usually more severely affected than small ruminants such as sheep and goats (Service, 2001). However, pigs can be severely affected by *T. simiae* and *T. vivax* (Hoare, 1972). Exotic breeds of livestock are often more severely affected than indigenous breeds, which frequently show a degree of resistance to the effects of infection (trypanotolelance) (Trail *et al.*, 1991; Taylor and Mertens, 1999). Animals are often infected with more than one species of trypanosome (Service, 2001). Human African trypanosomosis is caused by two different *Trypanosoma* species: *Trypanosoma brucei gambiense* found in Central and West Africa which causes a chronic infection and *T.b. rhodesiense* which cause acute infection in Southern

and Eastern Africa. African Animal Trypanosomosis (AAT) is caused by T. *vivax, T. uniforme, T. congolense, T. simiae, T. brucei* and *T. suis* (WHO, 2001). Domestic and wild animals are a major reservoir of *T.b.rhodesiense. Trypanosoma vivax* affects cattle, sheep and goats and is often found beyond the belts in Central and South America where it is transmitted mechanically by biting flies or contaminated needles, syringes and surgical instruments (WHO, 2001).

2.2 Tsetse fly vectors

Tsetse flies are haematophagous insects of the genus *Glossina* (Diptera: Glossinidae). Based on their morphology, ecology and behaviour, tsetse flies can be divided into 3 main groups of species: The *G. fusca* group, which is restricted to equatorial forests. They contain a few species that transmit nagana to livestock, but no important vectors of sleeping sickness. The *Glossina morsitans* group or savannah flies contain important vectors of both sleeping sickness and nagana such as *G. morsitans* and *G. pallidipes*. The *Glossina palpalis* group or riverine and forest fly contains important vectors of sleeping sickness and nagana like *G. palpalis* and *G. tachinoides* (Service, 2001; Mira and Ralph, 1989).

2.3 Life cycle and transmission of African trypanosomes

The life cycle of the parasitic African trypanosomes (Euglenozoa: Kinetoplastida) in their insect vector, the tsetse fly (Diptera: Glossinidae), begins when the fly feeds from an infected mammalian host (Hao *et al.,* 2001). For successful

transmission, the parasite undergoes two stages of differentiation in the fly: firstly, establishment in midgut and followed by maturation in the mouthparts or salivary glands (Yan *et al.*, 2001). In the fly's midgut (gut lumen), the parasite transforms into procyclic trypomastigotes, multiplies by binary fission, loses their variant surface glycoprotein and expresses a new coat composed of procyclic protein (Ruepp *et al.*, 1997). The procyclics migrate to the salivary glands and transform into epimastigotes. This migration is through the proventriculus, where the peritrophic membrane is soft and allows the parasites to penetrate. This is the establishment phase of the infection in the tsetse fly. The subsequent maturation phase occurs in the salivary gland for the subgenus *Trypanozoon (T. brucei*). Here, they first differentiate into attached proliferating epimastigote forms, which then yield the infective, free living metacyclic forms that are transmitted to the next host during subsequent feed (Vickerman *et al.*, 1988). In the case of *T. vivax,* the life cycle is restricted within the fly's mouthparts.

In the midgut, the parasite encounters a physiologically hostile environment that includes various digestive enzymes (Cheeseman and Gooding, 1985), lectins (Welburn *et al.*, 1989), agglutinins and trypanolytic proteins (Stiles *et al.*, 1990) and possibly other yet unknown factors. Most of the parasites are killed by one or more of these factors and survivors are those that transform into procyclic midgut forms (Stiles *et al.*, 1990). The successful establishment of the parasite is dependent on its transformation from bloodstream to procyclic forms. This

involves complex morphological, biochemical and physiological changes that enable the parasite to adapt to radically different environment within the fly's midgut (Englund *et al.*, 1982). These changes include loss of surface coat, cessation of synthesis of the variant surface glycoprotein, activation of mitochondrial enzymes, induction of procyclin synthesis and displacement of the position of the nucleus and kinetoplast (Roditi and Pearson, 1990).

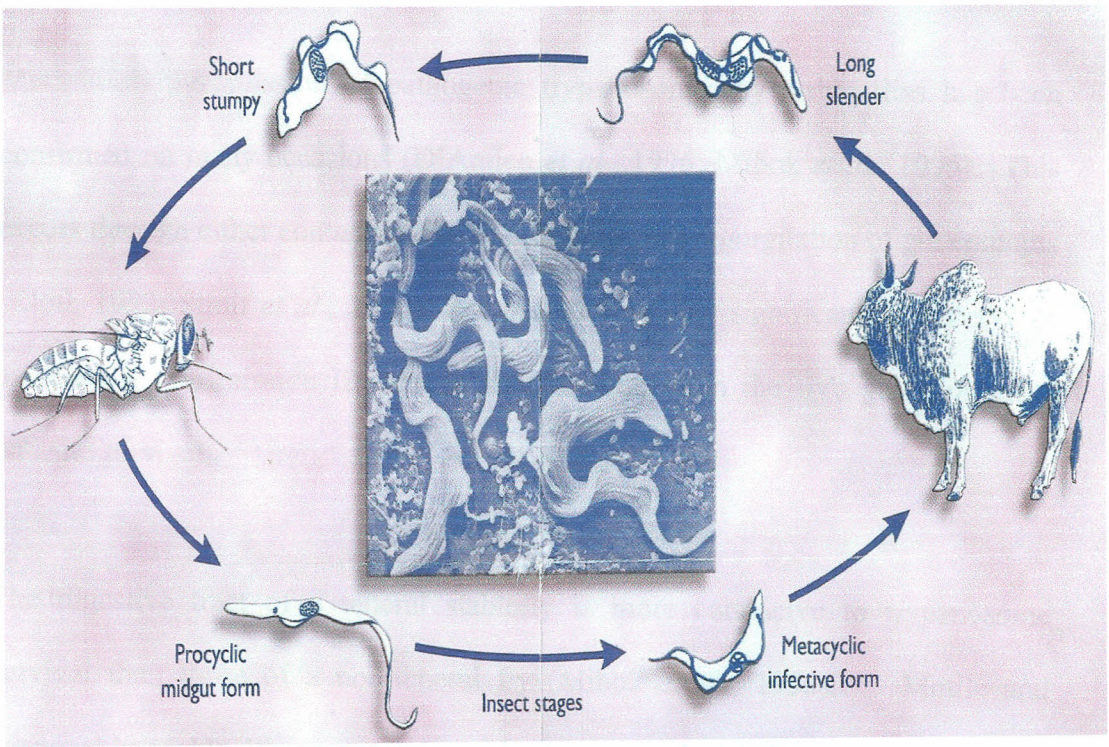

Fig. 1: The life cycle of African trypanosomes. (Adopted from Hursey, 2001).

2.4 Mechanical transmission of trypanosomes

Tsetse flies and other blood sucking arthropods through the transfer of blood from one animal to another also mechanically transmit Trypanosomosis. The most important mechanical vectors are flies of the genus *Tabanus*, but *Haematopota, Liperosia, Stomoxys* and *Chrysops* flies have also been implicated. In Africa, both *T. vivax* and *T. b. brucei* have spread beyond the "tsetse fly belts" where transmission is principally by tabanid and hippoboscid flies (Roder *et al.*, 1984).

Mechanical transmission of pathogenic trypanosomes by stable flies has been confirmed on many occasions (D`Amico *et al.*, 1996; Mihok *et al.*, 1995). This occurs through either contamination of mouthparts or regurgitation of gut contents (Kloft, 1992; Straif *et al.*, 1990). Regurgitation is only significant as a model of mechanical transmission if there is an environment in the fly's gut supporting parasite survival.

The digestive tract of a teneral stablefly is more conducive to trypanosome survival than those of a non-teneral fly (Mihok *et al.*, 1995). Motile and presumably viable trypanosomes remain in or on the proboscis for about 5-7 minutes after feeding (Mihok *et al.*, 1995). Large numbers of parasites survive only for the first 2 hrs, hence facilitating mechanical transmission by regurgitation (Schneider *et al.*, 1987). This suggests that stable fly midgut, regardless of species or prior feeding history is generally hostile to trypanosomes and therefore,

does not provide the sort of permissive environment observed in teneral or non-teneral tsetse flies (Welburn and Maudlin, 1992).

Most biting flies feed on both sugar and blood with direct transfer to the midgut during feeding (Friend, 1991). Previous meal of nectar, honeydew or plant sap may, however, influence both the processing of fluids and the ultimate survival of parasites for example in sandflies (Schlein and Jacobson, 1994). *Stomoxys* have been reported to transmit *T. congolense* and *T. evansi* mechanically (16 % by *S. taeniatus* and 25 % by *S. niger niger*) (Sumba *et al.*, 1998). The digestive tract of *S. calcitrans* contains many proteases and lectins, all presumably inimical to trypanosomes (Schneider *et al.*, 1987). These compounds are induced following a bloodmeal and return to low levels during starvation, hence providing scope for variation in levels of factors that have adverse effects towards trypanosomes in the midgut similar to tsetse flies. There is a great relationship in the role played by digestive enzymes produced in the midgut of most bloodsucking arthropods. For instance, lectins secreted in *Glossina* midgut prevents the establishment of midgut infections while in sandfly's gut, they prevent *Leishmania* establishments (Wallbanks *et al.*, 1986).

2.5 Tsetse control measures

Trypanosomes are transmitted cyclically by the tsetse fly, of which there are some 36 species and subspecies, each adapted to different climatic and ecological conditions (Ford, 1971). Considerable effort has been invested in control of this disease by the use of trypanocidal drugs, management of the vector and exploitation of the genetic resistance exhibited by indigenous breeds such as N'Dama cattle and Djallonké sheep (d'leteren and Kimani, 2003; Curtis and Davis, 2001; Dyuck *et al.*, 2000). The use of trypanocidal drugs is well established and represents the most widely adopted approach to control trypanosomes. However, there is increasing cases of trypanosome resistance to current drugs, both in individual cases and regionally, especially in East and West Africa (Clausen *et al.*, 1992; d'Ieteren *et al.*, 1997; Rowlands *et al.*, 1993). There appears to be little hope for developing new trypanocidal drugs to benefit smallholder farmers in the short term. Given the actual or potential problem of drug resistance in many areas, drug usage clearly cannot be relied upon continuously as the sole method of trypanosome control.

The main method currently employed to control tsetse flies is the use of synthetic pyrethroid insecticides to impregnate traps and screens, sometimes additionally baited with odour attractants (Brightwell *et al.*, 1991). More recently, live animals that have been impregnated through spraying, dipping or by pour-on treatments have been used as live targets (Bauer *et al.*, 1992). One of the major

drawbacks to the sustainability of these methods is that they require the active participation of the majority of communities contributing to a relevant production system in a given environment or region, in order to be successful in the long term. Thus, they require major economic incentives to be accepted by farmers for collective action.

A potential vaccine against trypanosomes is an attractive option. However, there is little hope that a conventional, anti-infection vaccine will be produced in the near future due to the extensive antigenic variation phenomena displayed by trypanosomes in the mammalian host (Li *et al.*, 2001). Transgenesis, a recombinant DNA technique that aims to modulate vector competence of insects vectors by introducing and expressing foreign genes with anti-pathogenic properties that then interferes with the parasites viability, development or transmission has been proposed (Beaty, 2000). The eventual goal of this approach is to replace the naturally susceptible population with their engineered refractory counterparts in the field. However there are no proven mechanism yet, aimed at achieving this.

Trypanotolerance, the ability of some livestock species and breeds to survive, reproduce and remain productive under trypanosome risk without the aid of trypanocidal drugs, was recognized and exploited by farmers long before research on trypanotolerance began. The exploitation of trypanotolerant breeds is

practiced as a major (if not the only) option for sustainable livestock production in 19 countries in the most humid parts of West and Central Africa (d'leteren *et al.*, 1998). In 11 countries, trypanotolerant cattle (mainly N'Dama) were either moved into the highest risk areas or were imported from other countries. While significant differences in resistance to trypanosomes also occur among various zebu (*Bos indicus*) types (Ismael and Njogu, 1985; Njogu *et al.,* 1985; Dolan *et al.*, 1994; Mwangi *et al.*, 1994), most *Bos indicus* cattle in tsetse fly-infested areas still require regular treatment. However, there is a continued perception that because of their small size, trypanotolerant livestock are less productive than other breeds (Holmes, 1997).

2.6 Tsetse- trypanosome interactions

Tsetse flies are in general refractory to parasite transmission. The refractoriness of most flies in a population is a reflection of the ability of their immune system to remove invading trypanosomes from their guts by secreting a specific lectin among other factors that binds to procyclic trypanosomes resulting in lysis and cell death (Maudlin, 1991). Refractoriness is mediated by midgut lectins that induce procyclic tyrpanosome death (Maudlin, 1991). However, some tsetse flies are susceptible to trypanosome infections. This susceptibility to midgut infection is a maternally inherited character in tsetse and lines of flies refractory and susceptible to infection can be selected (Moloo *et al.,* 1998; Maudlin, 1982).

Maternally inherited susceptibility is as a result of inherited bacterial endosymbiont, *Sodalis glossinidius* (ricketttesia-like organisms), which produces chitinase that degrades chitin to produce lectin inhibitory sugars (Dale and Maudlin, 1999; Maudlin, 1991). Maternally inherited susceptibility has also been shown to be responsible for Variation in susceptibility between teneral and non-teneral flies (Woolhouse and Hargrove, 1998). This susceptibility is promoted by endochitinase activity of a midgut bacterium which, degrades chitin during pupation resulting in a build up of inhibitory oligosaccharides, which inhibits the midgut lectin, and predispose teneral flies to midgut infection (Maudlin and Welburn, 1987; Welburn *et al.*, 1993). The high levels of lectin activity in non-teneral flies were not inhibited sufficiently by normal symbiont activity, resulting in refractoriness (Welburn *et al.*, 1994). A similar lectin-based system appear to operate in other vector-parasite systems for example in sandfly vector of *Leishmania major*, specific inhibitory sugars increase infection rates (Volf *et al.*, 1998).

Different *Glossina* species have been reported to differ in their susceptibilities to *Trypanozoon* and *Nannomonas* infections. The *Morsitans* group is more susceptible compared to the *Palpalis* group (Reifenberg *et al.*, 1997; Moloo *et al.*, 1998; Moloo and Kutuza, 1988a). Mixed infections have been reported to be more prevalent within the susceptible *Morsitans* group. This variation may be as

a result of a second midgut lectin molecule in *G .p. palpalis* and *G. p. pallidipes*, which is not detectable in *G. m. morsitans* (Welburn *et al.*, 1994).

2.7 Peritrophic membrane and vector competence

Peritophic membrane (pm) lines the gut of most insects at some stage in their development. It is composed of a cellular material containing chitin, integrate Proteins (peritophins) and proteoglycans. Information on the function, formation and constituents of the pm is scarce, but multiple roles have been proposed ranging from partitioning of the digestive enzymes and food, digestion, protection of the gut epithelial cells from viral and parasitic invasion (Casu *et al.*, 1997; Eismann *et al.,* 1994). Peritrophic membrane is categorized in to 2 classes, based on the their mode of synthesis. Many adult dipteran insects have a type 1 pm whose components are secreted by the entire midgut digestive epithelium in response to a bloodmeal (Peters, 1992). This type of pm surrounds the food bolus and is degraded by the chitinase enzyme produced by the gut cells at the end of digestion period (Peters, 1992). Tsetse adults however have a type 11 pm, which is typically present in the gut of the larval developmental stages of the diptera (Peters, 1992).

Type 11 pm is constitutively synthesized by cells in the proventriculus in the foregut prior to the bloodmeal and forms a sleeve like amorphous structure throughout the gut. Based on electron microscopy analysis, tsetse has a single pm

composed of 3 chitinous layers of about 30nm in thickness, and based on biochemical studies, it is interpread with glycosaminoglycans and glycoproteins (Lehane *et al.*, 1996; Lehane and Msangi, 1991). The pm is thought tom play a central role in the vector competence of disease vectors and particularly malaria. The malaria parasites find itself enveloped in a chitinous sac (type 1 pm), produced by the mosquito midgut in response to midgut distensions, and find s its way out of this sac by secreting a chitinase to digest its way through the pm (Billingsley and Sinden, 1997). In tsetse, proventriculus and pm are vital components of the gut. During the course of infection, trypanosomes must at some stage cross the pm barrier eventually to pass into the anterior gut to continue with their normal development cycle. Trypanosomes bypass the pm by escaping through the hindgut where the pm is disrupted, before migrating and multiplying in the ectoperitrophic space. Salivarian trypanosomes have been reported to be capable of active penetration of the pm and gut epithelial cells in the central two-thirds of the tsetse midgets (Evan and Ellis, 1983). This suggests that a fully formed pm may not necessarily be a potential barrier to trypanosome penetration (Welburn and Maudlin, 1999).

2.8 Transformation of trypanosomes

Transformation of bloodstream trypanosomes into procyclic forms within the midgut of tsetse fly is a crucial first step in the establishment of an infection (Maudlin, 1991; Roditi and Pearson, 1990). A number of factors induce this

transformation. The best characterised factors include trypanoagglutinins (lectins), trypsins, trypsin-like enzymes (Imbuga *et al.*, 1992 a,b; Maudlin, 1991) and host blood (Nguu *et al.*, 1996 ; Mihok *et al.*, 1993). Most recent work has provided evidence of the involvement of a lectin-trypsin molecule in trypanosome differentiation and lysis (Abubakar *et al.*, 2003). Since transformation is a key factor in the establishment of trypanosome infections, additional information on tsetse fly gut genes whose proteins play a role in transformation process, and their expression upon blood feeding will enhance understanding of the interactive biology of tsetse flies and trypanosomes.

2.9 Tsetse fly midgut enzymes

Insect gut enzymes are involved in digestion of dietary proteins. Additionally, they are implicated in the process of pathogen establishment in several insects including tsetse flies (Yan *et al.*, 2001). The midgut digestive enzymes are secreted in response to increased protein contents in the gut following a bloodmeal (Gooding, 1974). Six proteolytic enzymes have been biochemically characterized in tsetse flies: trypsin, carboxypeptidase A, carboxypeptidase B, aminopeptidase, a trypsin – like enzyme and a chymotrypsin-like enzyme (Cheeseman and Gooding, 1985).

The regulation of expression for digestive enzymes varies widely in different insects. In the mosquito *A. aegypti*, the synthesis of early trypsin appears to be regulated at the translational level since, early trypsin mRNA can be detected in unfed fly guts while no activity is found in gut cells prior to the bloodmeal (Noriega *et al.*, 1996). Late trypsin mRNA and protein is detectable after 8 hours and reaches its maximum level after about 24 hours (Noriega *et al.*, 1994; Barillas-Mury *et al.*, 1991). In *Stomoxys*, regulation of digestive enzymes is at the post- transcriptional stage with protein levels reaching a maximum shortly after obtaining a bloodmeal (8-10hrs) (Mofatt *et al.*, 1995).

Serine proteases have been shown to be involved in bloodmeal digestion in haematophagous insects. In *G. m. morsitans* two bloodmeal - induced serine proteases have been studied: serine protease 1 and 2. Their trypsin and chymotrypsin activitity levels change extensively during the digestive cycles. The protease activity in gut cells accumulates and increases to maximal levels about 24 hrs following a bloodmeal. This suggests their regulation at the translational level due to high levels of mRNA corresponding to these gene products before the bloodmeal, with only low level of corresponding protein activity (Yan *et al.*, 2001). In this regard, it appears that an optimal enzyme concentration is crucial for efficient transformation of the trypanosomes since high trypsin concentration resulted in complete lysis of the parasite (Imbuga *et al.*, 1992 a).

2.9.1 Midgut lectins

Lectins belong to a class of proteins of non-immune origin that bind carbohydrates specifically and covalently (Sharon and Lis, 1989; Liener *et al.*, 1986). They are not confined to plants as originally believed, but are ubiquitous in nature, being frequently found on cell surface and intracellular particles (Sharon and Lis, 1989). In insects and other invertebrates, lectins are believed to be involved in defence mechanisms that contribute to both cell-mediated and humoral immunity (Ingram and Molyneux, 1990; Natori, 1990).

Lectins secreted in the *Glossina* midgut prevent the establishment of midgut infections and induce maturation of established midgut trypanosomes. Inhibition of tsetse fly's midgut lectins by glucosamine and N-acetyl-glucosamine increased the midgut infection rates of *T. congolense* and *T. brucei* but blocked subsequent maturation of infections and anterior migration of trypanosomes (Maudlin and Welburn, 1994). The addition of glucosamine to the bloodmeal significantly increased midgut infection rates with *Trypanosoma spp.* (Maudlin and Welburn, 1994; Mihok *et al.*, 1992). Sandfly's gut lectin controls susceptibility to *Leishmania* infections (Wallbanks *et al.*, 1986). Galactosamine specifically inhibits sandfly midgut lectin activity *in vitro* (Volf *et al.*, 1998). This inhibition prevented the normal decrease of parasite loads in control flies on day 6 post-infection (Volf *et al.*, 1998). In *A. aegypti* the addition of N-acetyl-glucosamine

increased the number of *Brugia pahangi* microfilariae successfully migrating through the midgut wall (Ham *et al.*, 1991).

2.9.2 Lectin-trypsin molecule

Lectin-trypsin molecule is a bloodmeal induced molecule with both lectin and trypsin activities. Using a two-step ion exchange column chromatography procedure, a bloodmeal-induced agglutinnin with proteolytic activity has been purified from *Glossina longipennis* midgut extracts (Osir *et al.*, 1995). This molecule, aptly called the lectin-trypsin complex, was shown to be a glycoprotein with a native apparent molecular weight (M_r) of ≈ 61000 ± 3000 Da (Abubakar, 1995). It consists of two non-covalently linked subunits: α subunit (M_r ≈27000 Da) having tryptic activity and a β subunit (M_r ≈ 33 000 Da) with glycosyl residues (Osir *et al.*, 1995). The native molecule was capable of agglutinating both bloodstream form and procyclic trypanosomes as well as rabbit blood cells. This activity was specifically inhibited by D (+) glucosamine (Osir *et al.*, 1995). The native molecule also induced transformation of *T. brucei brucei, in vitro*. Recently, a similar molecule from the midguts of *G. f. fuscipes* has been isolated (Abubakar *et al.*, 2003). The molecule (native M_r ~ 65700) has 2 non-covalently linked subunits, M_r ~ 28800 and M_r ~ 35700. The native molecule was found to be capable of inducing differentiation of bloodstream-form trypanosomes into procyclic forms *in vitro* (Abubakar *et al.*, 2003). This induction was specifically inhibited by D-glucosamine.

A *Glossina fuscipes fuscipes* cDNA midgut expression library has been constructed and a putative proteolytic lectin gene identified (Abubakar, 2003; Abubakar *et al.,* 2003). The cDNA encoded a putative mature polypeptide with 274 amino acid designated *Glossina* proteolytic lectin 1 (*Gpl*1). The deduced amino acids sequence includes a hydrophobic signal peptide and a highly conserved N-terminal present with the conserved residue surrounding it and 3 pairs of cysteine residues for disulphide bridges. Expression of this gene in bacterial expression system yielded a protein with a molecular mass of ≈32500 Da. The recombinant protein bound D (+) glucosamine and caused agglutination of bloodstream form trypanosomes and rabbit red blood cells. In addition, the protein was capable of inducing transformation of bloodstream-form trypanosomes into procyclic forms *in vitro* (Abubakar, 2003). Since this gene has a key role in transformation of trypanosomes, studies on its expression levels at different time interval post feeding and its expression in other blood sucking arthropods would enhance further understanding of the regulation of this gene within the vector.

3.0 Gene expression

Gene expression covers the entire process from transcription through protein synthesis. The final measure of whether or not a gene is expressed is if the protein is produced, since it is the proteins that ultimately carry out the function

specified by the gene. For gene expression, prokaryotes require one kind of RNA polymerase in addition to 3 regulatory elements: promoter sequences, termination sequences and operator sequences. For eukaryotes, they require 3 RNA polymerises: RNA polymerase 1 that synthesises ribosomal RNA and needs accessory factors to initiate transcription. RNA polymerase 1 is species specific. RNA polymerase 11 which syntesises pre-messenger RNA's which requires accessory proteins, and it is not species specific and RNA polymerase 111 which is responsible for synthesis of tRNA, 5SRNA and small nuclear RNA (Orphanides and Reinberg, 2002).

RNA polymerase 1 and 111 have specific termination signal on the DNA which directs the RNA polymerase when to stop the transcription process and release the RNA. RNA polymerase 11 has no specific termination sequence. In this case, RNA polymerase continues until it 'falls off' the precursor RNA is terminated at a specific site to produce the final pre-mRNA product. The process of RNA synthesis in the nucleus and its transportation to the cytoplasm for protein synthesis is regulated by a regulating system (Orphanides and Reinberg, 2002).

3.1. Eukaryotic gene regulation

At the genomic level, majority of regulation encompasses modification of the chromatin structure of and around target gene. These modifications occur at the level of chromosome, nucleosomal array, individual nucleosides and the histones.

These modifications are orchestrated by the transcription factors and executed by transcriptional coregulators (An *et al.*, 2002). Transcriptional; level involves the mRNA, rRNA or tRNA synthesis. This is carried out by 3 distinct RNA polymerases: RNA polymerase 1,11 and 111. This regulation is governed by the action of transcription factors (Lee and Young, 2000). Post-transcriptional regulation (RNA processing) involves additional of a methylated guanosine cap (7-methylguanosine cap) to the 5' of mRNA, cleaving of the pre-mRNA about 15 nucleotides downstream from the recognition site and addition of adenosines to the 3' end by poly (A) polymerase (Lee and Young, 2000. Translational level control depends on mRNA localization, mRNA translation and stability. The information that governs the cytoplasmic localization of mRNA is located in the 3'untranslated region (UTR). This mechanism of RNA localization is thought to be mediated by proteins that recognise localization sequences in the mRNA (Muchard *et al.*, 2002). Regulation of gene expression at the translational level is achieved by masking RNA so that it cannot be translated, phosphorylating eLF2 and preventing translation or by specific repressors that may bind to the 5'UTR or the mRNA. Control at the RNA stability is achieved by regulating the survival of mRNA through degradation of mRNA by the poly (A) nuclease. Post-transational control includes modification of protein: proteolytic cleavage, phosphorylation and glycosylation (Orphanides and Reinberg, 2002).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Experimental arthropods

All experimental arthropods used in the experiments were 24 h post emergence except *Stomoxys calcitrans* and *Anopheles gambiae* that were field collected and 5 day old respectively.

3.1.1. Tsetse flies

Glossina fuscipes fuscipes used in the study were obtained from ICIPE Animal Rearing and Quarantine Unit (ARQU). They were maintained at 24-27° C with 55 % humidity and fed once on pig blood using an artificial membrane system (Sonnstrahl, Handelsges, Wein, Goldschlagstr). They were sampled at 1, 6, 12, 24, 36, 48, 72, 96 and 120-h post feeding.

3.1.2. Mosquitoes

Female *Anopheles gambiae s.s* (Ifakara strain) was obtained from ARQU colony mosquitoes. They were maintained at 28° C, 80 % Relative Humidity (RH) 12: 12 darkness/ light photoperiods. Five-day-old female mosquitoes used in the experiments were fed once on mice blood and starved for 72 h before sampling.

3.1.3. Sand flies

Female *Phlebotomus duboscqi* used in the experiment were obtained form the KEMRI colony of sandflies. They were maintained at 25° C and 80 % relative humidity. They were fed on anaesthetized hamster and starved for 72 h before sampling.

3.1.4. Ticks

Rhipicephalus appendiculatus used in the experiment were obtained from ARQU, ICIPE colony ticks. They were maintained at 28 ^{0} C and 85 % relative humidity. They were fed once on blood from restrained rabbit ears and starved for 72 h before sampling.

3.1.5. Stable flies

Stomoxys calcitrans were obtained from Marurui animal farm (Nairobi). They were fed once on pig blood soaked in sterile cotton wool and starved for 72 h before sampling.

3.2. Sample preparation

Midguts of all experimental arthropods were carefully dissected, dipped several times in RNase free phosphate-buffered saline (PBS; 0.1 M sodium phosphate (pH 8.0) containing 0.15 M sodium chloride) to remove any haemolymph and then stored at -70° C in 1.5 ml eppendorf tubes until RNA extraction.

3.3. Total RNA extraction

Midguts preserved at -70° C were used for total RNA isolation using RNAgents® Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer instruction. Briefly, denaturing solution was added to the tissue and subsequently disrupted using a polypropylene pestle for 10 min until no fragment tissue cells were visible. Two molar sodium acetate (pH 4.0) was added and mixed by inverting the tube 4 -5 times. Phenol: chloroform: isoamyl alcohol was then added and mixed by inversion 3-5 times, shaken vigorously for 10 sec and chilled on ice for 15 min. The mixture was centrifuged (10,000 rpm, 20 min, 4° C) in a bench top microfuge. The top aqueous phase containing RNA was carefully transferred into a fresh sterile microcentrifuge tube. An equal volume of isopropanol was added to the aqueous phase to precipitate and re-suspend RNA. The sample was incubated for 30 min at -70° C. Pelleting of RNA was carried out by centrifugation (10,000 rpm, 10 min, 4° C). The pellet containing the RNA was re-suspended in denaturing solution and vortexed until RNA dissolved. The RNA was then precipitated using isopropanol as described above and pelleted by centrifugation. The RNA was washed once using ice-cold nuclease free 75 % ethanol. The RNA was dissolved in nuclease free water. The integrity of the isolated RNA was assessed using an RNA denaturing gel. The RNA sample was separated by electrophoresis on a 1.4 % sodium phosphate/agarose gel and visualized by EtBr staining alongside the RNA marker.

3.4. Complementary DNA (cDNA) synthesis

The RNA was reverse-transcribed using SMART™ PCR cDNA synthesis kit (Clontech Laboratories, Inc, USA) according to the manufacturers instructions. Briefly, first strand oligo (dT) - Primed cDNA was synthesized using 3 µl of the RNA, 1 µl SMART IV oligonucleotide (10 µm) and 1 µl CDS 111 (10 µM) in a sterile 0.5 ml microcentrifuge tube. The tube contents were mixed, centrifuged briefly in a microcentrifuge and incubated at 72° C for 2 min. This was followed by a snap cooling on ice for 2 min. The tube contents were centrifuged briefly and 2 µl of 5x first strand buffer, 1 µl DTT (20 mM), 1.0 µl dNTP mix (10 mM) and 1. 0 µl Powerscript Reverse Transcriptase were added to make a total of 10 µl. The tube content were re-centrifuged briefly, overlaid with a drop of mineral oil and incubated at 42° C for 1 hr. Cooling the tube contents on ice terminated first strand synthesis.

3.5. Reverse transcriptase Polymerase Chain Reaction (RT-PCR).

Complementary DNA products (2µl) were amplified with 1 unit of Taq polymerase (recombinant, MBI Fermentas), 2 mM dNTPs, 2 mM $MgCl_2$ together with 0.4 µg of *Glossina* protease lectin (*GPl*) specific primer set (*Gpl* F:$^{5'}$ TTT GGA TCC ATG AAG TTT GCA GTG TTC GC $^{3'}$ and R:$^{5'}$ CGG TAG TAA GCT TAC AAA AGT TGC GCA TAG $^{3'}$). The PCR amplification conditions were 94° C for 2 min, 39 cycles of 1 min at 94° C, 1 min at 50° C and 1 min at 72° C in a MJ Research PTC-100 thermocycler. The amplification products were

analyzed by electrophoresis on 1% agarose gels and visualized by EtBr staining. The gels were photographed using gel documentation system (Biosystematica, UK).

3.6. Determination of expression levels by semi -quantitative RT-PCR

The expression levels of *Gpl* gene in *G. f. fuscipes* was determined by semi-quantitative RT-PCR method as described by Manore *et al.*, (2001) with slight modifications. For each primer set, preliminary PCR amplifications were carried out to determine the number of cycles necessary to obtain visualisable PCR products while avoiding saturation. Two microlitre of the cDNA products were amplified with 0.15 µg of *Gpl* specific primer, 0.4 µg of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) primer, 2.5 mM dNTP, 1 unit of Taq polymerase and 2mM $MgCl_2$ in a total volume of 25 µl. The amplification conditions were 94° C for 2min followed by 33 cycles of 94° C for 1 min, 50° C for 1 min, 72° C for 1 min and a final extension at 72° C for 5 min. The products were separated by electrophoresis on 1.5 % agarose gels and visualized by EtBr staining. The gels were photographed using gel documentation system (Biosystematica, UK).

3.7. Determination of expression levels by multiplex titration RT - PCR.

The expression levels of *Gpl* gene in *G. f. fuscipes* was also assessed using multiplex titration RT-PCR as described by Nebenfuhr and Lomax, (1998) with slight modifications. For each primer set, preliminary PCR amplifications were carried out under standard conditions to determine the number of cycles necessary to obtain visualizable PCR products while avoiding saturation. The PCR program used was as described for semi- quantitative RT-PCR. A 1:2 15-fold dilution was carried out on the original concentration of cDNA templates. Multiplex titration RT-PCR was performed with 2 µl of the cDNA in a total volume of 25 µl in the presence of 2.5-mM dNTPs, 1 units of Taq polymerase, 2 mM $MgCl_2$, 0.15 µg of *Gpl* primers including GAPDH primers as the internal control. Twelve µl of the PCR products were separated by electrophoresis on 1.5% agarose gel in TAE buffer. The gels were visualized and bands analysed on a UV transilluminator and dilution step of the last visible band noted. The gels were photographed using a gel documentation system (Biosystematica, UK).

3.8. Screening for the presence of *Glossina* proteolytic gene in other haematophagous arthropods.

Screening was carried out following the method of Pytela *et al.*, (1994) with slight modifications. The cDNAs were diluted (1:5) and subjected to PCR amplification with *Gpl* specific primers. The PCR amplification conditions were 94 ^{o} C for 2 min, 39 cycles of 1 min at 94 ^{o}C, 1 min at 50 ^{o}C and 1 min at 72 ^{o}C in a MJ

Research PTC - 100 thermocycler. The amplification products were analyzed by electrophoresis on a 1% agarose gel and visualized by EtBr staining. The gels were photographed using a gel documentation system (Biosystematica, UK).

3.9 Data analysis

Differences in number of bands visible under UV as a function of time for each sampling time in multiplex titration RT-PCR were analyzed by analysis of variance (ANOVA) using GLM procedure of SAS for PC (SAS, 1999). Student Neuman- Keuls separated means at different sampling times at $p < 0.05$.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Reverse transcription PCR was carried out using cDNA synthesized from the midguts of *G .f. fuscipes* sampled at different time intervals post- bloodmeal to ascertain the expected fragment sizes while using Gpl specific and GAPDH primer sets. Polymerase chain reaction data revealed that the sizes were 900 bp for cDNA amplified using Gpl specific primers (Fig.2) and 400 bp for cDNA amplified using GAPDH primers (Fig. 3). Using the Gpl specific primers, faint bands of teneral gut cDNA and for those starved for 120-hrs post feeding were obtained (Fig. 2, lane 1 and 10) as opposed to the bands obtained with GAPDH primers.

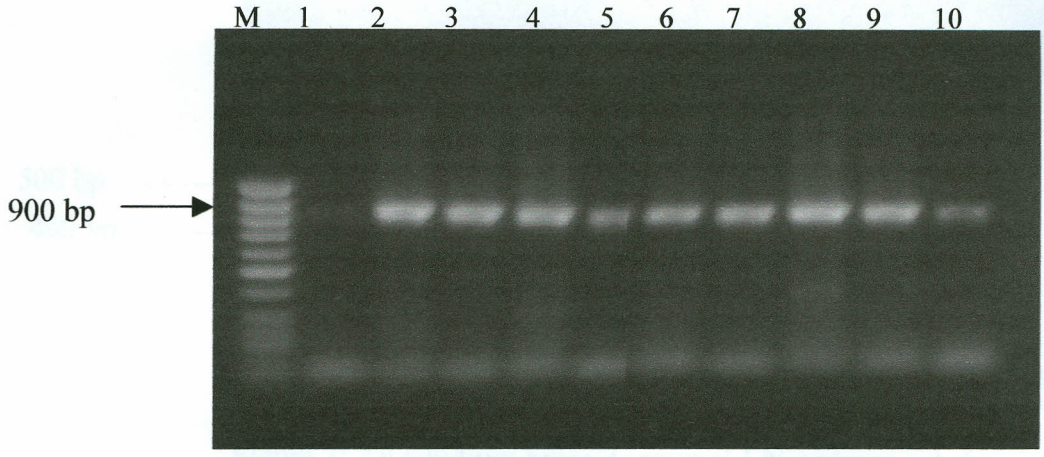

Figure.2: An ethidium bromide stained 1 % agarose gel showing bands obtained after RT-PCR using *Gpl* specific primers. Lane M-50 bp ladder DNA marker; 1, teneral gut cDNA; 2, 1 h post-feeding (pf); 3, 6 h pf; 4, 12 h pf; 5, 24 h pf; 6, 36 h pf; 7, 48 h pf; 8, 72 h pf; 9, 96 h pf; 10, 120 h pf.

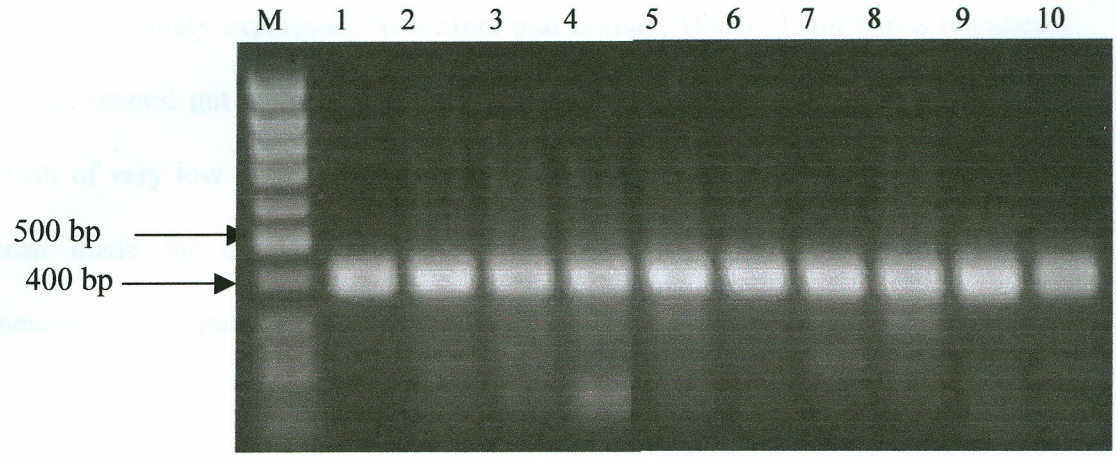

Figure.3: An ethidium bromide stained 1 % agarose gel showing bands obtained after typical RT-PCR using GAPDH primers. Lane M-50 bp ladder DNA marker; 1, teneral gut cDNA; 2, 1 h post-feeding (pf); 3, 6 h pf; 4, 12 h pf; 5, 24 h pf; 6, 36 h pf; 7, 48 h pf; 8, 72 h pf; 9, 96 h pf; 10, 120 h pf.

4.1 1. Typical Reverse Transcriptase - PCR

Typical RT-PCR using *Gpl* and GAPDH primers individually yielded 900 and 400 base pair fragments respectively showing that the annealing of the primers to the region of interest was achieved. The fragment size obtained using *Gpl* specific primers was close to that which was obtained from the expression library from which the *Gpl* gene was first discovered (Abubakar, 2003). This was expected since these primers were designed from the sequence obtained from the positive clone of the expression library (Abubakar, 2003). The bands obtained using GAPDH primers were of the same intensity unlike those obtained using *Gpl* primers. This was as a result of GAPDH gene being housekeeping genes, which

are constitutively expressed (Nebenfuhr and Lomax, 1998). Faint bands obtained for the teneral gut cDNA amplification using *Gpl* specific primers may be as a result of very low levels of *Gpl* transcript in unfed gut. Similar observation has been made for other bloodmeal induced genes like carboxypeptidase and metalloprotease genes in *G. m. morsitans* (Yan *et al.*, 2002).

4.2 Determination of *Gpl* expression levels in *G .f. fuscipes*

Expression levels of *Glossina* poteolytic lectin gene in *G. f. fuscipes* were evaluated in the midgut tissue as a function of time after the emergence of flies and during the digestion cycle. For each time-point evaluated, total RNA was prepared from 5 single midguts. The mRNA for each gut was reverse transcribed into cDNA using powerscipt reverse transcriptase. The cDNA was analysed using two methods: semi- quantitative RT-PCR and multiplex titration RT-PCR. In both methods, *Gpl* specific primers were used together with internal control primer (GAPDH) for normalization of the reaction. Results of the semi-quantitative RT-PCR, which measures transcript levels, based on the band intensity of amplification products at different time post feeding relative to an internal control is shown in (Fig. 4). There were low levels of expression of *Gpl* in teneral flies as demonstrated in Fig. 4 lane marked 1. *Glossina* proteolytic lectin gene expression was rapidly induced as early as 1 h post bloodmeal acquisition. The transcript abundance remained high throughout the digestion process and peaked between 48 and 72 h. The transcript abundance then

decreased from 96 h of starvation. Further decrease was observed after 120 h of starvation but did not return to levels close to those present in teneral guts (Fig. 4).

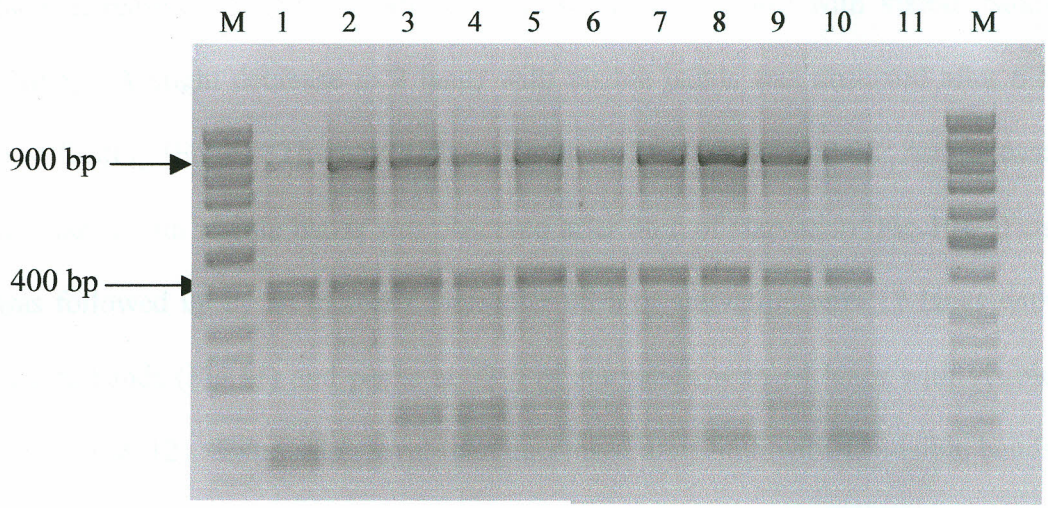

Figure 4: An ethidium bromide stained 1.5 % agarose gel showing *Gpl* expression by semi quantitative RT-PCR. Lane M-50 bp ladder DNA marker; 1, teneral gut cDNA; 2, 1 h post-feeding (pf); 3, 6 h pf; 4, 12 h pf; 5, 24 h pf; 6, 36 h pf; 7, 48 h pf; 8, 72 h pf; 9, 96 h pf; 10, 120 h pf; 11, negative control –the template was not included in the reaction mix.

Determination of expression levels was also carried out using multiplex titration RT-PCR. In this approach, amplification was carried out on a dilution series of 1:2-15 fold of the original template. The dilution at which the template becomes limiting for PCR is used as an indicator of transcript abundance (McDowell *et al.*, 1996). Multiplex titration RT-PCR data showed low levels of expression in teneral guts with only 4 lanes with visible bands (Fig.5). The expression level then increased 1 h after bloodmeal acquisition to 10 lanes with visible bands (Fig.6). A slight decrease to 8 lanes with visible bands was observed after 6 h post feeding (Fig.7). There was plateuing up to 24 h (Figs.8 and 9). An increase to 9 lanes with visible bands was observed after 36 h of starvation (Fig.10). This was followed up by an increase even after 48 h of starvation with 12 lanes with visible bands (Fig.11) and peaks at 72 h of starvation with 14 lanes with visible bands (Fig. 12). Expression level then decreased after 96 h with 11 visible bands (Fig.13) and decreased further after 120 hours, which had only 7 lanes with visible bands (Fig. 14). The low levels of expression after 120 hours of starvation were higher than the expression levels observed in teneral guts. Analysis of 5 midguts in each time point revealed similar profile with low expression levels being observed in teneral flies (mean 3.75 bands) and reaching a maximum between 48 and 72 h, mean 11.5 and 12 bands respectively (Fig.15). The expression means were significant (df, 9,30; F, 12.47; $P < 0.001$).

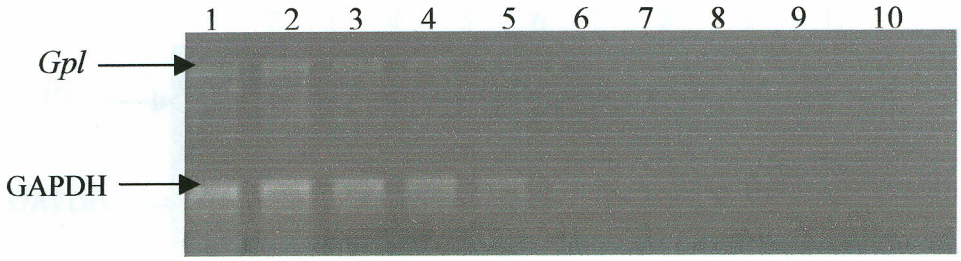

Figure.5: An ethidium bromide stained 1.5 % agarose gel showing MTRP on teneral gut cDNA. Lanes 1-10 shows the 1:2 serial dilutions carried out on the original template. Lanes 1-4 shows the *Gpl* bands that were visible under UV light. Lane 5 shows the dilution at which the template became limiting for PCR.

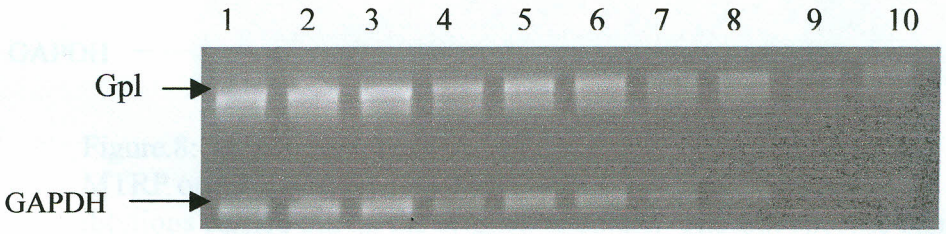

Figure.6: An ethidium bromide stained 1.5 % agarose gel showing MTRP on 1 h post feeding gut. Lanes 1-10 shows 1:2 serial dilutions carried out on the original concentration of the template. *Glossina* proteolytic lectin specific band was visible up to the tenth dilution.

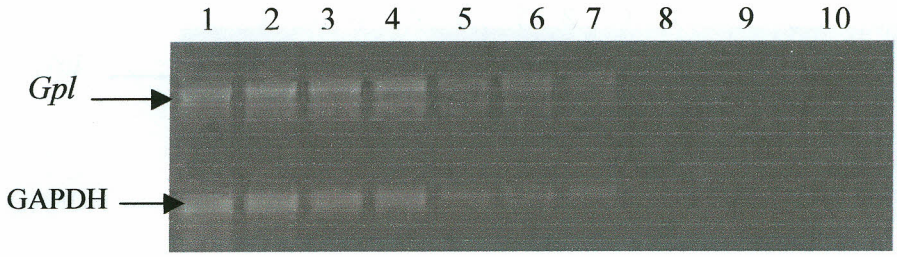

Figure.7: An ethidium bromide stained 1.5 % agarose gel showing MTRP on 6 h post feeding gut. Lanes 1-10 shows 1:2 serial dilutions carried on the original template. The *Gpl* specific band was visible up lane number 8 under UV light.

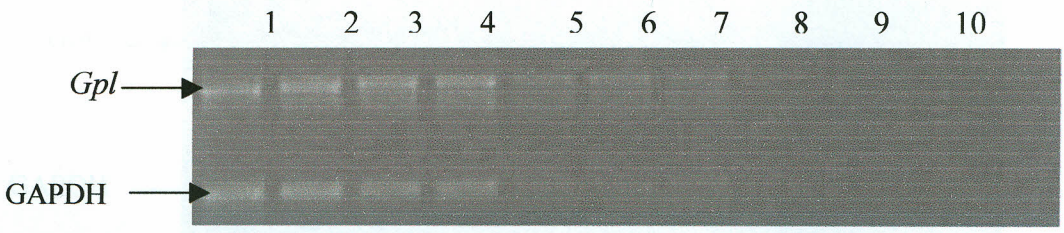

Figure.8: An ethidium bromide stained 1.5 % agarose gel showing MTRP on 12 h post feeding gut. Lanes 1-10 shows 1:2 serial dilutions carried out on the original template. The *Gpl* specific band was visible up to lane number 8.

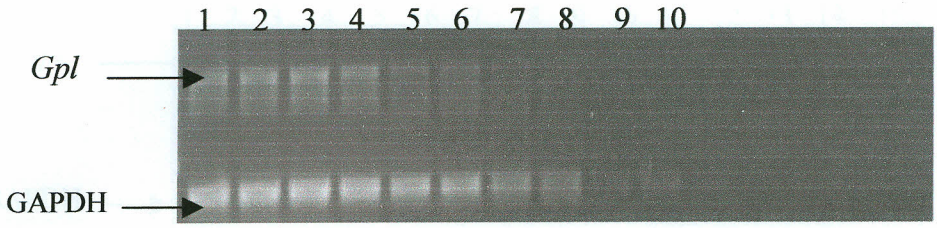

Figure.9: An ethidium bromide stained 1.5 % agarose gel showing MTRP on 24 h post feeding gut. Lanes 1-10 shows 1:2 serial dilutions carried out on the original template. The *Gpl* specific band was visible up to lane number 8 under UV light.

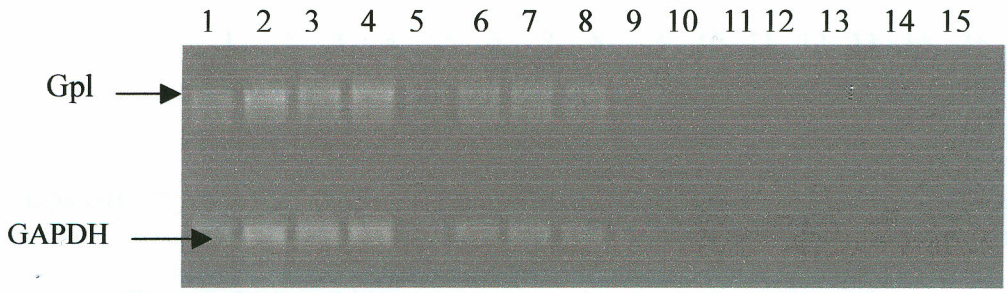

Figure.10: An ethidium bromide stained 1.5 % agarose gel showing MTRP on 36 h post-feeding gut. Lanes 1-15 shows 1:2 serial dilutions carried out on the original template. The *Gpl* specific band was visible up to lane number 9 under UV light.

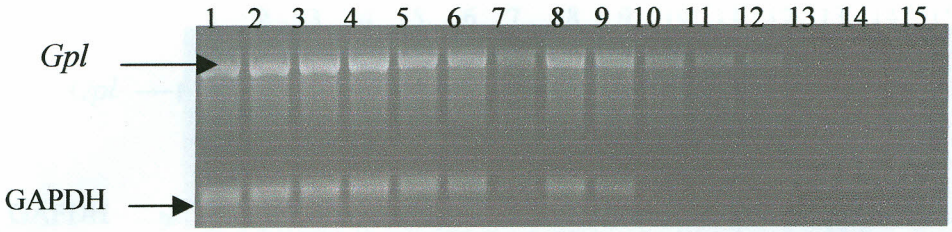

Figure.11: An ethidium bromide stained 1.5 % agarose gel showing MTRP on 48 h post feeding gut. Lanes 1-15 shows 1:2 serial dilutions carried out on the original template. The *Gpl* specific band was visible up to lane number 12 under UV light.

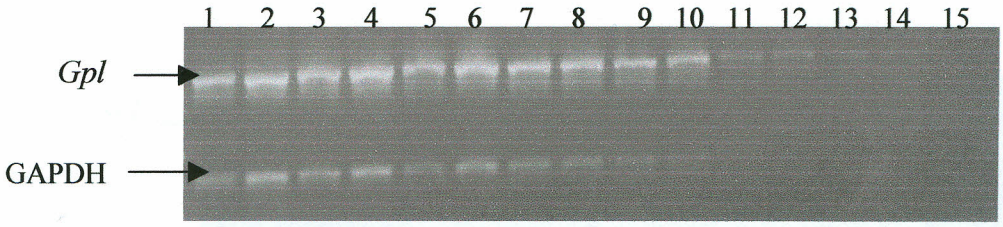

Figure.12: An ethidium bromide stained 1.5 % agarose gel showing MTRP on 72 h post-feeding gut. Lanes 1-15 shows 1:2 serial dilutions carried out on the original template. The *Gpl* specific band was visible up to lane number 14 under UV light.

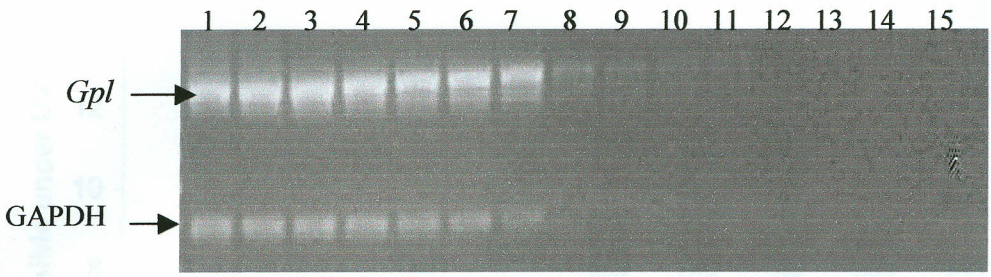

Figure.13: An ethidium bromide stained 1.5 % agarose gel showing MTRP on 96 h post feeding gut. Lanes 1-15 shows 1:2 serial dilutions carried out on the original template. The *Gpl* specific band was visible up to lane number 11 under UV light.

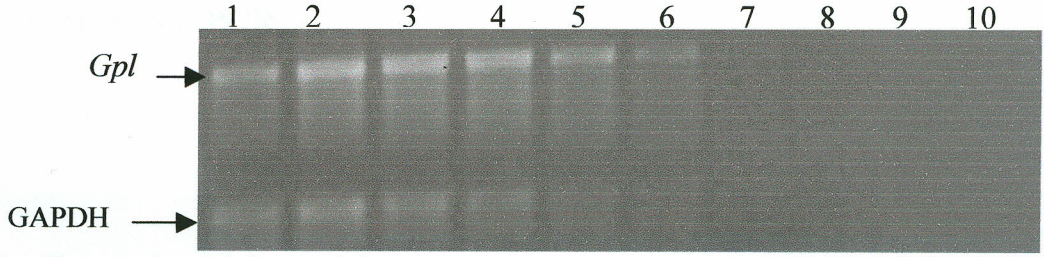

Figure.14: An ethidium bromide stained 1.5 % agarose gel showing MTRP on 120 h post feeding gut. Lanes 1-10 shows 1:2 serial dilutions carried out on the original template. The *Gpl* specific band was visible up to lane 7 under UV light.

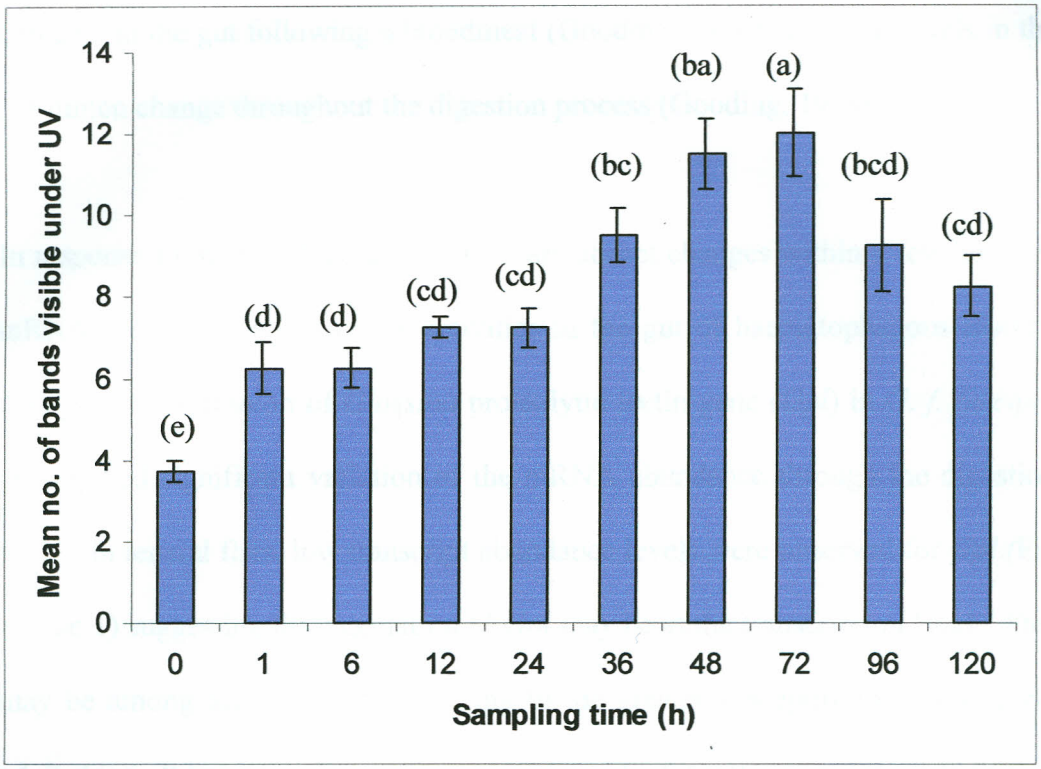

Fig. 15. A bar graph showing expression of *Gpl* by multiplex titration RT-PCR

4.2.1 Expression of *Gpl* in *Glossina fuscipes fuscipes*

Insect gut enzymes are involved in digestion of dietary proteins. Additionally, they are implicated in the process of pathogen establishment in several insects including tsetse flies (Yan *et al.*, 2001). In tsetse flies, they are thought to mediate differentiation of bloodstream forms to procyclic (midgut) forms band also restrict their transmission in the majority of flies (Welburn and Maudlin, 1999). The midgut digestive enzymes are secreted in response to increased protein

contents in the gut following a bloodmeal (Gooding, 1974), and their levels in the gut lumen change throughout the digestion process (Gooding, 1974).

In response to the first bloodmeal, there are abrupt changes within a few hours in mRNA abundance and enzyme activities in the gut of haematophagous insects. Analysis of expression of *Glossina* proteolytic lectin gene (*Gpl*) in *G. f. fuscipes* gut showed significant variation of the mRNA abundance through the digestion cycle. In teneral flies, low transcript abundance levels were observed for *Gpl (*Fig 4, lane 1) suggesting that regulation of *Gpl* may be at the transcription level. This may be among the factors contributing to the greater susceptibility at this early adult stage. It has been shown that tsetse flies which receive parasites in their first bloodmeal are more susceptible than those which receive an infectious meal as non –teneral adults (Welburn and Maudlin, 1999). This susceptibility is promoted by endochitinase activity of a midgut endosymbiont, which degrades chitin during pupation resulting in a build up inhibitory glucosamine, which subsequently inhibits the midgut lectin and predisposes teneral flies to midgut infections (Welburn *et al.*, 1993; Maudlin and Welburn, 1987). Similar findings have been observed in the analysis of other bloodmeal-induced genes. Yan *et al* (2002) reported very low transcript levels of carboxypeptidase and metalloprotease genes in *G. m. morsitans* in teneral flies gut tissue. In contrast, high levels of cathepsin B gene were detected in teneral guts (Yan *et al.*, 2002). Yan *et al.*,

(2001) also reported the same findings in the analysis of 2 serine proteases (Gsp1 and Gsp2) expressed in gut tissue of *G. m. morsitans.*

Transcript abundance increased rapidly by 6- folds as early as 1 h after bloodmeal acquisition. Transcript levels remained high throughout the digestion cycle and peaked between 48 and 72 h (ba,a) of starvation post bloodmeal acquisition. Transcript abundance then decreased after 96 h of starvation. Further decrease after 120 h of starvation was observed but this was still higher than for teneral gut (cd and e respectively). This mode of expression may suggest that *Gpl* is regulated at the transcription level.

Insects with continuous digestive systems such as *Stomoxys* and *Glossina,* store and dehydrate the blood in a specialized region of the midgut (reservoir) before it is passed through the gut where different regions are responsible for digestion (opaque zone) and absorption (lipoid zone) activities (Jordao *et al.*, 1996). The opaque zone cells are found to contain many vesicles where the digestive enzymes are stored and are released quickly to the gut lumen in response to the bloodmeal (Jordao *et al.*, 1996). This could explain the increase in transcript abundance observed 1 h after bloodmeal.

Analysis from mosquitoes indicates the non-transcriptional regulation of key digestive enzymes. For example, in *Aedes aegypti* the synthesis of early trypsin appears to be regulated at the translational level since, early trypsin mRNA can be detected in unfed fly guts while no activity is found in gut cells prior to the bloodmeal (Noriega *et al.*, 1996). In contrast, late trypsin and protein is detectable only after 8 h and reaches its maximum level after about 24 h (Noriega *et al.*, 1996; Barillas-Mury *et al.*, 1991). It is thought that the digested products of the bloodmeal from the activity of the early trypsin provide the signals for inducing the transcription of the late trypsins (Barillas-Mury *et al.*, 1995; Barillas-Mury and Wells, 1993).

The expression of carboxypeptidase gene in *Stomoxis vittatum* (Ramos *et al.*, 1993) and in *A. aegypti* (Edwards *et al.*, 2000) reaches a peak at 16-20 h post-feeding, while in *A. gambiae*, it increases markedly and rapidly after 3-4 h (Edwards *et al.*, 1997). In all the cases, however, the transcript abundance was found to return to the preblood-fed state after 24-30 h. In *Stomoxys*, regulation of digestive enzymes is at the post-transcriptional level with protein levels reaching a maximum after obtaining a bloodmeal (8-10 h) (Mofatt *et al.*, 1995). Analysis of 2 serine proteses Gsp 1 and Gsp 2 in *G. morsitans* showed a constitutive mode of expression during the digestion cycle, indicating that they are regulated at the translation level (Yan *et al.*, 2001). The levels of their corresponding trypsin and chymotrypsin activities in the gut lumen, however, increased upon blood feeding

and changed significantly throughout the digestion cycle indicating a post-transcriptional mechanism of these gene products (Yan *et al.*, 2001).

The expression of induced *Gpl* remained high throughout the digestion process. The expressed transcript peaked between 48 and 72 h of starvation. This may mean that the actual digestion takes place after the peak (between 72 and 96 hours post feeding) after the translation of the message to the protein of interest. Although the transcript abundance decreased as from 96 h post-feeding, the transcript levels were higher than in teneral guts even after 120 h of starvation. Similar findings have been reported in other bloodmeal induced genes where the level of trypsin in the gut lumen was higher than that found in teneral flies even after the end of the digestive cycle (96 h) (Yan *et al.*, 2001). This may reflect the obligate haematophagous nature of tsetse flies, where both male and female feed on blood throughout their life stages. As much as the transcript levels remained high throughout the digestion cycle, the mode of expression was not constitutive.

This non-constitutive mode of expression of *Gpl* may be attached to the functional role played by its protein in tsetse flies. *Glossina* proteolytic lectin gene encodes a protein with both lectin and trypsin activities (Abubakar *et al.*, 2003). Abubakar *et al.*, (2003) reported that this protein is able to induce transformation of bloodstream form trypanosomes *in vitro,* as well as agglutination of both bloodstream form trypanosomes and rabbit red blood cells. Non- constitutive

expression may also reflect the refractory nature to trypanosomes transmission reflected in nature by the *fusca* group to trypanosomes. The 3-tsetse group *Morsitans, Fusca* and *Palpalis* exhibits different vector competence abilities for the transmission of the pathogenic trypanosomes. While the genetic basis of vector competence in tsetse is largely unknown, the *Morsitans* subgroup has been shown to be highly susceptible, whereas *Fusca* and *Palpalis* are increasingly refractory for the transmission of the *T.brucei* complex parasites (Moloo and Kutuza, 1988a, b).

While digestive functions of the guts tissue are central to insect viability, the gut is also the first site of interaction between tsetse and trypanosomes and therefore influences the disease outcome (Yan *et al.*, 2002). It has been found that the N-terminal domains of all procyclins, the surface coat of the procyclic trypanosome, are quantitatively removed by proteolysis in the fly, but not in culture, indicating a close interaction between gut enzymes and parasites (Acosta-Serrano *et al.*, 2001). Low levels of trypsin or trypsin like enzymes in the guts of tsetse flies cleaves off the trypanosome surface coat and this may provide the signal for the parasites to transform from bloodsteam to midgut forms (Yabu and Takayanagi, 1988; Van den Abbeele and Decleir, 1991; Imbuga *et al.*, 1992a; Osir *et al.*, 1993). On the other hand, high levels of these enzymes result in lysis of the parasites (Van den Abbeele and Decleir, 1991; Imbuga *et al.*, 1992).

Recent studies on immune responsive genes showed that tsetse immune system could discriminate not only between molecular signals specific for bacteria and trypanosome infections but also between different life stages of trypanosomes (Hao *et al.*, 2001). Midgut lectin is switched on in response to serum and is essential for both lysis and maturation of the parasites within the midgut region (Maudlin and Welburn, 1987). The insect immune system has been implicated in other vector–borne diseases and lectins from sandfly gut extracts have been shown to agglutinate leishmania species (Wallbanks *et al.*, 1986).

Initial studies on *Gpl* gene showed that it is similar to *G. m. morsitans* serine protease 1 (Gsp 1) gene (Abubakar, 2003). Serine proteases are major insect gut enzymes involved in digestion of dietary proteins (Yan *et al.*, 2001). In addition, they have been implicated in the process of pathogen establishment in several insect vectors including tsetse fly (Yan *et al.*, 2001). Previous work on the protein encoded by *Gpl* gene-showed that it was capable of inducing transformation of trypanosomes *in vitro* (Abubakar *et al.*, 2003).

4.3 Screening for the expression of *Gpl* in other haematophagous arthropods

The expression of *Gpl* in other haematophagous arthropods: *Anopheles gambiae, Stomoxys calcitrans, Phlebotomus dubosci, Rhipicephallus appendiculatus, Glossina fuscipes fuscipes* and *Glossina austeni* was assessed by RT-PCR method using specific *Gpl* primers. For each arthropod used, cDNA was synthesized

from guts of both unfed and also fed and starved for 72 h. *Glossina* proteolytic lectin gene was expressed only in *Glossina* species but not in other haematophagous arthropods (Fig. 16). In *Glossina* species, the expression was found to be more pronounced guts of the fed as compared to unfed ones. In fed *Glossina fuscipes fuscipes,* the transcript abundance was higher as compared to the fed *G. austeni* guts. For the unfed *Glossina* species, the transcript abundance intensity was very little as compared to the fed guts. This was depicted by the 900 bp fragments that were visualized for the unfed *Glossina* guts as opposed to the bright bands observed for the fed guts (Fig.16, lanes 10 and 12).

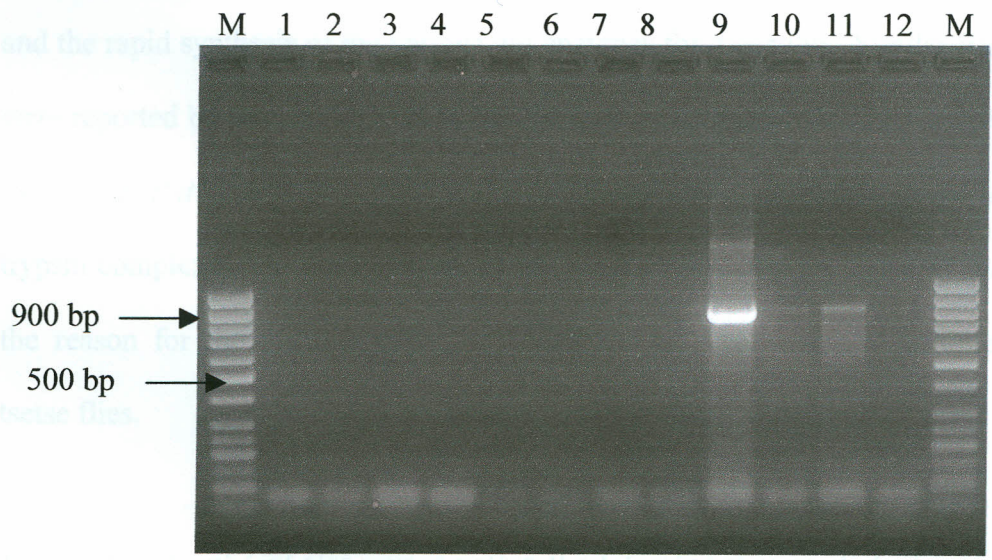

Figure.16: An ethidium bromide stained 1.5 % agarose gel showing screening of *Gpl* expression in other haematophagous arthropods. Lanes M, 50 bp ladder DNA marker; 1 & 2, fed & unfed *A. gambiae*; 3 & 4, fed & unfed *R. appendiculatus*; 5 & 6, fed & unfed *P. duboscqi*; 7 & 8; fed & unfed *S. calcitrans*; 9 & 10, fed & unfed *G. f. fuscipes*; 11 & 12, fed & unfed *G. austeni*.

4.3.1 Expression of *Gpl* in other haematophagous arthropods

Expression of *Gpl* in *Anopheles gambiae, Stomoxys calcitrans, Phlebotomus dubosci, Rhipicephallus appendiculatus, Glossina fuscipes fuscipes* and *Glossina austeni* was studied using the more sensitive RT-PCR method. Results from the study showed that *Gpl* gene is only expressed in *Glossina* species and not in other blood feeders. This may be the reason why these other haematophagous arthropods only participate in trypanosome transmission as mechanical vectors due to lack of some of the factors that are key to the establishment of trypanosomes in the arthropod midgut. This may also reflect the tsetse fly's strict haematophagous nature where both males and females rely on vertebrate blood and the rapid synthesis of the various gut enzymes for digestion. Similar findings were reported by Osir *et al.*, (1995) who observed no cross reactivity with midgut extracts of *P.duboscqi*, *A.aegypti* and *S.calcitrans* using antibodies to the lectin-trypsin complex. The conservation of this gene in *Glossina* species only may be the reason for the cyclic mode of transmission of trypanosomes exhibited by tsetse flies.

The expression of *Gpl* also varied within the *Glossina* species studied, with *G. f .fuscipes* expressing a higher transcript level than *G. austeni*. This may be related to the greater refractoriness of *G. f. fuscipes* to trypanosomes infection as compared to *G. austeni.* The two species belong to 2 different groups of *Glossina Fusca* and *Palpalis*, which differ in their vectorial capacities. Since *Gpl* encodes

a protein with lectin and trypsin activities, the high transcript levels observed for *G .f. fuscipes* may prevent the survival of trypanosomes within the gut. This is so since high levels of trypsin have been shown to lyse bloodstream form trypanosomes and the transformation of trypanosomes is a key feature for the establishment of primary infections in the fly gut. Similar variations between the tsetse groups have been reported. For example, the midgut trypsin activity levels were shown to be higher in *G. fuscipes* than in *G. morsitans* and *G. centralis* (Kongoro *et al*., 2002).

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

The expression of *Glossina* proteolytic lectin gene was studied in tsetse flies and other haematophagous arthropods: *Anopheles gambiae, Stomoxys calcitrans, Phlebotomus dubosci* and *Rhipicephallus appendiculatus* using RT-PCR methods. The study showed that *Gpl* gene is only expressed in *Glosina* species and not in *Anopheles gambiae, Stomoxys calcitrans, Phlebotomus dubosci* and *Rhipicephallus appendiculatus* that were also studied. The expression of *Gpl* was found to vary between different species of *Glossin*a, with *G.fuscipes* clearly having higher expression of the transcript than *G.austeni* suggesting that control of trypanosomes should focus on the vectors that are able to transmit trypanosomes cyclically than the mechanical transmitters.

Study of the expression of *Gpl* carried out in *G. fuscipes* showed varied expression levels at different time intervals following points post bloodmeal. While teneral guts had very low expression, transcript abundance increased abruptly shortly after bloodmeal and peaked between 48 and 72 h of starvation. Further starvation resulted in a decrease of the expressed transcript level. Even after 120 h of starvation, the transcript level was higher than the levels expressed by teneral guts, suggesting that the regulation of *Gpl* is at the transcription level.

From this study however, it is not possible to predict whether *Gpl* expression would be the same for other *Glossina* species. The expression profiles of *Gpl* should be analysed in other *Glossina* species in order to understand of its regulation and transcript abundance. The lectin and trypsin activities exhibited by *Gpl* should also be studied in order to ascertain whether they would vary in the same manner. In addition, the expression profiles of *Gpl* in infected flies amongst all the *Glossina* groups should be evaluated since additional information on tsetse gut genes and the regulation of the expression of their products stands to enhance the understanding of the interactive biology of tsetse and trypanosomes. It would also be important to find out if this gene is expressed in other body tissues of the tsetse fly other than the midgut.

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