SUSCEPTIBILITY OF THE TSETSE FLY
(GLOSSINA MORSITANS MORSITANS)
TO TRYPANOSOME INFECTIONS

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This work is dedicated to my mother and my late father
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAT</td>
<td>African Animal Trypanosomiasis</td>
</tr>
<tr>
<td>BTC</td>
<td>Belgian Technical Cooperation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>DFMO</td>
<td>α-difluoromethylornithine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organisation</td>
</tr>
<tr>
<td>GlcN</td>
<td>D-glucosamine</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetyl glucosamine</td>
</tr>
<tr>
<td>HAT</td>
<td>Human African Trypanosomiasis</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>IVC</td>
<td>Intrinsic Vectorial Capacity</td>
</tr>
<tr>
<td>NO</td>
<td>Nitrous Oxide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RLOs</td>
<td>Rickettsia-Like Organisms</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RTQ-PCR</td>
<td>Real time quantitative PCR</td>
</tr>
<tr>
<td>SIT</td>
<td>Sterile Insect Technique</td>
</tr>
<tr>
<td>STATAcorp</td>
<td>Stata Corporation statistical software</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
</tbody>
</table>
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Chapter I
General Introduction
1.1 Introduction

Tsetse flies (Diptera: Glossinidae) are vectors of several species of pathogenic trypanosomes in sub-Saharan Africa. In 36 countries of this region, tsetse-transmitted trypanosomes affect both humans [causing sleeping sickness or Human African Trypanosomiasis (HAT)] and animals [causing African animal Trypanosomiasis (AAT)]. Sleeping sickness is caused by two subspecies of *Trypanosoma brucei*, namely *Trypanosoma brucei rhodesiense* in East and Southern Africa where the disease is zoonotic with both wildlife and domestic animals being important reservoirs of the parasite, and *T. brucei gambiense* in West and Central Africa where human are the main reservoir of the parasite. In livestock, *T. vivax* and *T. congolense* are normally regarded as major pathogens of cattle and other ruminants. *Trypanosoma brucei brucei* affects all livestock but causes severe effects in equine and dogs. *Trypanosoma simiae* causes high mortality in domestic pigs.

African trypanosomiasis continues to have a profound effect on sustainable development in rural sub-Saharan Africa as it affects not only the well-being of the poor and compromises their ability to produce food efficiently, but also their livestock on which their livelihoods are heavily dependent. According to the World Health Organisation (WHO), over 60 million of people are exposed to the risk of sleeping sickness and about 300 000 cases of the disease were estimated to occur annually in the recent years. However, with the current free distribution of drugs and support in disease control activities by WHO in partnership with pharmaceutical companies, the disease prevalence has fallen drastically over the last two years to about 50 000 cases. On the other hand, the Food and Agriculture Organisation (FAO) attributes 3 million cattle deaths to the disease and current losses in cattle production alone amounting to between US$ 1.0 – 1.2 billion annually.

1.2 The trypanosomes

Trypanosomes are unicellular, flagellated, elongated and usually slightly curved protozoan parasites. Those infecting livestock vary in size (15-50µm). Within the host, the parasites are extracellular and are mostly found within the blood circulation. In some species e.g. *T. brucei s.l.*, the parasites can also colonise the spinal fluid or can be found localised in host tissues. Trypanosomes mainly reproduce by binary fission, while sexual reproduction of *T. brucei* in the tsetse fly midgut (Kooy *et al.*, 1989) and salivary glands (Jenni *et al.*, 1986; Gibson, 1996; Gibson *et al.*, 2006) has been reported.
African trypanosomes belong to the section Salivaria of the family Trypanosomatidae, order Kinetoplastida, class Zoomastigophora. Within this section, the parasites are classified into subgenera and furthermore into different subspecies. Classification is based on parasite morphology and site of development in the vector. Table 1.1 lists three important subgenera of trypanosomes according to site of development in tsetse.

**Table 1.1:** The developmental sites of various *Trypanosoma* species in *Glossina* (after Aksoy, 2003).

<table>
<thead>
<tr>
<th>Subgenus</th>
<th>Species</th>
<th>Trypomastigotes</th>
<th>Epimastigotes</th>
<th>Metacyclics</th>
<th>Infective medium</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Duttonella</strong></td>
<td><em>T. vivax</em></td>
<td>Proboscis</td>
<td>Proboscis</td>
<td>Proboscis</td>
<td>Saliva</td>
</tr>
<tr>
<td><strong>Nannomonas</strong></td>
<td><em>T. congolense</em></td>
<td>Midgut</td>
<td>Proboscis</td>
<td>Proboscis</td>
<td>Saliva</td>
</tr>
<tr>
<td></td>
<td><em>T. simiae</em></td>
<td>Midgut</td>
<td>Proboscis</td>
<td>Proboscis</td>
<td>Saliva</td>
</tr>
<tr>
<td></td>
<td><em>T. godfreyi</em></td>
<td>Midgut</td>
<td>Proboscis</td>
<td>Proboscis</td>
<td>Saliva</td>
</tr>
<tr>
<td><strong>Trypanozoon</strong></td>
<td><em>T. b. brucei</em></td>
<td>Midgut</td>
<td>Salivary glands</td>
<td>Salivary glands</td>
<td>Saliva</td>
</tr>
<tr>
<td></td>
<td><em>T. b. rhodesiense</em></td>
<td>Midgut</td>
<td>Salivary glands</td>
<td>Salivary glands</td>
<td>Saliva</td>
</tr>
<tr>
<td></td>
<td><em>T. b. gambiense</em></td>
<td>Midgut</td>
<td>Salivary glands</td>
<td>Salivary glands</td>
<td>Saliva</td>
</tr>
</tbody>
</table>

**1.3 Life cycle**

Pathogenic tsetse-transmitted trypanosomes of mammals (*T. brucei* s.l., *T. congolense*, *T. simiae* and *T. vivax*) are transmitted during feeding on an infected host. Their life cycle includes vertebrate and invertebrate (tsetse fly) hosts, and their change from one to the other, involves deep morphological, biochemical and physiological transformations (Fig 1.1). In the vertebrate host, the infective metatrypanosomes undergo development and multiplication at the site of infection causing a swelling (for *T. brucei*) and, eventually, trypomastigotes are released into blood circulation via the lymphatic system.

In the tsetse fly, trypanosomes undergo cycles of development and multiplication, involving different parts of the alimentary tract depending on the trypanosome species. Development in the tsetse fly may take from a few days to a few weeks depending on the trypanosome species. During this development, trypanosomes of the subgenera *Trypanozoon* and *Nannomonas* undergo substantial morphological and metabolic changes to survive in the tsetse midgut. This transformation is necessitated by drastic changes in the parasite’s living conditions i.e. from a stable temperature and oxygen-rich environment in the vertebrate bloodstream where glucose is used for energy to a fluctuating temperature and oxygen-
deficient environment in the tsetse where proline becomes their source of energy (Vickerman, 1985; Vickerman et al., 1988; Leak, 1999).

Figure 1.1: Life cycle of Trypanosoma brucei. Source: TDR/Wellcome Trust (http://www.who.int/tdr/diseases/tryp/lifecycle.htm).

Trypanosoma vivax has the simplest lifecycle in the tsetse fly, normally developing in the proboscis (Gardner, 1989). Development is complete within a period of 5-13 days (Leak, 1999). Trypanosoma congolense develops first in the midgut and then in the proboscis of the tsetse fly. Its lifecycle is longer than for T. vivax (about 14 days, but variable ranging from 7-40 days) (Hoare, 1970; Harley and Wilson, 1968; Dale et al., 1995). Development of T. brucei spp. trypanosomes takes place in the midgut and culminates in the metacyclic trypomastigote form in the vector’s salivary glands. Full development takes about 30 days but may range between 17-45 days in extreme cases (Hoare, 1970).

Within few hours after ingestion by the tsetse fly, viable trypanosomes expressing a new surface coat (procyclin) appear in the midgut and begin to multiply exponentially (Turner, 1988; Van den Abbeele et al., 1999; Gibson and Bailey, 2003). At around three days, a process of attrition is evident leading to the elimination of the infection in a large proportion of the flies (Gibson and Bailey, 2003). This is evidenced by the low frequency of
trypanosome infections in the midgut following experimental administration of an infectious blood meal. Various digestive factors including midgut lectin(s) (Welburn et al., 1989, 1994), agglutinins (Ibrahim et al., 1984) and lectin-like activities (Ingram and Molyneux, 1988, 1990) are involved in the destruction of the parasite in the midgut. In addition, innate immune response products have been implicated to contribute to this refractoriness (Hao et al., 2001).

Procyclic infections move to the ectoperitrophic space by contouring or directly penetrating the peritrophic membrane where they actively divide. From here they move to the proventriculus where they cease to divide (Evans and Ellis, 1983; Gibson and Bailey, 2003). After passing through the proventriculus, mesocyclic trypanosomes reinvade the endoperitrophic space (Vickerman, 1985). In the subgenus Nannomonas they migrate through the oesophagus to become attached to the wall of the hypopharynx where they complete their development. In the Trypanozoon subgenus, the trypanosomes move through the oesophagus and mouthparts to the salivary glands and acquire the variable antigen coat to become mature metacyclics (Vickerman, 1985; Van den Abbeele et al., 1999).

1.4 The tsetse fly and its distribution

Only tsetse flies are biological vectors of trypanosomes i.e. capable of cyclically transmitting African trypanosomes whilst other biting insects may transmit them mechanically (Desquesnes and Dia, 2003). Both female and male tsetse flies can carry trypanosomes. Tsetse flies constitute a small, ancient taxon of exclusively hematophagous insects that reproduce slowly and viviparously. They are quite easy to identify with their forward projecting proboscis and the typical hatchet shaped cell formed by the wing venation. There are 31 species and subspecies of tsetse flies (Glossina, Glossinidae) restricted to sub-Saharan Africa. Based on morphological and ecological considerations the genus has been divided in three groups or subgenera namely morsitans group (subgenus Glossina), palpalis group (subgenus Nemorhina) and the fusca group (subgenus Austenina). Tsetse flies are distributed discontinuously over 10 million square kilometres of sub-Saharan African land (14°N and 29°S), with each group of tsetse restricted to a relatively specific habitat. Flies belonging to the fusca group are mostly found in the forest or dense riparian forest while those of the palpalis group are found in riverine forest but also inhabit the rain forest. The morsitans group flies are mostly restricted to the savannah woodlands. Figure 1.2 shows the distribution of tsetse flies in Africa. In general the fusca and palpalis groups occur in West and Central Africa while the morsitans group is widely distributed throughout the drier zones.
1.5 The mammalian host

Both male and female tsetse flies are hematophagous insects and feed on a very large range of vertebrates, including reptiles. Mammalian hosts include a wide range of wild (bushpig, warthog, bushbuck, buffalo, kudu) and domestic animals (cattle, sheep, goats, donkey, dogs, pigs) as well as humans. Host preference may vary according to the species of tsetse but this preference may also depend on host availability.

Wild animals usually do not express severe clinical signs but may become carriers for some of the trypanosome species including *T. b. rhodesiense* and constitute an important reservoir of trypanosome infection for the susceptible hosts. In livestock, the impact of the infection depends on various factors including host susceptibility (which in turn, depends on but is not limited to species, breed, physiological condition, presence of other disease) and trypanosome species involved in the infection. In susceptible animals, the disease can be highly devastating. Infection is mostly characterised by destruction of red blood cells (anaemia) and loss of body condition (emaciation) in chronic cases. Animals may become
immunosuppressed and become more susceptible to other infections. Such animals will also mount a poor response to vaccination. The disease can cause high herd mortality, reduction in reproduction, reduction in livestock off-take of animal proteins and other products (hides, manure) and loss of draught power.

In humans the disease is generally characterised by fever and general malaise, swelling of glands, pruritus associated skin rash, swelling of eyelids, cardiovascular disturbances. As the disease progresses, nervous system injury becomes obvious and neurological disorders set in. These are commonly reported with *T. b. gambiense* as the disease duration is longer (years) while the infection with *T. b. rhodesiense* is more acute (with a duration of 6-9 months) and the terminal coma usually arrives before marked neurological symptoms (drowsiness, tremors and unsteadiness) can set in.

### 1.6 Control of Trypanosomiasis

Over several decades considerable effort has been invested in controlling the disease. Control of the disease has mainly been aimed at either the parasite in the mammalian host or removal of the vector from the environment.

#### 1.6.1 Parasite control in the host

Chemotherapy to control the parasite in the mammalian host though effective has its own drawbacks. In sleeping sickness the most widely used drugs are Suramin, Pentamidine, Melarsoprol and Eflornithine (α-difluoromethylornithine, DFMO). These current therapies are unsatisfactory for various reasons, including unacceptable toxicity, poor efficacy, undesirable route of administration, drug resistance and high cost (reviewed by Fairlamb, 2003). In the majority of African countries affected by animal trypanosomiasis, a handful of trypanocidal drugs remain the choice of controlling the disease. One of the drugs, isometamidium chloride, has prophylactic activity whilst two other trypanocidal drugs, homidium and diminazene aceturate, only have short-term therapeutic activity. There has been widespread incorrect use of these drugs which has lead to development of drug resistance by the parasite (Geerts et al., 2001).

The use of trypanotolerant livestock such as N’Dama cattle in tsetse-infested areas has been successful and has, to some extent, provided relief from the disease in West Africa (D’leteren et al., 1998). The method is, however, difficult to disseminate because of the low performance of these trypanotolerant animals. Moreover, under heavy tsetse challenge and physiological stress these animals can succumb to the disease. Finally, acceptance of those
breeds of cattle in other tsetse-infested parts of Africa is another issue that needs addressing (Chadenga, 1994).

The development of vaccines has, up to now, been unsuccessful because of the antigenic variation displayed by the parasites during their infection of the mammalian host.

1.6.2 Vector control
In the absence of effective vaccines and affordable drugs, and because of wildlife reservoirs, disease control relies heavily on controlling the vector. Historically, attempts to control the vector were based on strategies such as game elimination, creation of fly barriers and widespread bush clearing to destroy habitats for tsetse breeding and survival. Ground and aerial insecticide spraying with highly persistent and toxic chlorinated hydrocarbons such as (Dichlorodiphenyltrichloroethane) DDT have also been used in the past. Reduction of the tsetse populations has been successful to some extent but the sustainability of results is still of major concern. Even traditional methods of control are difficult to sustain (Peter et al., 2005). Targets and tsetse traps have been intensively and successfully used in localised settings to control tsetse flies. In addition, direct application of insecticides to animals (e.g. pour-ons or dips) has met with considerable success in tsetse control. The sterile insect technique (SIT) has successfully eradicated tsetse flies from the island of Zanzibar (Vreysen et al., 2000), but the choice of this technique largely depends on the isolation of the area, the level of reinvasion from neighbouring populations being a major problem.

Recent research in vector-parasite interaction at the molecular level shows a potential for the development of novel control strategies aiming to inhibit the vector’s capacity to transmit the parasite (Aksoy, 2003). One of these approaches, transgenesis, would involve blocking the vectorial capacity of tsetse by synthesizing parasite inhibitory molecules by symbiotic bacteria (endosymbionts or Rickettsia-like organisms [RLOs]) the flies harbour in their midgut.
Chapter II

Literature review: Host-parasite interactions in trypanosome infections in the tsetse fly
2.1 Introduction

Tsetse flies have been associated with the transmission of African trypanosomes since the discovery of the animal disease called “Nagana” by Bruce over a century ago (Bruce, 1895). However, it is only over the past few decades that numerous attempts have been made toward understanding the interactions between the tsetse fly and the African trypanosomes they transmit cyclically (reviewed by Aksoy et al., 2003). These interactions between trypanosomes and their invertebrate hosts are of considerable importance in the epidemiology of trypanosomiasis. Full understanding of tsetse and trypanosome interactions could potentially lead to the successful disruption of the transmission cycle. Such a disruption in the parasite’s development could become a novel trypanosomiasis control approach and be incorporated in control strategies for trypanosomiasis management (e.g. Rio et al., 2004).

In general, the majority of tsetse flies are refractory to trypanosome infections, though the reasons for this phenomenon are only partially understood. There are two key stages in the development of Trypanosoma brucei or T. congolense in tsetse. These are: i) the initial establishment of infection in the midgut and ii) the subsequent maturation of trypanosomes to produce infective metacyclics. Barriers and various factors influence both the establishment and the maturation, and as a consequence, the prevalence of trypanosome infections in the midguts of experimentally infected tsetse and proportion of mature infections usually is low.

In this chapter, the most important information concerning trypanosomes, their interaction with tsetse flies, the vectorial capacity of tsetse and the epidemiological implications of the interaction between tsetse and trypanosomes are reviewed.

2.2 Endogenous factors associated with tsetse vectorial capacity

Many investigations have been carried out to determine the barriers responsible for the low infection rates in tsetse. These barriers or factors interfere with the trypanosome colonisation of the midgut and further migration to the salivary. Le Ray (1989) referred to the intrinsic vectorial capacity (IVC) of tsetse by which he meant the intrinsic capability of a tsetse fly to develop a metacyclic infection. He proposed that the IVC of a given fly population be the product of colonisation and migration:

\[ \text{IVC} = p \times m \]

where
-p is the proportion of tsetse allowing bloodstream trypanosomes to establish as procyclic forms in the gut (n procyclic flies/n fed flies)
and
-m is the proportion of tsetse flies infected with procyclic trypanosomes that allowed the trypanosomes to migrate to the proboscis or salivary glands (n metacyclic flies/n procyclic flies)

2.2.1 Tsetse/trypanosome species and genetic variation between individual tsetse flies
Trypanosome infection rates differ between fly species. Tsetse flies belonging to the morsitans group, except Glossina austeni, are naturally good vectors for most of trypanosome species. Tsetse flies belonging to the palpalis group are described as poorer vectors (Leak, 1999, Harley and Wilson, 1968; Moloo and Kutuza, 1988; Ndewga et al., 1992) although they can be very important vectors of human trypanosomes (e.g. Wijers, 1974; Gouteux et al., 1993).

Laboratory colonies of tsetse may also differ in terms of their susceptibility to trypanosome infection. Distelmans (1985) found salmon eye colour mutation in G. m. morsitans to be associated with increased susceptibility to T. congolense. Susceptibility to trypanosome infection in different tsetse laboratory colonies was thought to be dependent on the presence of RLOs in the midgut (Baker et al., 1990; Maudlin et al., 1990). However, Moloo and Shaw (1989) and Shaw and Moolo (1991) failed to demonstrate this link in laboratory colonies of G. m. centralis and several other species.

When susceptible and refractory lines of G. m. morsitans were selected on the basis of infection rate with T. congolense, divergence of the two phenotypes was already apparent in the F1 generation (Maudlin, 1982). As the male parental phenotype did not affect the progeny, it appeared that susceptibility was an extra-chromosomally inherited character. The maternally inherited characteristic only applied to establishment of midgut infections in teneral flies. It was later shown that RLOs in the midgut were the extra-chromosomal factor (Maudlin and Ellis, 1985).

Transmissibility varies among trypanosomes species as well as between strains or stocks within a species (reviewed in Leak, 1999). Recently, Masumu et al. (2006) found differences in transmissibility by G. m. morsitans between virulent and non virulent strains of T. congolense. Flies infected with extremely virulent T. congolense strains had a significantly higher proportion of infection than those infected with strains with moderate or low virulence. In addition, Van den Bossche et al. (2006), using three isogenic clones of T. congolense with
different susceptibility to isometamidium chloride found a significantly higher proportion of infected flies infected with a *T. congolense* clone with the highest level of resistance. It is therefore possible that other genetic factors can affect the transmissibility to tsetse.

2.2.2 Sex of the fly
There are contradictory and perhaps unreliable results on the influence of the sex of tsetse on their susceptibility to trypanosomes. Some early experiments failed to take into account differences in longevity between males and females, a fact which becomes important in trypanosome species with a long developmental cycle in the fly (Burtt, 1946a; Fairbairn and Culwick, 1950). In addition females live longer than males and therefore have a greater likelihood of feeding on an infected host and picking up and maturing an infection. Males have shown a higher rate of maturation when compared to females, although females may sometimes start with a higher midgut infection rate (Distelmans *et al*., 1982; Mwangelwa *et al*., 1987; Maudlin, 1991; Mihok, 1992). The question of whether sex of a fly influences infection rates has still not been answered. Kazadi *et al*., (1991) and Welburn and Maudlin (1992) found no significant differences in trypanosome infection rates in laboratory infected male and female tsetse.

2.2.3 Age at time of infective feed and age structure of the tsetse population
Teneral flies i.e. soft bodied newly emerged flies that have not yet taken a bloodmeal after eclosion are generally considered to be most susceptible to a trypanosome infection (Buxton, 1955; Wijers, 1958; Distelmans, 1982; Mwangelwa *et al*., 1987; Gibson and Ferris, 1992; Welburn and Maudlin, 1992). However, for some fly and trypanosome species combinations this has proven not to be true (Dipeolu and Adam, 1974; Moolo *et al*., 1994). Makumyaviri *et al*., (1984), while investigating the vectorial capacity of *G. m. morsitans* for *T. b. brucei*, found a significant effect on the infection rates in flies of both sex and age at the time of the infective feed. Males that had taken their infective feed within 32 hours after eclosion had highest metacyclic infections. These findings are in agreement with earlier findings of Wijers (1958), Gingrich *et al*., (1982) for *T. b. brucei* and of Distelmans (1982) for *T. congolense*.

The high susceptibility of teneral flies has led to the assumption that in its teneral state, the fly gut offers less resistance to invading trypanosomes than that of a fed fly. Several suggestions have been put forward to explain this difference e.g. the condition of the peritrophic membrane (Lehane and Msangi, 1991), the concentration of midgut lectins (Maudlin and Welburn, 1987; Welburn and Maudlin, 1992) or proteases, the release of anti-
trypanosomal factors (midgut trypanolysin and trypano-agglutinins) (Molyneux and Stiles, 1991) or the accumulation of N-acetyl glucosamine (GlcNAc) during the larval development. Available evidence on the effect of starvation on the fly’s subsequent vector competence is sparse and contradicting (Wijers, 1958; Gingrich et al., 1982; Gooding, 1988; Welburn and Maudlin, 1992).

However, field observations on the prevalence of trypanosome infections in various age categories of tsetse show a significant increase in the proportion of infected flies with age well above the increase that would be expected when taking into account differences in susceptibility between teneral and non-teneral flies (Woolhouse et al., 1993; 1994; Leak and Rowlands, 1997). This may be the result of the acquisition of new trypanosome infections by non-teneral flies.

2.2.4 Concurrent infection with endosymbionts

Micro-organisms with different ultrastructual characteristics have been isolated from various tissues of tsetse (Pinnock and Hess, 1974; Shaw and Moloo, 1991). Tsetse flies are known to harbour three phylogenetically-distinct bacterial endosymbionts which are transmitted maternally to the progeny (Beard et al., 1993). Wolbachia spp. are found in ovarian tissues and have been implicated in fecundity and cytoplasmic incompatibility of tsetse (Cheng et al., 2000). Wigglesworthia glossinidia is the primary endosymbiont of tsetse having a well defined history of co-evolution with the tsetse and residing within specialised cells called mycetocytes in the anterior part of the midgut (Aksoy, 1995). Sodalis glossinidius is the secondary endosymbiont of tsetse, residing both inter- and intracellularly in a wide range of tissue including haemolymph, fat body and midgut (Dale and Maudlin, 1999). These organisms (earlier referred to as RLOs) are intimately related to the physiology of tsetse. For example, their elimination renders flies sterile and they have also been implicated in vector competence (reviewed by Dale and Maudlin, 2001; Aksoy et al., 2003).

Considering the close association of these endosymbionts with the host’s biology and their localisation in tissues, they can be exploited for drug and vaccine delivery (Hooper and Gordon, 2001), or for foreign gene expression designed to block the development of other pathogens (Aksoy et al., 2001). Since the symbionts live in close proximity to developing trypanosomes in the midgut, anti-pathogenic products expressed and secreted from these cells could adversely affect parasite transmission. Thus using transgenic engineering, tsetse endosymbionts may have the potential of being utilised to control trypanosomiasis by creating parasite-refractory tsetse.
2.2.5 Role of lectins
Lectins are a group of proteins, or glycoproteins with a range of properties, notably that of binding specific carbohydrates, causing agglutination. In tsetse, lectins play an important role in their defence against trypanosome infections (Welburn and Maudlin, 1999). A lectin specific for D-glucosamine (GlcN) and with lesser affinity for GlcNAc from the midgut of G. m. morsitans is capable of binding trypanosomes (Ibrahim et al., 1984; Ingram and Molyneux, 1988). Feeding lectin-inhibitory sugars, either GlcN (Maudlin and Welburn 1987, 1988; Mihok et al., 1992, 1994; Peacock, 2006) or GlcNAc (Welburn et al., 1993, 1994; Peacock, 2006) increased infection rates in tsetse. Based on the effects of plant lectin concanavalin A (Con A) on trypanosomes, it has been suggested that midgut lectins kill trypanosomes by a process termed proto-apoptosis (Welburn et al., 1999; Pearson, 2000). However, supporting data for the direct role of lectins in the tsetse/trypanosome interaction are still limited since a trypanocidal lectin has not been purified yet from tsetse, though a gene that encodes a proteolytic lectin has recently been isolated from cDNA libraries of G. f. fuscipes (Abubakar et al., 2006) and G. austeni (Amin et al., 2006). The protein encoded by this gene was expressed in bacteria and was shown to agglutinate trypanosomes. This agglutination was strongly inhibited by GlcN.

Feeding sugars at high concentration may affect other aspects of the fly midgut physiology such as efficiency of midgut digestion and hence affecting the establishment of trypanosome infections (Moloo and Shaw, 1989; Osir et al., 1993; Mihok et al., 1994, 1995; Peacock et al., 2006).

In addition to killing trypanosomes, lectins are purported to initiate the process of maturation in the fly midgut (Maudlin and Welburn, 1988; Welburn and Maudlin, 1989, 1999). However other studies have failed to demonstrate any effect of GlcN or GlcNAc on maturation (Mihok et al., 1992; Welburn, et al., 1993; Peacock et al., 2006).

2.2.6 Role of the peritrophic matrix
The peritrophic matrix, secreted by midgut epithelial cells in the annular pad of the proventriculus, consists of an annular sheet of chitin with associated proteins and forms a sleeve-like barrier throughout the midgut. Lehane (1997) reviewed the peritrophic matrix structure and its physiological function. It may protect the midgut epithelium from mechanical damage and insult from pathogens, toxins, and other damaging chemicals. Its role as a barrier to infection with T. brucei and T. congolense has been under considerable discussion since Wigglesworth (1929) demonstrated its involvement in the trypanosome
lifecycle. Moloo et al. (1970) investigated the matrix ultrastructure and its possible penetration by trypanosomes in relation to its structure. The role of the peritrophic matrix as a physical barrier to trypanosome infection as suggested earlier (reviewed by Molyneux, 1977; Leak, 1999) was discounted by Welburn and Maudlin (1992) when they showed that addition of GlcN to blood meals of tsetse restored their teneral state. Their finding suggested that the underdeveloped status of the peritrophic matrix in teneral flies could not be solely responsible for the higher trypanosome susceptibility observed in these flies. Indeed, it has been shown that trypanosomes are actually able to penetrate the peritrophic matrix in the middle region of the gut as one possible route of migration to the ectoperitrophic space (Evans and Ellis, 1977, 1983; Gibson and Bailey, 2003). This is in addition to the accepted classical route of migration around the open posterior end of the peritrophic matrix (Welburn and Maudlin, 1999).

2.2.7 Role of tsetse innate immune response
Most of the knowledge on the innate immune system of insects and its interaction with parasites has been derived from studies with Drosophila melanogaster and Anopheles gambiae, respectively, while studies of other insect-pathogen models are revealing novel and complimentary aspects on insects’ immune system (reviewed by Dimopoulous, 2003; Hoffmann 2003; Hetru et al., 2003). An insect can clear invading pathogenic agents with a robust immune system using a diverse set of mechanisms ranging from phagocytosis to activation of proteolytic cascades, such as coagulation and melanisation, and the production of various antimicrobial peptides (Barillas-Mury et al., 2000; Dimopoulos et al., 2001). Insects rely on the recognition of conserved molecules common to specific groups of pathogens to initiate a systemic response to infection in their fat body, where a variety of proteins and peptides with antimicrobial activity are synthesized (reviewed by Lehane et al., 2004). The activity spectrum of immune peptides is diverse and can be specific to a pathogen or to various pathogen groups. Thus with the fat body being a key centre in metabolism and biochemistry of the tsetse innate immune response, physiological changes in tsetse affecting the fat body such as nutritional stress may result in down regulation of the immune function in the insect (Schmid-Hempel, 2005).

The tsetse fly’s immune system has been found to play an important role in determining the efficiency with which trypanosomes establish infections in the fly. Hao et al. (2001) studied the role of a systemic response in tsetse during trypanosome transmission by looking at transcriptional regulation of the immunity genes defensin, attacin and dipterycin.
The trypanosome infection rate in tsetse midgut was substantially lowered by providing an infectious blood meal after the stimulation of the immune system of teneral flies by microinjection of either *Escherichia coli* or the LipoPolySaccharide bacterial component (Hao *et al.*, 2001). Contrary to this finding, trypanosomes failed to elicit such a strong response in the immune response tissues of teneral flies. However, adult *G. m. morsitans* with established midgut infections showed transcriptional activation of the immunity genes *attacin* and *defensin* in the fat body and proventriculus (Hao *et al.*, 2001, 2003), *cecropin, attacin* and *defensin* in hemolymph (Boulanger *et al.*, 2002) and cecropin in the fat body (Attardo *et al.*, 2006). These findings suggest that mammalian-stage trypanosomes, present in the early phase of infection, do not induce an immune response but after differentiating to procyclic forms they are recognised as foreign and elicit responses in the fat body, proventriculus and hemolymph. It is possible that reactive intermediates such as nitric oxide (NO) and hydrogen peroxide (H$_2$O$_2$), which are produced in the proventriculus might function as immunological signals mediating molecular communication between the different tsetse compartments (Hao *et al.*, 2001). As the response elucidated late in the infectious process fails to clear the parasite infections, trypanosomes might either be resistant to actions of the immune peptides, though trypanolytic activity has been demonstrated for tsetse Attacin peptide (Hu and Aksoy, 2005) or they fail to reach and harm the parasites in their midgut niche.

The identification of 68 tsetse fly genes with potential implication on its immune system in a tsetse genome discovery project (Lehane *et al.*, 2003) has opened the way for more detailed investigation of tsetse responses to *Trypanosoma* infections.

More recently, Hu and Aksoy (2006) have provided the first direct evidence on the involvement of antimicrobial peptides in trypanosome transmission in tsetse. By knocking down attacin and relish expression in tsetse, they obtained significantly higher midgut and salivary gland trypanosome infection rates as well as a high intensity of midgut infections. In addition, immunopeptides might not necessarily eliminate the trypanosomes but affect their intensity and maintain the parasites at homeostatic levels in the gut such that they do not impact tsetse fitness adversely.

2.2.8 Nutritional status of tsetse
The fat body is not only involved in the synthesis of proline - the energy source for flight in tsetse (Bursell, 1963) - but is also important in the immune response of tsetse to a trypanosome infection (reviewed by Aksoy, 2003).
The existing evidence on the effect of nutritional status or starvation on tsetse trypanosome midgut and mature infection rates is not clear. Wijers (1958), while investigating the effect of time of infective bloodmeal after emergence, obtained highest proportion of T. gambiense infected G. palpalis flies when infected less than 30 hours old after emergence, but the proportion of infected flies fed thereafter reduced significantly. Welburn and Maudlin (1992) obtained fewer maturing midgut infections of T. congolense in starved non teneral flies than in teneral G. m. morsitans. Gingrich et al. (1982), having starved flies for four days before a T. b. rhodesiense infective meal, obtained mature infection rates in adult G. m. morsitans comparable to teneral flies. However, it should be noted that the infective feeds used in this experiment were serum free. Serum in a bloodmeal is thought to trigger the secretion of trypanocidal lectins in addition to stimulating digestive enzyme secretion (reviewed by Aksoy, 2003). Gooding, (1988) did not find any significant differences in prevalence of T. b. brucei infections in G. m. centralis starved for five days after the infective feed. Thus, the effect of starvation on the susceptibility of tsetse to trypanosome infection needs to be clarified further. Furthermore, considering that the amount of fat body may depend on the nutritional status of the fly, the immune response of tsetse flies to trypanosome challenge under various levels of nutritional stress needs to be investigated.

2.3 Ecological factors affecting tsetse trypanosome infections

2.3.1 Temperature

In laboratory studies, infection rates are positively correlated with the maintenance temperature to which puparia and adult flies are subjected to. This also influences the duration of the development cycle within tsetse (Fairbairn and Culwick, 1950; Hoare, 1970). Kinghorn and Yorke (quoted by Leak, 1999) found that T. b. rhodesiense developed more readily in G. morsitans at higher temperature. Burtt (1946b) showed that G. morsitans flies that emerged from puparia incubated at high temperature (30°C) are more readily infected with T. b. brucei and transmitted the parasite more readily. Fairbairn and Watson (1955) demonstrated a similar temperature effect for T. vivax infection in G. palpalis. Ndegwa et al. (1992) also obtained higher infection rates with T. congolense in G. m. morsitans and G. brevicalpis emerging from pupae incubated at 29°C. With more controlled temperature conditions, Dipleou and Adam (1974) using G. morsitans infected with T. brucei found no differences in midgut infection rates when flies where kept at three different temperatures. However,
maintenance of flies or pupae at high temperature (31°C) greatly enhanced the proportion of infections that matured. Infections in flies kept at temperatures below 20°C did not mature.

Although field studies have shown a similar effect of temperature on trypanosome infection rates, the effect is less clear because other factors may play a role in the uncontrolled natural environment. Ford and Leggate (1961) found a positive correlation between infection rates (all species combined) in tsetse and distance from the median (7°S) of the Glossina belt in Africa. They associated this positive correlation with increasing mean annual temperature.

2.3.2 Relationship between tsetse and trypanosome infection in host (preference and availability)

It is generally accepted that a single trypanosome in the blood meal may be enough for a tsetse fly to become infected. However, the number of infected tsetse and the species of trypanosome with which they are infected are affected by the host species. Jordan (1965, 1974) positively correlated the overall rate of infection with trypanosomes in tsetse with the proportion of blood meals taken on bovids. An examination of the relationships between trypanosome infection rates and natural hosts of three tsetse species (Moloo et al., 1971) showed that vivax -type infections originated from bovids while conglobense-type infections came from bovids and bushpig. Ashcroft (1959), discussing the feeding habits of tsetse in relation to wild animals as reservoirs of trypanosomes, suggested that the number of tsetse carrying trypanosomes and the relative proportion of the different trypanosome species may be closely related to the host animals on which they feed. The availability of the host can influence infection rates in tsetse. At the Kenyan coast, G. pallidipes had higher rates of trypanosome infections in areas with high densities of domestic animals than in areas with wild animals only (Tarimo et al., 1984). This was attributed to higher parasitaemias in domestic animals compared to wild animals. Allsopp (1972) investigated the role of game animals in the maintenance of trypanosomiasis in the Lambwe valley in Kenya and found an overall trypanosome infection rate of only 16% in the wild animals. However, 90% of bushbucks, the preferred host of G. pallidipes, were infected thereby illustrating the importance of the host on the probability that a tsetse fly picks up a trypanosome infection.

2.4 Field observation on tsetse infection rates

Most of the existing field observations on tsetse infection rates are based on trapping tsetse, dissection and microscopic examination of gut and mouthparts according to the method of Lloyd and Johnson (1924). However, with the introduction of the more accurate and sensitive
DNA based methods, the accuracy of this method has come under question. For example Morlais et al., (1998) revealed the extent to which microscopical examination fails with 38.2% of PCR positive flies being missed by microscopy. Furthermore, using the dissection method it is difficult to discriminate different species within a subgenus of trypanosomes (e.g. Kukla et al., 1987, Majiwa et al., 1994) as well as detect and characterise new trypanosome species (e.g. Majiwa et al., 1993; McNamara et al., 1994). Furthermore, wrongly identified infections according to site of location by microscopy and mixed infections could now be correctly identified by these new methods (Masiga et al., 1996; Lehane et al., 2000).

Despite these drawbacks, nearly all the data on field infection rates are based on this dissection and microscopy method. Nevertheless, certain conclusions can still be drawn from those data. First, the prevalence of trypanosome infections in field caught tsetse is typically low despite the high prevalence of disease in humans and livestock. Secondly, the prevalence of infection is found to increase with age (Harley, 1966, 1967; Woolhouse et al., 1993, 1994). Woolhouse and Hargrove 1998, using various published field data from several surveys, developed a theoretical model to explain dynamics of fly infection in which both trypanosome prevalence (from dissections) and age of female flies (ovarian aging) were included. A simple model of increasing prevalence with age fitted the data for vivax-type infection well but not that of T. congolense-type infections for which a best fit could only be obtained assuming that only a proportion of flies, variable between survey sites was susceptible. But since infections increase with age, flies must continuously acquire infections even as non-teneral.

Using highly sensitive and species-specific diagnostic tools, several workers have also shown that, in the field, a substantial proportion of infected tsetse carry mixed trypanosome infections (Majiwa and Otieno, 1990; McNamara et al., 1995; Masiga et al., 1996; Woolhouse et al., 1996; Morlais et al., 1998; Lehane et al., 2000; Jamonneau et al., 2004). Laboratory evidence has shown that such mixed infections can be contracted simultaneously from a single animal supporting a mixed infection (Van den Bossche et al., 2004) or could be a result of sequential infections over a number of feeds on different infected animals (Moloo et al., 1982; Gibson and Ferris, 1992). The question remains on how the presence of an established midgut or mature trypanosome infection in older tsetse flies affects the development of a new, secondary trypanosome infection.
Chapter III

Objectives of the thesis
The general objectives of this thesis were to study different aspects affecting the susceptibility of tsetse flies, *Glossina morsitans morsitans*, to develop an infection with *Trypanosoma congolense* or *T. brucei* and to estimate the importance of changes in this susceptibility in the epidemiology of human and animal trypanosomiasis. In order to achieve these objectives the study comprised of the following specific objectives:

- To investigate age/prevalence relationship of trypanosomal infections in field *G. m. morsitans*;
- To determine the effect of tsetse fly’s age on its susceptibility to infection with *T. congolense* and *T. brucei*;
- To determine the effect of tsetse fly starvation on its susceptibility to infection with *T. congolense* and *T. brucei*;
- To investigate whether tsetse fly starvation affects the course of trypanosome development in the tsetse midgut;
- To assess the possible role of the tsetse fly immune response in the modification of the tsetse susceptibility to trypanosome infection after nutritional stress;
- To determine whether the presence of an established midgut or mature trypanosome infection in older flies affects the development of a new secondary trypanosome infection;
- To investigate whether the trypanocidal drug isometamidium chloride can affect tsetse susceptibility to a trypanosome infection.
Chapter IV
Age prevalence of trypanosomal infections in female *Glossina morsitans morsitans* (Diptera: Glossinidae)

Adapted from:
4.1 Introduction

The complex epidemiology of animal trypanosomosis is greatly affected by the number of infected tsetse flies, transmitting the disease (Lambrecht, 1980). Many ecological and physiological factors have been suggested to affect tsetse infection rates (Lambrecht, 1980). Of particular epidemiological interest is the age prevalence of metacyclic trypanosomal infections in tsetse. This age/prevalence relationship has been described by various authors for different tsetse species (e.g. Harley, 1966). Recently, a field study conducted in the Zambezi Valley (Zimbabwe) described this relationship for Glossina pallidipes using a mathematical model (Woolhouse et al., 1993). At the same time, this model provided estimates of the developmental period of trypanosomes in tsetse and age-dependent susceptibility to infection.

This paper describes a study conducted in the Eastern Province, Zambia investigating the age/prevalence relationship of trypanosomal infections in another tsetse species, G. m. morsitans. Whereas previous studies (Woolhouse et al., 1993, 1994) described this age/prevalence relationship for mature or metacyclic infections, this study gives an important additional dimension to the analysis by also investigating the age/prevalence relationship of immature or midgut infections. In an attempt to clarify the age-dependent maturation process of immature infections the fitted age/prevalence models of mature and immature infections are compared.

4.2 Materials and Methods

4.2.1 Study site

The study was conducted, between 1992 and 1994, in an area situated between 31°47′-31°55′E and between 13°55′-14°12′S in Katete District, Eastern Province, Zambia. It is a highly cultivated area with a dense cattle population (about 8 head of cattle/km²). Bovine trypanosomosis, transmitted by G. m. morsitans, is one of the major constraints for agricultural development.

4.2.2 Tsetse sampling

Tsetse flies were captured using man-walked fly rounds (Potts, 1930) and F3-traps (Flint, 1985). The odour bait used was acetone at a release rate of 250mg/hour. Tsetse flies were sampled in different vegetation types (Brachystegia, riverine and Combretum woodland) during different seasons (rainy, cold dry and hot dry).
4.2.3. Fly dissection

Flies were dissected within 4 h after collection. Physiological age-determination of females was conducted as described by Saunders (1960) and Challier (1965). Each fly was assigned to an ovarian age category (0-7) depending on its ovarian configuration. Ovarian age categories 0-1 correspond to ages 0-8 and 9-16 days, respectively; ovarian categories 2-6 correspond to additional intervals of 9 days, from 17-25 to 53-61 days, respectively. Depending on the content of the uterus, ovarian category 1-7 was subdivided into A (egg or first instar larva), B (second instar larva) or C (third instar larva). Ovarian category 0 was subdivided into A (immature egg) and B (mature egg). The ovarian age categories were transformed into days corresponding to the pivotal age of each category.

Mouthparts, salivary glands and midgut dissections were performed using the method described by Lloyd and Johnson (1924). Infections in the tsetse flies were identified according to the site of trypanosomal infestation. Infections in the proboscis alone were recorded as vivax-type, in the proboscis and the midgut as congolense-type and in the midgut alone as immature. The salivary glands were examined for mature brucei-type infections.

Throughout the analysis it was assumed that midgut or immature infections either mature into congolense-type infections or remain immature for the rest of the fly life. Changes in the age/prevalence relationship of immature infections are thus due to either maturation of immature infections into congolense-type infections, acquisition of new midgut infections or a combination of both. The age/prevalence relationship of the sum of immature and congolense-type infections, on the other hand, is only influenced by the development of new midgut infections. Consequently, the maturation of midgut infections or transmissibility was expressed as - congolense-type infections/ immature infections+congolense-type infections (%) per ovarian age category.

4.2.4 Statistical data analysis

The raw fly data consist of three dichotomous response variables indicating, for each fly, the presence or absence of congolense-type, vivax-type and immature infections and a set of three explanatory variables i.e. the year and month of fly capture and the ovarian age of the fly. The data are expressed in the form of prevalence of infection.

The first part of the analysis explored the effect of the three explanatory variables on the prevalence of the different infection-types. It was assumed that the responses are from underlying binary distributions and consequently logistic regression was used to model the
prevalences. Hypothesis testing was done by means of $\chi^2$-tests. The significance level was set at 0.01.

Since there was no significant interaction between the effects of age and time (year, month) for any of the infection-types, the analysis of the age-prevalence curves proceeded using the pooled data.

The second part of the analysis estimated the per capita rate ($\lambda$) at which flies become infected and the developmental period ($\tau$) of the trypanosomes in the tsetse fly. Since logistic regression fits prevalences that are bounded between 0 and 1 it was not appropriate for this purpose. Instead we described the age/prevalence relationship for mature infections by the model used by Woolhouse et al., (1993):

$$y(a) = 1 - \exp[-\lambda(a-\tau)] \quad \text{or} \quad \ln(1-y(a)) = \lambda \tau - \lambda a \quad \text{for } a > \tau;$$

$$y(a) = 0 \quad \text{for } a \leq \tau$$

The age/prevalence relationship for immature infections was described using the model

$$\ln(1-y(a)-z(a)) = \lambda^\prime a$$

Where $y(a)$ is the proportion of infected flies at age $a$ ($a$ is the pivotal age of each of the ovarian categories) and $z(a)$ is the proportion of tsetse with immature infections at age $a$. The log-linear model was fitted to the age-prevalence data using least squares i.e. with the assumption of approximate normality of $\ln(1-y)$ or $\ln(1-y-z)$. Polynomial terms were added when they significantly improved the model fit. The model’s assumptions are discussed by Woolhouse (1989). The per capita rate at which tsetse become infected with immature infections ($\lambda^\prime$) was compared with the per capita rate at which tsetse become infected with mature infections ($\lambda$) using a t-test. The GLIM statistical software package was used for all statistical analyses.

4.3 Results

Over the two year study period, 4416 female *G. m. morsitans* were sampled, screened for the presence of trypanosomal infections and aged using the ovarian dissection method. The yearly totals were 2162 and 2254 flies for 1992 and 1993, respectively. Monthly sample sizes, pooled over both years, ranged from 175 in January to 599 in May. *Congolense*-type infections,
identified in 212 flies (4.8 %), were the dominant infection-type. A total of 81 (1.8 %) flies had a vivax-type infection. The difference in prevalence is significant ($\chi^2=61, p<0.01$). A total of 295 (6.8 %) flies had immature or midgut infections. One fly had a brucei-type infection (0.02 %). Brucei-type infections were not included in the analysis.

Table 4.1: Logistic regression analysis of deviance of terms affecting the prevalence of congolense-type, vivax-type and immature + congolense-type infections in female G. morsitans morsitans.

<table>
<thead>
<tr>
<th>Terms included</th>
<th>Terms added</th>
<th>$\chi^2$-value</th>
<th>d.f.</th>
<th>p-value</th>
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<td>-</td>
<td>year</td>
<td>3.2</td>
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<td>0.07</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>year</td>
<td>3.5</td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td>-</td>
<td>month</td>
<td>25.7</td>
<td>11</td>
<td>0.007$^a$</td>
</tr>
<tr>
<td>month</td>
<td>month.year$^b$</td>
<td>13.9</td>
<td>12</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>age</td>
<td>14.2</td>
<td>1</td>
<td>&lt;0.001$^a$</td>
</tr>
<tr>
<td>age</td>
<td>year</td>
<td>3.5</td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>month</td>
<td>25.0</td>
<td>11</td>
<td>0.009$^a$</td>
</tr>
<tr>
<td>age</td>
<td>age.month$^b$</td>
<td>11.9</td>
<td>11</td>
<td>0.37</td>
</tr>
<tr>
<td>immature + congolense-type infections</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>year</td>
<td>1.6</td>
<td>1</td>
<td>0.20</td>
</tr>
<tr>
<td>-</td>
<td>month</td>
<td>39.9</td>
<td>11</td>
<td>&lt;0.001$^a$</td>
</tr>
<tr>
<td>month</td>
<td>month.year$^b$</td>
<td>18.3</td>
<td>12</td>
<td>0.10</td>
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<tr>
<td></td>
<td>age</td>
<td>19.5</td>
<td>1</td>
<td>&lt;0.001$^a$</td>
</tr>
<tr>
<td>age</td>
<td>year</td>
<td>31.7</td>
<td>1</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>month</td>
<td>36.4</td>
<td>11</td>
<td>&lt;0.001$^a$</td>
</tr>
<tr>
<td>age</td>
<td>age.month$^b$</td>
<td>6.9</td>
<td>11</td>
<td>0.8</td>
</tr>
</tbody>
</table>

$^a$ significant at $p=0.01$

$^b$ interaction term only

$^c$ main effect+interaction term

4.3.1 Temporal variation
In 1992, the overall prevalence of trypanosomal infections was 277 flies out of a total of 2162 dissected flies (12.8 %). In 1993, a total of 311 flies, out of 2254 dissected flies (13.8 %), were
infected. The difference in prevalence between the two years was not significant ($\chi^2_{1}=0.93$, $p>0.01$). Average monthly prevalence of trypanosomal infections ranged from 8 % in June to 17.5 % in July. This variation was significant ($\chi^2_{11}=30$, $p<0.01$). The shape of the monthly variation did not significantly differ between both years (month.year interaction: $\chi^2_{11}=19$, $p>0.01$).

The effects of year and month on the prevalences, allowing for the effect of the age structure of the flies, were explored for *congolense*-type, *vivax*-type infections and the sum of immature and *congolense*-type infections. Results are presented in Table 4.1.

There was no significant yearly variation in the prevalences of any of the infection-types and no effect of year on the shape of the monthly variation of any of the infection-types. For *congolense*-type infections, only age had a significant effect on prevalence. Monthly variation in prevalences were not significant. For *vivax*-type infections both age and month, but not their interaction, were significant factors. The monthly prevalence of *vivax*-type infections varied from 0.7 % in October to 3.6 % in December. For the sum of immature and *congolense*-type infections both age and month, but not their interaction, were significant factors explaining the variation in the prevalence. Average estimates of infection prevalences with age, for the whole of the study period, were obtained ignoring the effects of month and year.

4.3.2 Age-prevalence relationship
The observed age/prevalence relationship for *congolense*-type and *vivax*-type are shown in Fig. 4.1A and B.

The prevalence of *congolense*-type infections ranged from 0 % (ovarian age category 0a and 0b) to 9.5 % (ovarian age category 6b). For *vivax*-type infections the prevalence varied between 0 % (ovarian category 0a) to 6.1 % (ovarian category 6b). The model $\ln(1-y)=\lambda \tau - \lambda a$ was fitted to the age prevalence data of the *congolense*-type and *vivax*-type infections to obtain estimates of the per capita infection rate ($\lambda$) and developmental period ($\tau$). The fitted curves are listed in Table 4.2 and plotted, together with the observed prevalences, in Fig. 4.1A and B. For the *congolense*-type infections, the model included a cubic term of age which significantly improved the fit ($F_{1,16}=5.95$, $p<0.01$). The model explained 87 % and 71 % of the variation in $\ln(1-y)$ for *congolense*-type and *vivax*-type infections, respectively. The parameter estimates (± standard error) for *congolense*-type infections were $\tau=7.9\pm3.7$ days and $\lambda=0.00261\pm0.00043$/fly/day at age=$\tau$. For *vivax*-type infections, the parameter estimates (± standard error) were $\tau=9.4\pm6.5$ days and $\lambda=0.00085\pm0.00013$ /fly/day at age=$\tau$. 
Table 4.2: Parameter estimates of age-prevalence models for three trypanosomal infection-types in female *G. morsitans morsitans*.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>s.e.</th>
<th>parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>congolense-type</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-0.02107</td>
<td>0.009337</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>-0.002672</td>
<td>0.0004328</td>
<td>age</td>
<td></td>
</tr>
<tr>
<td>2.292 e-07</td>
<td>9.391 e-08</td>
<td>age^3</td>
<td></td>
</tr>
<tr>
<td><strong>vivax-type</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.007950</td>
<td>0.005302</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>-0.0008496</td>
<td>0.0001323</td>
<td>age</td>
<td></td>
</tr>
<tr>
<td><strong>Immature + congolense-type</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-0.00448</td>
<td>0.0003929</td>
<td>age</td>
<td></td>
</tr>
<tr>
<td>0.00000032</td>
<td>0.00000012</td>
<td>age^3</td>
<td></td>
</tr>
</tbody>
</table>

The prevalence of immature infections varied between 0 % (ovarian category 0a) to a maximum of 12.2 % (ovarian category 5c). The observed prevalences for immature only and the sum of immature and *congolense*-type infections are presented in Fig 4.1C.
Figure 4.1: Variations in prevalence of trypanosomal infections in female *Glossina morsitans morsitans* with fly age (by ovarian category), (A) *congolense*-type. The best fit model (line) is $\ln(1-y)=0.02107-0.002672\text{age}+2.297\times10^{-7}\text{age}^3$. (B) *vivax*-type. The best fit model (line) is $\ln(1-y)=0.007950-0.0008496\text{age}$. (C) (●) *congolense*-type + immature The best fit model (line) is $\ln(1-y)=-0.004448\text{age}+0.00000032\text{age}^3$ and (□) immature only.
The model for immature + *congolense*-type infections was fitted without intercept ($F_{1,16} = 0.01, p>0.05$). A cubic term significantly improved the fit ($F_{1,17}=6.1, p<0.05$). This model explains 91.5% of the variation of the prevalence of immature+$congolense$-type infections. The fitted curve is plotted, together with the observed prevalences, in Fig. 4.1C. The parameter estimates of the fitted model are given in Table 4.2. The *per capita* rate ($\lambda$) at which flies become infected was estimated at $0.00447\pm0.00039$/fly/day at day 0. The estimate of $\lambda$ at age $\tau$ for mature infections is significantly lower ($t_{33}=2.958; p=0.0028$) than the estimate of $\lambda'$ for immature infections at age 0.

The observed and fitted ratio of *congolense*-type infections/ immature infections + *congolense*-type infections, for each of the age groups, is plotted in Fig. 4.2. The ratio increases rapidly to a value of about 0.5 at the age of about 30 days after which it remains almost constant.

![Figure 4.2: Variations in the ratio of *congolense*-type infections/ *congolense*-type+immature infections (●) of female *Glossina morsitans morsitans* with fly age (by ovarian category).](image-url)
4.4 Discussion

Except for studies conducted by Clarke (1969) and Woolhouse et al., (1994) little information is available on the prevalence of trypanosomal infections in Zambian tsetse species. Contrary to Clarke’s observations, congolense-type infections are the most prevalent in our study area. This is in accordance with the high bovine trypanosomosis prevalence, caused by T. conglobense, in the plateau area of the Eastern Province.

The per capita rate at which female G. m. morsitans acquire congolense-type infections, suggests that 0.26 % of the flies become infected per day. At a feeding interval of 4 days (Rogers, 1988) and a 75 % feeding preference for cattle (as is the case in the study area), this corresponds to a successful infection every 72 blood meals on cattle. The prevalence of Trypanosoma congolense infections in cattle is high in the study area. At a prevalence of 40 %, 3.5 % of the feeds on infected cattle develop and mature in female G. m. morsitans. This figure is slightly higher than the 2.5 % obtained by Rogers & Boreham (1973) from an analysis of data on G. swynnertoni in Tanzania.

The estimated developmental period of congolense-type infections in G. m. morsitans corresponds well with the, average 8 to 9 days observed by Elce (1971). Nantulya et al. (1978), however, observed development periods up to 40 days. The estimated developmental period for vivax-type infections, agrees with the 5 to 13 days reported by Davies (1977).

Results from this study show that the prevalence of vivax-type infections rises approximately linearly with fly age. This is what could be expected and reinforces the observations that tsetse flies can readily infect themselves with T. vivax throughout their life. This is most likely related to the relative simple developmental cycle of T. vivax in the tsetse fly.

The age/prevalence curve of immature+congolense-type infections strongly suggests that, in accordance with laboratory observations (Welburn and Maudlin, 1992), wild tsetse also acquire new trypanosomal midgut infections at any age. Consequently, infected blood meals can give rise to immature infections even when these blood meals are taken by non-teneral flies.

There is some conflicting evidence concerning the ease with which non-teneral tsetse flies can infect themselves with T. congolense. Many authors, using both laboratory reared tsetse and tsetse emerging from wild pupae, have described the significantly higher susceptibility of teneral, compared to non-teneral flies, to T. congolense infections (Owaga,
1981; Distelmans et al., 1982; Mwangelwa et al., 1987; Welburn and Maudlin, 1992; Dale et al., 1995). Harley (1967), on the other hand, found an increasing T. congoense infection prevalence with increasing age in females of several tsetse species. It is nevertheless generally accepted that tsetse, once fed, are relatively but not completely refractory to trypanosomal infections. Consequently, infections acquired by non-terenal wild flies are expected to play a minor role in the epidemiology of tsetse-transmitted trypanosomosis.

According to our observations, the prevalence of congoense-type infections increases substantially, though not linearly, with increasing age. The fit of the model to the age/prevalence data was improved by an additional cubic term. This age-prevalence model can be explained by: variations in incubation time of infections obtained in young flies, age-dependent decrease in susceptibility to infection or increased mortality of tsetse infected with a metacyclic T. congoense infection (Woolhouse et al., 1993; Dale et al., 1995).

The developmental or incubation period of congoense-type infections can vary (Dale et al., 1995). Midgut or immature infections, however, must develop immediately after the ingestion of the infected blood meal. Assuming that maturation is restricted to infections obtained during the first blood meal, the prevalence of midgut infections in the youngest age categories can not be lower than the maximum prevalence of mature, congoense-type infections in the subsequent age categories. This could not be confirmed by our observations. The maximum congoense-type infection prevalence (8.6 % in ovarian category 6) is higher than the maximum midgut infection prevalence in the first three age categories (6 %). Furthermore, our results show that between ovarian age category 2 and 6 the congoense-type infection prevalence almost doubles from 4.5 % to 8.6 %. This increase in congoense-type infections can not be due to retarded maturation of trypanosomal infections obtained during the first blood meal since the prevalence of immature infections in the first age categories only varies between 0 and 4 %. Consequently, the increase in the congoense-type infection prevalence with increasing age is a result of maturation of newly acquired midgut infections.

Moreover, if few of the newly acquired midgut infections in non-terenal tsetse would mature, one would expect a decreasing ratio of congoense-type infections/ immature + congoense-type infections once the developmental period of the infections acquired during the first blood meal is over. This is not the case.

Although our results indicate that maturation of trypanosomal congoense-type infections is not restricted to those obtained during the first blood meal, the shape of the ratio of congoense-type infections/ immature infections + congoense-type infections curve suggests that trypanosomal infections obtained early in life contribute more to the congoense-
type prevalence than those acquired at a later age. Moreover, there is a significant difference between the force-of-infection for immature infections ($\lambda^*$) and the force-of-infection for mature *congolense*-type infections ($\lambda$). This implies that immature infections do not all mature to become mature infections some weeks later. There are two obvious explanations: either there is an increased mortality of immature infected flies, or there is some recovery from infections. Welburn *et al.*, (1989) showed that, under laboratory conditions, it can take up to 7 days to remove an incoming infection form the midgut of a tsetse fly. Consequently, some of these “non-permanent” midgut infections will be detected and classified as midgut infection for a particular age group but will be removed from the midgut in a higher age group.

Observations described in this paper confirm the age-prevalence relationship of trypanosomal infections in wild tsetse reported in other recent studies and another tsetse species (Woolhouse *et al.*, 1993, 1994). Moreover, our results clearly indicate that the increase in the prevalence of *congolense*-type infections with increasing age is due to the maturation of midgut infection acquired by non-teneral tsetse flies. Contrary to laboratory observations (Welburn and Maudlin 1992), these newly acquired infections substantially contribute to the overall *congolense*-type infection rate of the tsetse population.
Chapter V

The effect of starvation on the susceptibility of teneral and non-teneral tsetse flies to trypanosome infection

Adapted from:

5.1 Introduction

Tsetse-transmitted trypanosomiasis is a vector-borne disease that poses a serious threat to human and animal health in large parts of sub-Saharan Africa. Tsetse flies occur on about 10 million km\(^2\) of the African continent. The epidemiology of the disease in humans and animals depends, among other factors, on the proportion of infected flies in a tsetse population. A wide range of intrinsic and extrinsic factors are proposed to determine the tsetse vector competence (reviewed by Leak, 1999). More recently, several studies have suggested that specific immune responses of the tsetse fly against the trypanosome parasite possibly interfere with the establishment and maturation of the trypanosome in the tsetse (reviewed by Aksoy et al., 2003). In most trypanosomiasis transmission models, the age-specific susceptibility of tsetse to a trypanosome infection is taken as an important parameter. Tsetse flies are believed to be most susceptible at their first bloodmeal (Wijers 1958; Buxton 1955; Distelmans et al., 1982; Welburn and Maudlin 1992) and subsequent trypanosome-infected blood meals contribute little to the overall infection rate of the tsetse population. However, field observations on the prevalence of trypanosome infections in various age categories of tsetse show a significant increase in the proportion of infected flies with age well above the increase that would be expected when taking into account the differences in susceptibility between teneral and non-teneral flies (Woolhouse and Hargrove, 1998). These observations clearly indicate that a substantial proportion of the adult tsetse population still has the ability to pick up a trypanosome infection that matures into the mammalian infective stage. A few laboratory studies have already suggested that adult tsetse flies can acquire a mature *Trypanosoma brucei* infection and that, in some cases, starvation increased their susceptibility to such an infection (Gingrich et al., 1982; Gooding, 1988; Welburn et al., 1989). To further clarify the potential role of the adult tsetse population in the transmission dynamics of African trypanosomiasis, the age-related ability of tsetse flies to develop a mature infection of the *T. congolense* and of *T. brucei brucei* was reconsidered. Moreover, this study has attempted to clarify the effect of starvation on the modulation of this vectorial ability.
5.2 Materials and methods

5.2.1 Tsetse flies and trypanosome strains
Male *Glossina morsitans morsitans* Westwood from the colony maintained at the Institute of Tropical Medicine (Antwerp, Belgium) were used in all experiments. The origin of the colony and its maintenance has been described by Elsen *et al.* (1993).

A *T. b. brucei* strain derived from the stock EATRO 1125 AnTAR1 (Le Ray *et al.*, 1977) and *T. congolense* IL 1180, a strain originating from Serengeti in Tanzania (Geigy and Kauffman, 1973) were the trypanosome strains used in the experiments.

5.2.2 Experimental design
Male *G. m. morsitans* of various ages (teneral and non-teneral flies) were given an infective bloodmeal with either *T. congolense* or *T. b. brucei*. In this context we define teneral flies as newly emerged soft bodied flies that did not receive any blood meal prior to the infective feed while non-tenerals received at least one blood meal prior to the infective blood meal. For the single infective feed, a batch of 40 flies was fed on an anaesthetized mouse showing a parasitaemia of *T. congolense* or *T. b. brucei* of approximately $10^{8.4}$ trypanosomes/ml in the blood. Only fully engorged flies were retained and maintained on clean rabbits. To avoid re-infection of the flies, these rabbits were replaced at weekly intervals. For each experimental series, 3 to 5 batches of flies were infected separately.

Twenty days (for *T. congolense* infected flies) and thirty days (for *T. b. brucei* infected flies) after the infective feed, surviving flies were dissected according to the method described by Lloyd and Johnson (1924). The midgut and mouthparts or salivary glands were examined for the presence of trypanosomes.

In the first experiment, the age-specific susceptibility to infection of teneral and non-teneral male *G. m. morsitans* was determined. For this purpose, 0-2 days old teneral flies and 4-20 days old non-teneral *G. m. morsitans* were infected and their infection status determined. In a subsequent experiment, the effect of starvation on susceptibility to infection was assessed by infecting teneral flies and 20 days old adult flies after starving them for 1 to 4 days and for 2, 3, 5 or 7 days, respectively.

The proportion of midgut procyclic infections was calculated as the proportion of dissected flies that developed a trypanosome infection at the midgut level. The proportion of mature or metacyclic infections was calculated as the proportion of dissected flies that developed an infection in the proboscis (for infections with *T. congolense*) or the salivary
glands (for infections with *T. b. brucei*). The maturation of an infection was calculated as the proportion of midgut infections that developed into a mature infection. In the first experiment, infection proportions and maturation in the teneral and non teneral flies were compared using chi-square tests. The second experiment on the effect of starvation (categorical explanatory variable) on the proportions of infected flies and maturation was analysed using logistic regression. STATA Release 8.0 (Stata Corp., College Station, TX, U.S.A.). Three logistic regressions were calculated for each trypanosome species. The response variables were the proportion of flies infected in the midgut, the proportion of metacyclic infections and the maturation. When a logistic regression failed because of proportions equal to 0 or 1, the exact method was used to calculate a confidence interval. Animal ethics approval for the experimental infections was obtained from the Animal Ethical Commission of the Institute of Tropical Medicine, Antwerp, Belgium (Ref DG001-PD-M-TT).

5.3 Results

5.3.1 Age-specific susceptibility to infection

The uptake of a single bloodmeal prior to the infective bloodmeal significantly reduced the tsetse fly ability to establish a procyclic midgut infection with *T. congoense* or *T. b. brucei* (*p*<0.001) (Table 5.1 and Figs. 5.1a and b.). Moreover, the maturation of *T. brucei* midgut infection was also significantly lowered in the non-teneral flies. Despite this, a substantial proportion of the flies infected in the non-teneral status could still acquire a mature infection, i.e. 11% and 5% of the flies given an infective bloodmeal with *T. congoense* and *T. b. brucei*, respectively.
Table 5.1: Overall midgut infection and maturation rate of male *G. m. morsitans* infected with *T. congolense* or *T. b. brucei* in a teneral (no bloodmeals prior to infection) or non-teneral status (one or more bloodmeals prior to infection).

<table>
<thead>
<tr>
<th></th>
<th>Proportion (no.) of infected flies</th>
<th>Teneral flies</th>
<th>Non-teneral flies</th>
<th>X² test</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td><em>T. congolense</em></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Midgut</td>
<td>0.37 (57/153)</td>
<td>0.11 (88/772)</td>
<td>p&lt;0.001*</td>
<td></td>
</tr>
<tr>
<td>Proboscis</td>
<td>0.37 (57/153)</td>
<td>0.11 (86/772)</td>
<td>p&lt;0.001*</td>
<td></td>
</tr>
<tr>
<td>Maturation rate</td>
<td>1.0 (57/57)</td>
<td>0.98 (86/88)</td>
<td>p=0.25</td>
<td></td>
</tr>
<tr>
<td><em>T. b. brucei</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Midgut</td>
<td>0.66 (139/212)</td>
<td>0.16 (103/653)</td>
<td>p&lt;0.001*</td>
<td></td>
</tr>
<tr>
<td>Salivary gland</td>
<td>0.29 (61/212)</td>
<td>0.05 (31/653)</td>
<td>p&lt;0.001*</td>
<td></td>
</tr>
<tr>
<td>Maturation rate</td>
<td>0.44 (61/139)</td>
<td>0.30 (31/103)</td>
<td>p=0.029*</td>
<td></td>
</tr>
</tbody>
</table>

* Significant at p=0.05
Figure 5.1: Midgut and mature infection proportions of male *G. m. morsitans* given an infective feed with (A) *T. congolense* IL 1180 or (B) *T. b. brucei* AnTAR 1 in a teneral status (days 0-2 after emergence; no bloodmeals prior to infection) or at various ages after emergence (days 4-20 one or more bloodmeals prior to infection).
5.3.2 Starvation and susceptibility to infection

In non-teneral flies, an extreme starvation period of 7 days resulted in a significant increase in the proportion of flies that developed a mature infection with *T. congolense* or *T. b. brucei* (Tables 5.2 and 5.3). For *T. congolense*, this was exclusively due to the increased ability of the flies to develop an established procyclic midgut infection. In contrast, for a *T. b. brucei* infection, the starvation of the adult flies did not have any effect on the establishment of a midgut infection but resulted in an increased maturation rate -i.e. a higher proportion of midgut infections giving rise to mature, metacyclic infections in the salivary glands.

**Table 5.2:** Proportion of male *G. m. morsitans* infected with *T. congolense* after a period of starvation.

<table>
<thead>
<tr>
<th>Flies</th>
<th>Days of starvation</th>
<th>Number of flies dissected</th>
<th>Proportion of infected flies/maturation with 95% CIs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Midgut Proboscis Maturation</td>
</tr>
<tr>
<td>Teneral flies</td>
<td>0</td>
<td>27</td>
<td>0.22 (0.10-0.41) 0.22 (0.10-0.41) 1.00 (0.61-1.00)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>75</td>
<td>0.29 (0.20-0.40) 0.29 (0.20-0.40) 1.00 (0.87-1.00)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>89</td>
<td>0.22 (0.15-0.32) 0.21 (0.14-0.31) 0.95 (0.72-0.99)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>71</td>
<td>0.56 (0.44-0.67) 0.56 (0.44-0.67) 1.00 (0.93-1.00)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>34</td>
<td>0.56 (0.39-0.71) 0.50 (0.34-0.66) 0.89 (0.66-0.97)</td>
</tr>
<tr>
<td>20-day-old</td>
<td>2</td>
<td>75</td>
<td>0.13 (0.07-0.23) 0.09 (0.04-0.18) 0.70 (0.38-0.90)</td>
</tr>
<tr>
<td>adults</td>
<td>3</td>
<td>71</td>
<td>0.10 (0.05-0.19) 0.08 (0.04-0.18) 0.86 (0.42-0.98)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>103</td>
<td>0.17 (0.10-0.25) 0.17 (0.10-0.25) 1.00 (0.84-1.00)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>77</td>
<td>0.49 (0.38-0.60) 0.48 (0.37-0.59) 0.97 (0.83-0.99)</td>
</tr>
</tbody>
</table>

The effect of starvation on the susceptibility of tsetse flies was also clearly demonstrated in teneral flies. Here, starvation for 3 or 4 days prior to the infective bloodmeal significantly increased the proportion of flies that established a *T. congolense* or *T. b. brucei* midgut infection (Table 5.2 and 5.3).
Table 5.3: Proportion of male *G. m. morsitans* infected with *T. b. brucei* after a period of starvation.

<table>
<thead>
<tr>
<th>Flies</th>
<th>Days of starvation</th>
<th>Number of flies dissected</th>
<th>Proportion of infected flies/maturation with 95% CIs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Midgut</td>
</tr>
<tr>
<td>Teneral flies</td>
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<td>69</td>
<td>0.45 (0.34-0.57)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>71</td>
<td>0.42 (0.31-0.54)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>68</td>
<td>0.37 (0.26-0.49)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>52</td>
<td>0.65 (0.52-0.77)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>44</td>
<td>0.77 (0.63-0.87)</td>
</tr>
<tr>
<td>20-day-old adults</td>
<td>2</td>
<td>95</td>
<td>0.21 (0.22-0.40)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>96</td>
<td>0.14 (0.08-0.22)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>67</td>
<td>0.22 (0.14-0.34)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>64</td>
<td>0.25 (0.16-0.37)</td>
</tr>
</tbody>
</table>

95%CI=95% confidence interval.

5.4 Discussion

These data confirm that the age of a tsetse fly at the time of the infective bloodmeal drastically affects the vectorial ability of the fly for *T. congolense* as well as for *T. b. brucei* as earlier observed by others (Wijers 1958; Distelmaes *et al.*, 1982; Welburn and Maudlin 1992). However, we did not observe a significant decrease in the proportion of infected flies observed by Wijers (1958). This vectorial ability is highest in newly emerged flies and decreases substantially when a tsetse fly has taken a single blood meal before the infective one. Nevertheless, the ability of adult flies to acquire a mature infections remains relatively high, especially for *T. congolense*. This is in line with the field observations made by Woolhouse *et al.* (1993, 1994) on the age-specific infection rates of *G. pallidipes* with *T. congolense*. Contrary to findings of Welburn and Maudlin (1992), non-teneral flies matured midgut infections comparable to teneral flies for *T. congolense*. 
The nutritional status of the tsetse fly at the time of the infective blood meal affects its vectorial ability for both *T. congolense* and *T. b. brucei*. Indeed, an extreme period of starvation (3 to 4 days for tenereal flies, 7 days for adult flies) lowers the developmental barrier for a trypanosome infection, especially at the midgut level of the tsetse fly. This suggests that the physiological factors that constitute this barrier are suppressed when the fly is under a nutritional stress. Starvation possibly reduces the immunological responses of the fly to invading micro-organisms such as trypanosomes. Indeed, components of the insect immune system are involved in the natural refractoriness observed in the tsetse fly (reviewed by Lehane *et al.*, 2004). Recent studies have demonstrated an induced expression and synthesis of several antimicrobial peptides when flies are offered a trypanosome-infected bloodmeal (Hao *et al.*, 2001; Boulanger *et al.*, 2002). The fat body constitutes the tsetse energy reserve but also functions as a key centre of metabolism and biochemistry of the fly innate immune response. The energy cost for the insect of using this immune defence machinery is considerably high (Schmid-Hempel, 2005). Hence, when a tsetse fly is exposed to nutritional stress up to a level that results in a serious depletion of its energy reserves, it is plausible that the tsetse immune functions are down-regulated leading to a less hostile midgut environment for the ingested trypanosomes. This could explain the increase in the midgut infection rates that was observed for both trypanosome infections in tenereal flies and in 7-days starved adult flies for *T. congolense*. Although this was not observed for the infection of 7-days starved adult flies with *T. b. brucei*, the significant higher trypanosome maturation rate observed in these flies could also be explained by this less hostile midgut environment since the growth of the procyclic trypanosome population in the tsetse midgut will be less hindered, increasing the probability of the infection to progress to the tsetse salivary glands (Dale *et al.*, 1995). The relationship between nutritional stress and immune response may also explain why the effect of starvation on the tsetse’s susceptibility to infection is only observed after the flies have been starved for several days i.e. after the lipid reserve has been depleted sufficiently to affect immune responses. Moreover, the reverse phenomenon could explain why feeding tenereal flies results in a higher developmental barrier for trypanosomes at the tsetse midgut level. Indeed, tenereal flies are physiologically immature and have limited energy reserves and need a bloodmeal (or several) to make all physiological systems fully operational, including their immune response.

In conclusion, more experimental evidence is needed to clarify the role of the immune system in trypanosome development and the impact of nutritional stress on this defence mechanism. Although our observations are based entirely on laboratory experiments the
implications of these findings on our understanding of the epidemiology of animal and human trypanosomiasis may be considerable. First, the proportion of infected flies in a population may not be determined only by the proportion of teneral flies that take a bloodmeal on an infected host but also by environmental factors such as high ambient temperature that may significantly reduce the tsetse’s energy reserve. It is thus plausible that during certain periods of the year the overall infection rate of a tsetse population is determined to a large extent by a substantial number of non-teneral flies that acquire a mature infection. Furthermore, our findings may have repercussions for the distribution and prevalence of infections with trypanosome species that are difficult to transmit, for example, *T. b. rhodesiense* (Welburn et al., 1995). It could be hypothesised that the focal nature of the distribution of ‘Rhodesiense’ human sleeping sickness is due to local conditions that enhance the susceptibility of non-teneral flies to infection and thus substantially increase the proportion of flies in that particular population that can become infected and transmit the parasite.
Chapter VI
The effect of starvation on the development of *Trypanosoma brucei* in the tsetse fly’s midgut and preliminary investigations of the immune response of starved tsetse flies
6.1 Introduction

The proportion of infected flies in a tsetse population is an important determinant in the epidemiology of human and animal trypanosomiasis. Tsetse flies are generally regarded as being most susceptible to infection during their first bloodmeal (Buxton, 1955; Wijers, 1958; Distelmans et al., 1982; Welburn and Maudlin 1992). Hence, teneral flies are expected to contribute most to the infection rate of a tsetse population. Nevertheless, infection experiments using starved tsetse flies have shown that starvation can significantly increase the susceptibility to trypanosome infections of teneral and non-teneral flies (Gingrich et al., 1982; Welburn et al., 1989; Kubi et al., 2006). The reasons for this increase in susceptibility are not clearly understood.

Many factors and mechanisms, operating at different levels and time points, determine the fate of trypanosomes contained in a bloodmeal in the tsetse midgut (reviewed by Aksoy et al., 2003). The development process of *Trypanosoma brucei* in the tsetse fly has been described in detail (Vickerman, 1985; Van den Abbeele et al., 1999; Gibson and Bailey, 2003). In short, after ingestion by the tsetse fly and an initial period of attrition during which 99% of the trypanosomes are eliminated, the infection establishes in the tsetse’s midgut (Van den Abbeele et al., 1999). This establishment requires the differentiation from the bloodstream forms to procyclic forms followed by rapid multiplication, initially in the midgut lumen and then the ectoperitrophic space. Differentiation into procyclic forms involves the replacement of the variant surface glycoprotein (VSG) coat, which protects the parasite from the mammalian host’s immune response, with procyclins. These are the major surface glycoproteins of midgut forms of the parasite. According to internal amino acid repeats, two types of procyclins can be distinguished i.e. the three EP isoforms (EP1-EP3) and the GPEET procyclin (reviewed by Roditi et al., 1998). They are expressed at the onset of differentiation with the GPEET predominating in the early procyclic form. However, in the later course of infection this GPEET becomes repressed and only the EP-procyclins remain at the trypanosome surface (Vassella et al., 2000; Acosta-Serrano et al., 2001). During midgut colonization, the procyclic trypomastigote population expands in the ectoperitrophic space from the posterior towards the anterior midgut (Van den Abbeele et al., 1999).

Insects are able to initiate a systemic immune response to pathogens in their fat body, where a variety of proteins and peptides with antimicrobial activity are synthesized (reviewed by Lehane et al., 2004). Thus with the fat body being a key centre in the metabolism of tsetse
immune response, physiological changes in tsetse affecting it, such as nutritional stress may result in down-regulation of the immune function (Schmid-Hempel, 2005). The role of the systemic response in tsetse during trypanosome invasion has been assessed by transcriptional regulation of the immunity genes defensin, attacin, diptericin and cecropin (Hao et al., 2001, 2003; Boulanger et al., 2002; Attardo et al., 2006). The possible modification of the tsetse fly’s immune response to pathogen challenge as a result of nutritional stress needs to be investigated.

Since trypanosome establishment in the midgut involves drastic changes in the density, the localization and the surface coat of the trypanosome population, these three processes were examined in starved and non-starved tsetse flies to clarify how starvation affects the establishment of a midgut infection. Furthermore, the possible role of tsetse immune response in the modification of the tsetse fly’s susceptibility as a result of nutritional stress was investigated.

6.2 Materials and methods

6.2.1 Tsetse flies and trypanosomes
Male Glossina morsitans morsitans from the colony maintained at the Institute of Tropical Medicine, Antwerp (Belgium) were used in all the experiments. The origin of the colony and its maintenance are described by Elsen et al. (1993)

Four experimental groups of tsetse flies were used i.e. (Group 1) teneral flies (newly emerged flies less than 24 hours old); (Group 2) teneral flies starved for 4 days; (Group 3) 20 day old adult flies starved for 2 days and (Group 4) 20 day old adult flies starved for 7 days. For the single infective feed, batches of 40 flies were fed on anaesthetized mice showing a parasitaemia of approximately $10^{8.4}$-$10^{8.7}$ trypanosomes/ml blood of the T. b. brucei AnTAR1 strain, derived from the stock EATRO 1125 (Le Ray et al., 1977). This strain was used to determine trypanosome density, localisation and the expression of GPEET and EP procyclins. A clone of this strain transfected with a construct carrying the gene for Red Fluorescent Protein (RFP) under the control of procyclin promoter (provided by Prof. I. Roditi, University of Bern) was used to study the differentiation from bloodstream to procyclic forms.

On days 2 to 6 (daily interval), 8 or 10 after infection, the midguts of flies from the different experimental series were dissected to quantify the trypanosome population, to localize these parasites in the midgut and to determine the expression of the GPEET- and EP-
procyclin at the trypanosome surface. Flies that were dissected six days post infection were fed once on a clean rabbit.

6.2.2 Trypanosome quantification during development in the tsetse midgut
To quantify trypanosome populations during their development, the entire midgut of a fly was isolated and placed in an Eppendorf tube containing 100µl phosphate buffered saline (PBS 10mM, pH 7.4 at 20°C). The trypanosomes were released from the midgut by mechanical disruption and counted using a haemocytometer. For each experimental group at least 20 flies were examined on the various days post-infection.

6.2.3 Localisation during development in the tsetse midgut
To locate the trypanosome infection in the midgut, whole midguts of infected flies from each experimental group and on the various days post-infection were dissected and placed in a drop of PBS on a slide. They were examined as wet mounts and the localization of the trypanosomes was determined by inspecting the (i) posterior midgut (ii) anterior midgut (part of gut between mycetome and proventriculus) and (iii) foregut.

6.2.4 Differentiation to procyclic forms during development in the tsetse midgut
To compare the differentiation of trypanosomes from bloodstream to procyclic forms, entire midguts of flies belonging to one of the four experimental groups were dissected on the various days post infection and placed in Eppendorf tubes containing 100µl phosphate buffered saline (PBS 10mM, pH 7.4 at 20°C). The trypanosomes were released from the midguts by mechanical disruption. At least 100 trypanosomes per fly were first examined under light microscopy (400X magnification) and the relative number of trypanosomes expressing the red fluorescent protein was determined using a fluorescence microscope (Leitz, Ortholux II).

6.2.5 Expression of GPEET and EP during development in the tsetse’s midgut
A drop of 5µl of the midgut trypanosome preparation from positive flies was spread on a glass slide. Cell smears were air dried and fixed with acetone for 10 min at -20°C. They were stored dry over silica gel at -20°C until analysis by immunofluorescence. To detect the presence of GPEET and EP on the trypanosomes, a double immunofluorescence was performed using a rabbit polyclonal anti-GPEET serum (diluted 1:250; kindly provided by I. Roditi, University of Bern) and the anti-EP monoclonal antibody TRBP1/247 (diluted 1:1000; Cedarlane). The secondary antibodies used in this study were respectively, a goat anti-rabbit Texas Red
(1:100) and a goat anti-mouse FITC (1:50) (Sigma). After the final labelling the slides were washed 6 times extensively with PBS. They were mounted with cover slips using a 50% mixture of PBS/glycerol and viewed using a fluorescence microscope (Leitz, Ortholux II). The number of trypanosomes expressing the different procyclins was expressed as a proportion of the total number of trypanosomes examined (at least 200 trypanosomes examined) on different days after infection.

6.2.6 Immunopeptide expression in tsetse in response to immune challenge
To evaluate the bacteria induced immune response in each of the four experimental groups (described above), batches of 40 flies belonging to each of the groups were microinjected either with 2 µl of PBS (control) or with 2µl of a *Escherichia coli* (DH10B strain) cells suspension (OD$_{620}$ : approx. 0.6). To evaluate the trypanosome induced immune response in these experimental groups, a batch of 40 flies of each group was fed either on *T. b. brucei* infected mice (parasitaemia of approximately 10$^{8.4}$ trypanosomes/ml) or uninfected mice (control); only fully engorged flies were retained.

Four days after the immune challenge, whole abdomens were dissected and pooled from three flies in each series (five sets of pooled samples were dissected in each series).

Total RNA was extracted using Tripure® reagent (Roche). Extracted total RNA product was quantified using a Nanodrop® (Nanodrop technologies) spectrophotometer. The samples were treated with DNA-free™ (Ambion) according to manufacturer’s instructions to remove any contaminating genomic DNA.

**First strand cDNA synthesis.** Total RNA (2 µl,[400 ng/µl]) was mixed with 100 pmol primer oligo(dT)$_{15}$ (Promega) and water to a total volume of 13.5 µl, incubated for 5 min at 65°C and immediately chilled on ice for 1 min. Then, 4 µl of Transcriptor RT reaction buffer (Roche), 2 µl of dNTPs (10 mM each) were added. Lastly 0.5 µl Transcriptor Reverse Transcriptase (10 units) (Roche) was added, reaction mixture incubated at 55°C for 30 min followed by an incubation at 85°C for 5 min and then chilled on ice.

**Real time Quantitative PCR.** Real time quantitative PCR (RTQ-PCR) was performed on 1 µl of cDNA reactions in 12.5 µl of iQ SYBR Green Supermix (Biorad) with immunopeptides primers *attacin I, attacin II, defensin* (700nM); *cecropin* (300 nM) and internal control gene primers actin (700 nM) and tubulin (300 nM). The primers used were: *defensin* (forward, 5’-TAGTTTTTGCTTTCTTACAC-3’ and reverse 5’-
CGACTACAGTATCCGCTCTTT-3'); \textit{cecropin} (forward, 5'-ATACTCGCTTTTCAGTCAG-3' and reverse, 5'-CTCTAACAGTAGCGGCAACA-3'); \textit{attacin I} (forward, 5'-TTTTTCAGTGCCACCCATT-3' and reverse, 5'-AAACGCTCTCTTGCAAATCC-3'); \textit{attacin II} (forward, 5'-TAATGTGGTGTCGGGTATGAT-3' and reverse, 5'-TTTGTCGTTGAAGAGGTGGG-3'); actin (forward, 5'-CGCTTCTGGTCGTACTACT-3') and reverse, 5'-CCGGACATCACAATGTTGG-3'); tubulin (forward, 5'-GATGGTCAAGTGCGATCCT-3') and reverse, 5'-TGAGAACTCGCCTTCTTCC-3'). The reactions were performed in a Biorad iCycler IQ detection system (Biorad) and analysed with its software version 3.1. The results of the RTQ-PCR were normalised to the tsetse house keeping genes, actin and tubulin and expressed relative to their respective unchallenged controls. Experiments were done in triplicate to calculate standard deviations.

6.2.7 Statistical analyses
Differences in trypanosomes counts and rate of decline in the different groups were compared using generalised negative binomial regression analyses while differences in differentiation were compared using logistic regression analysis taking into account the random effect of each fly (Stata corp., 2003).

Animal ethics approval for the experiments was obtained from the Animal Ethic Commission of the Institute of Tropical Medicine, Antwerp, Belgium (Ref DG001-PD-M-TT).

6.3 Results

6.3.1 Trypanosome quantification during development in the tsetse midgut
Since the parasitaemia of the infected bloodmeal varied between $10^{8.4}$-$10^{8.7}$ trypanosomes/ml and the average minimum quantity of blood ingested by a male G. \textit{m. morsitans} was about 20µl, we can estimate that each fly initially ingested at least 7.5 x $10^6$ bloodstream trypanosomes. During the first 4 days following this initial infective feed, parasite numbers in the tsetse midgut dropped in all the experimental series (Figure 6.1). With the exception of day three post-infection in the adult non-teneral flies where the reduction was borderline significant (p=0.057), the absolute number of trypanosomes in the midgut of the starved flies (both teneral as well as the adult flies) was significantly reduced (p<0.05) during the first 4 days after the infective bloodmeal. Since all tsetse flies ingested comparable numbers of trypanosomes during the infective bloodmeal, the rate of decline in trypanosome numbers
during the first four days of development was lower in starved flies compared to non starved flies for both non-teneral flies and teneral flies.

6.3.2 Localisation of trypanosomes during development in the tsetse midgut

The localization of trypanosomes on the various days following infection did not differ between the four experimental groups. In all series the development of the midgut trypanosomes progressed according to the scheme presented in Figure 6.2.

**Figure 6.1:** Number of *T. b. brucei* in the midgut of starved and non-starved teneral and non-teneral male *G. m. morsitans* on various days post-infection.

![Figure 6.1](image)

**Figure 6.2:** Location of trypanosomes in different parts of the digestive tract of starved and non-starved teneral and non teneral male *G. m. morsitans* infected with *T. b. brucei* on various days post-infection.

<table>
<thead>
<tr>
<th>Localisation in the digestive tract</th>
<th>Days post infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Posterior gut</td>
<td></td>
</tr>
<tr>
<td>Anterior midgut</td>
<td></td>
</tr>
<tr>
<td>Fore gut</td>
<td></td>
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</tbody>
</table>
6.3.3 Differentiation to procyclic forms during development in the tsetse midgut
Trypanosomes expressing RFP were observed from day three post-infection onwards (Figure 6.3). With the exception of trypanosomes in non starved non-teneral flies, more than 90% of the trypanosomes expressed RFP from eight days post-infection onwards. The proportion of trypanosomes expressing RFP was significantly higher \( (p<0.001) \) in the batches of starved flies.

**Figure 6.3:** Proportion of trypanosomes in starved and non-starved teneral and non-teneral male *G. m. morsitans* infected with *T. b. brucei* expressing the Red Fluorescent Protein on various days post-infection.

The difference in the proportion of trypanosomes expressing RFP between starved teneral and starved non teneral flies was statistically not significant \( (p=0.47) \). On the other hand, the proportion of trypanosomes expressing RFP in the non starved teneral flies was significantly higher \( (p<0.001) \) compared with the proportion of trypanosomes expressing RFP in the non-starved non-teneral flies. Within each experimental series significant \( (p<0.0001) \) differences in the proportion of trypanosomes expressing RFP were observed between individual flies.
Table 6.1: Proportion of trypanosomes in non starved teneral (group 1); starved teneral (group 2); non starved non-teneral (group 3) and starved non-teneral (group 4) male *G. m. morsitans* infected with *T. b. brucei* expressing surface EP and/or GPEET on various days post-infection.

<table>
<thead>
<tr>
<th>Day post-infection</th>
<th>Experimental group</th>
<th>Proportion of trypanosomes (in %) expressing</th>
<th></th>
</tr>
</thead>
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<td></td>
<td></td>
<td>EP</td>
<td>EP+GPEET</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
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<td>10</td>
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<td>100</td>
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*No trypanosomes were observed in this group on this day.*
6.3.5 Expression of GPEET and EP during development in the tsetse midgut
The expression of the two procyclins at the trypanosome surface during midgut development did not differ much between the four experimental groups (Table 6.1). Two days post-infection the majority of trypanosomes expressed both the GPEET- and EP-procyclin at their surface. However, six days post-infection all trypanosomes expressed only a high level of EP-procyclin but did not express GPEET (Table 6.1). Unexpectedly the repression of GPEET was sudden and not gradual as expected.

6.3.6 Immune response to immune challenge after nutritional stress
Levels of immunopeptides genes (attacin I, attacin II, defensin and cecropin) mRNA in non-starved and starved flies were measured using RTQ-PCR. These flies were previously exposed to immune challenge with either bacteria or with trypanosomes. Immune levels in starved teneral flies were generally lower when compared to non starved flies except for one out of two occasions for cecropin (Figure 6.4A and B). Similar results were observed for the non-teneral flies (Figure 6.5A and B). However the immune response levels where slightly higher in starved non-tenerals than starved teneral experimental series.

When flies were challenged with *E. coli* bacteria, immune response levels increased almost 100 fold compared to that of unchallenged flies with no marked differences between the non starved and starved experimental series (Figure 4A). The immune response level was even high (1000 fold) in the non-teneral experimental series (Figure 5A). However, again the response was comparable across the non-starved and starved experimental series (Figure 5A).

After challenge with *T. brucei* immune response levels were poorer than those seen when tsetse where challenged with bacteria. Nevertheless these immune response levels were only slightly higher (2-3 fold) than in unchallenged experimental series teneral flies, especially in non-starved flies but very poor (less than 1) in starved flies, except for cecropin (more than 2 fold) (figure 4B). When adult flies were challenged with *T. brucei* there was very low immune response, less than 1-fold in the non-starved response group and even much lower in the starved experiment series. The *cecropin* level was much lower than in the unchallenged group.
Figure 6.4: Relative normalised levels of attacin I, attacin II, defensin and cecropin measured with RTQ-PCR in starved (S) and non starved (NS) teneral male G. m. morsitans. (A) after challenge with E. coli, (B) after challenge with T. b. brucei and in control groups.
Figure 6.5: Relative normalised levels of *attacin I, attacin II, defensin* and *cecropin* measured with RTQ-PCR in starved (S) and non starved (NS) non-teneral male *G. m. morsitans*. (A) after challenge with *E. coli*, (B) after challenge with *T. b. brucei* and in control groups.
6.4 Discussion

A critical period in the development of trypanosomes in the tsetse’s midgut is the early phase of development. During this phase a large proportion of the trypanosomes that were ingested are eliminated in both the anterior and posterior portions of the tsetse midgut with the slender forms being killed more rapidly than the stumpy forms. (Turner et al., 1988; Van den Abbeele et al., 1999). About 99% of the ingested trypanosomes are eliminated in this phase and any factor reducing this elimination process may enhance the probability that the parasites develop in the fly’s midgut.

The outcome of the experiments described above show that starving teneral and nonteneral tsetse before infection significantly reduces the rate of trypanosome elimination during the early phase of infection. This finding suggests that the high infection rate observed in adult tsetse flies after a period of starvation can be attributed largely to a significant reduction in the process of trypanosome elimination immediately after the ingestion of the infected bloodmeal. If the trypanosomes survive this process of attrition in the early phase then they are able to colonise the midgut, with the procyclic trypomastigote population expanding in the ectoperitrophic space from posterior towards the anterior midgut (Vickerman, 1985; Van den Abbeele et al., 1999; Gibson and Bailey, 2003).

Our observations that there are no differences in the manner in which the trypanosomes colonise the midgut clearly indicates that if they survive the initial barrier to infection and become established the trypanosome development is similar in both starved and non starved teneral and non-teneral flies.

During the differentiation of stumpy bloodstream forms to procyclic forms, EP and GPEET were expressed in both starved and non starved teneral and non teneral flies as earlier reported (Vassella et al., 2000; Acosta-Serrano et al., 2001). However, changes in the midgut environment, in this case due to starvation do not seem to influence the expression of these procyclins on the trypanosome’s surface. Indications are therefore that expression of the procyclins might not be dependent on fly-related factors.

Immune response in tsetse is pathogen specific (Lehane et al., 2004). Our data confirm this finding with *E. coli* inducing a higher immune response than trypanosomes. Interpretation of the immunopeptides levels in challenged and non-challenged (control) teneral and non-teneral flies is difficult. However the results in the control groups after challenge with either *E. coli* or *T. brucei* are in agreement with those of Hao et al. (2001). The preliminary results of the analyses presented suggest that the immunopeptides expression is down-regulated in
starved teneral flies and with the exception of cecropin, which remains low after trypanosome challenge. Immunopeptide expression is also relatively low in non-teneral flies after challenge with *T. b. brucei*. Although there are indications that starvation may affect the relative normalised levels of various immunopeptides investigated in this study it is probably too early to draw conclusions from these preliminary results.
Chapter VII

Ability of trypanosome-infected tsetse flies (Diptera:Glossinidae) to acquire an infection with a second trypanosome species

Adapted from:

Journal of Medical Entomology 42, 1035-1038
7.1 Introduction

Tsetse-transmitted trypanosomiasis is a serious constraint to human and animal health in large parts of sub-Saharan Africa. Tsetse flies, the main vector of the trypanosomes, occur on \( \approx 10 \) million \( \text{km}^2 \) of the African continent. The epidemiology of the disease in humans and livestock is determined by various factors including the proportion of infected tsetse flies. The tsetse’s susceptibility to an infection with cyclically-transmitted trypanosomes is determined by a range of intrinsic and extrinsic factors (Maudlin 1991). Tsetse flies are usually more susceptible to infections with *Trypanosoma congolense*, a trypanosome species that has a shorter development cycle compared to *T. brucei s.l.* Using highly sensitive and species-specific molecular diagnostic tools several researchers have shown that in the field a substantial proportion of the infected tsetse flies carry mixed trypanosome infections (Majiwa and Otieno 1990; McNamara *et al.*, 1995; Masiga *et al.*, 1996; Woolhouse *et al.*, 1996; Morlais *et al.*, 1998; Lehane *et al.*, 2000; Jamonneau *et al.*, 2004). Such mixed infections can be contracted simultaneously from a single animal supporting a mixed trypanosome infection (Van den Bossche *et al.*, 2004) or could be the result of sequential infections over a number of feeds on different infected animals. Moloo *et al.*, (1982) and Gibson and Ferris (1992) have shown that teneral tsetse flies can become infected with different trypanosome species during consecutive bloodmeals. Question remains how the presence of an established midgut or mature trypanosome infection in older flies affects the development of a new, secondary trypanosome infection. Experiments were conducted to determine the competence of older, trypanosome-infected tsetse flies to acquire such a secondary infection.

7.2 Materials and Methods

7.2.1 Trypanosomes

A *T. brucei brucei* strain derived from the stock EATRO 1125 AntAR1 (Le Ray *et al.*, 1977) and *T. congolense* IL1180, a strain originating from the Serengeti region in Tanzania (Geigy and Kauffman, 1973), were used in the experiments.

7.2.2 Tsetse flies

Male *Glossina morsitans morsitans* (Westwood) from the colony maintained at the Institute of Tropical Medicine (Antwerp, Belgium) were used. The origin of the colony and rearing techniques are described by Elsen *et al.* (1993).
7.2.3 Experimental design

Six groups of flies were infected once or twice by feeding on infected mice. Flies were then dissected to determine their infection status according to the schedule presented in Table 7.1. After offering an infected meal, only fully engorged flies were retained. Infected flies were maintained on rabbits and offered the opportunity to feed three times weekly. The rabbits were replaced weekly to avoid cyclic transmission of the trypanosomes.

Prior to dissection, flies were starved for 48 h. The labrum, hypopharynx, salivary glands and the midgut were examined for the presence of trypanosomes. Trypanosome species identification was conducted using the method described by Lloyd and Johnson (1924). Flies of which only the midgut was infected were considered to have an immature infection. Flies with infections in the midgut or salivary glands were considered to have a mature infection. The mature infection rate was calculated as the proportion of dissected flies that developed a mature trypanosome infection. Differences in infection rates were compared using the Fisher Exact Test.
**Table 7.1:** Overview of infection experiments of *G. m. morsitans* with *T. congolense* IL1180, *T. b. brucei* AnTAR1, or both.

<table>
<thead>
<tr>
<th>Infection group</th>
<th>Age in days</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>$Tc_0$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Tc_0-Tb_{20}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Tb_{20}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Tb_0$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Tb_0-Tc_{30}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Tc_{30}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Teneral flies = day 0. *Tb*, infected with *T. b. brucei*; *Tc*, infection with *T. congolense*

**Table 7.2:** Midgut and mature infection rates of male *G. m. morsitans* with number of flies (n), infected according to the infection schedule presented in Table 7.1.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. dissected</th>
<th>Total midgut infection</th>
<th>$Tb$ mature only</th>
<th>$Tc$ mature only</th>
<th>$Tb + Tc$ mature</th>
<th>Total $Tb$ mature</th>
<th>Total $Tc$ mature</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Tc_0$</td>
<td>99</td>
<td>0.35 (35)</td>
<td>NA</td>
<td>0.35 (35)</td>
<td>NA</td>
<td>NA</td>
<td>0.35 (35)</td>
</tr>
<tr>
<td>$Tc_0-Tb_{20}$</td>
<td>212</td>
<td>0.46 (98)</td>
<td>0.038 (8)</td>
<td>0.32 (67)</td>
<td>0.0094 (2)</td>
<td>0.047 (10)</td>
<td>0.33 (69)</td>
</tr>
<tr>
<td>$Tb_{20}$</td>
<td>97</td>
<td>0.27 (26)</td>
<td>0.052 (5)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>$Tb_0$</td>
<td>90</td>
<td>0.51 (46)</td>
<td>0.21 (19)</td>
<td>NA</td>
<td>NA</td>
<td>0.21 (19)</td>
<td>NA</td>
</tr>
<tr>
<td>$Tb_0-Tc_{30}$</td>
<td>124</td>
<td>0.38 (47)</td>
<td>0.17 (21)</td>
<td>0.048 (6)</td>
<td>0.048 (6)</td>
<td>0.22 (27)</td>
<td>0.097 (12)</td>
</tr>
<tr>
<td>$Tc_{30}$</td>
<td>141</td>
<td>0.10 (14)</td>
<td>NA</td>
<td>0.092 (13)</td>
<td>NA</td>
<td>NA</td>
<td>0.092 (13)</td>
</tr>
</tbody>
</table>

*Tb, T. b. brucei; Tc, T. congolense; NA, not applicable*
7.3 Results

In total, of 763 flies, divided over the six experimental groups, were dissected. The infection results are shown in Table 7.2. The *T. b. brucei* or *T. congolense* infection rate of flies infected when respectively 20 and 30 days old (groups *Tb*20 and *Tc*30, respectively) differed little from the infection rate of the same parasites in flies that were previously infected with another trypanosome species (groups *Tc*0-*Tb*20 and *Tb*0-*Tc*30). The *T. congolense* infection rate of flies infected when teneral (*Tc*0) were comparable to the one observed in flies infected with *T. congolense* when teneral and subsequently infected at the age of 20 days with *T. b. brucei* (group *Tc*0-*Tb*20). Similarly, the *T. b. brucei* infection rate of flies infected when teneral (group *Tb*0) was similar to the one in flies that were infected with *T. b. brucei* when teneral and subsequently infected at the age of 30 days with *T. congolense* (group *Tb*0-*Tc*30). The *T. b. brucei* or *T. congolense* infection rate of flies infected when 20 or 30 days old were significantly lower (P < 0.001 for *T. b. brucei* or *T. congolense* infections) than the infection rate when infected as teneral (groups *Tb*20 compared to *Tb*0 and *Tc*30 compared to *Tc*0).

The *T. b. brucei* infection rate in *T. congolense* infected flies was 2.9% (two *T. b. brucei* infected flies out of a total of 69 *T. congolense* infected flies) (group *Tc*0-*Tb*20) compared to 5.2% (5 *T. b. brucei* infected flies out of a total of 97 infected flies) in flies that were infected for the first time at the age of 20 days (group *Tb*20). The differences were statistically not significant (P = 0.7). The *T. congolense* infection rate in *T. b. brucei* infected flies was 22.2% (6 *T. congolense* infected flies on a total of 27 *T. b. brucei* infected flies) (group *Tb*0-*Tc*30 compared to 9.2% (13 *T. congolense* infected flies out of a total of 141 infected flies) in flies that were offered an infective bloodmeal only at the age of 30 days (group *Tc*30). The observed differences in infection rates were again not statistically significant (P = 0.09).

7.4 Discussion

The results from our experiments show that an infective bloodmeal ingested by trypanosome-infected tsetse flies can result in the establishment of a mixed mature trypanosome infection. However, the tsetse’s susceptibility to pick up a trypanosome infection decreases substantially with age. Indeed, the *T. b. brucei* or *T. congolense* infection rates of flies that are offered an infective bloodmeal at 20 days or 30 days of age are
significantly lower than the infection rates of flies that ingested the parasites at teneral stage, just after emergence. This confirms previous reports (Wijers 1958; Distelmans et al., 1982; Mwangelwa et al., 1987; Welburn and Maudlin 1992) and clearly more research is required to determine the molecular background of age-specific changes in the tsetse’s susceptibility to trypanosome infections. Despite this, a substantial proportion of the population of adult tsetse flies can still acquire an infection, even when the fly is already carrying a trypanosome infection. This implies that, in the field, the proportion of infected flies will increase with increasing age as was already observed by various researchers (e.g. Woolhouse and Hargrove, 1998). Based upon our infection data, two important conclusions can be drawn with regard to the development of sequential trypanosome infections in tsetse. Firstly, a secondary infection with a different trypanosome species has no effect on an already established mature infection. Secondly, previous exposure to an infected bloodmeal or the presence of a mature or immature trypanosome infection has no effect on the development and maturation rate of the subsequent secondary infection. Hence, there seems to be little interaction between the two trypanosome species during their development in the midgut and subsequent migration from the midgut to the either the mouthparts or the salivary glands does not seem to be hindered by the presence of a mature infection. Moreover, our data seem to suggest that already infected flies are as susceptible to develop a secondary trypanosome infection compared to non-infected flies of the same age. Considering the small sample size in our experiment, new experiments with a larger sample size may be required to confirm this observation.

The underlying molecular factors that modulate the susceptibility of a tsetse fly for a trypanosome infection are still under debate. Among these factors, components of the insect immune system are assumed to be involved in the natural refractoriness observed in the tsetse fly. Prior studies have clearly demonstrated an induced expression and synthesis of several antimicrobial peptides when teneral flies are offered a trypanosome-infected bloodmeal (Hao et al., 2001; Boulanger et al., 2002). Moreover, it was shown that 20 day old midgut-infected flies have elevated expression levels of two important immune-peptides, attacin and defensin, compared to non-infected flies that had cleared the trypanosomes. This clearly demonstrated that these immune peptides did not affect an already established midgut trypanosome population. Based upon our results, we can hypothesize that this elevated level of defence peptides in midgut-infected flies also does not interfere with the establishment of a new, secondary infection in the midgut of a 20 or 30 day old fly when these flies ingest a new, infective bloodmeal. This would mean that the efficacy of the immune system to interfere with a secondary establishment of trypanosomes in the tsetse midgut is significantly
decreased when a fully grown procyclic trypanosome population is already present in fly’s midgut, although it was shown in prior studies that these flies are fully immunocompetent (Hao et al., 2001).

Notwithstanding the observed vector competence of non-teneral and infected tsetse flies, the prevalence of trypanosome infections in a natural tsetse population is usually low. The low infection rates in adult flies in our experiments confirm that a majority of tsetse flies will not develop a mature infection with neither T. b. brucei nor with T. congolense, even when the infected bloodmeal was offered to teneral flies. This indicates that a high proportion of the tsetse population is refractory to a trypanosome infection. This heterogeneity in the susceptibility to trypanosome infections in tsetse and the apparent absence of competition between trypanosome species infecting a fly may result in the aggregation of infections in susceptible part of the tsetse population and the large proportion of mixed infections observed in the field (e.g. Majiwa and Otieno 1990; McNamara et al., 1995; Masiga et al., 1996; Woolhouse et al., 1996; Morlais et al., 1998; Lehane et al., 2000; Jamonneau et al., 2004).
Chapter VIII

Effect of Isometamidium chloride treatment on the susceptibility of tsetse flies (Diptera: Glossinidae) to trypanosome infections

Adapted from:

Journal of Medical Entomology 43, 564-567
8.1 Introduction

Tsetse-transmitted trypanosomiasis is one of the major constraints to sustainable rural development in a large-part of sub-Saharan Africa. Recent work on the Island of Zanzibar has demonstrated that the release of sterile male tsetse flies can be an effective method to eradicate tsetse in isolated pockets and thus result in a permanent solution to the trypanosomiasis problem (Vreysen et al., 2000). However, a possible unwanted repercussion of the release of large numbers of sterile male flies is a significant increase in the number of potential vectors of trypanosomes in the initial phase of the control campaign. To prevent these sterile males from picking up trypanosome infections and acting as vectors, a first bloodmeal containing isometamidium chloride, a trypanocidal drug with prophylactic action, is usually given before release. The effect of isometamidium chloride on trypanosome infections in the tsetse fly has already been documented in the literature. Hawking (1963) demonstrated that isometamidium chloride administered at a dose of 100 µg/ml of blood was capable of clearing *Trypanosoma congolense*, *T. vivax* and *T. brucei* infections in tsetse. Other workers, using tsetse maintained on sheep treated with isometamidium chloride (Agu, 1984) or in an *in vitro* system of defibrinated cow blood containing 100 µg/ml of blood (Agu, 1985) showed that the drug was capable of eliminating mature and immature *T. vivax* infections in tsetse. Similarly, feeding infected *Glossina morsitans centralis* (Diptera: Glossinidae) on fresh pig blood containing isometamidium resulted in a significant reduction in the *T. congolense* and *T. brucei* infection rates (Jefferies and Jenni, 1987a, b). However, recent findings clearly show that a proportion of the tsetse population can still become infected with *T. congolense* trypanosomes after a first bloodmeal. Such infections could be prevented through prophylactic treatment of the released flies. The “prophylactic” effect of a trypanocide, administered to the first bloodmeal, on the ability of released flies to acquire a trypanosome infection has not been determined so far. Therefore, we performed a series of experiments to determine the efficacy of isometamidium chloride to prevent trypanosome development in the tsetse fly when administered to the fly’s first bloodmeal. In the first series of experiments, we determined the prophylactic effect of two different doses of the drug. In the second series of experiments the ability of the treated flies to transmit isometamidium chloride–resistant trypanosome strains was assessed.
8.2 Materials and Methods

8.2.1 Tsetse flies
Male *G. morsitans morsitans* (Westwood) (< 32h old), from the tsetse fly colony at the Institute of Tropical Medicine of Antwerp (Belgium), were used in the experiments. The origin of this tsetse colony and the conditions of maintenance are described by Elsen *et al.* (1993).

8.2.2 Trypanosomes
*Trypanosoma brucei brucei* AnTARI (stock EATRO 1125) originally isolated from a bushbuck in Uganda (Van Meirvenne *et al.*, 1975) and three isogenic clones of *T. congolense* IL 1180 were used in the experiments. The origin of these isogenic clones is described by Delespaux *et al.* (2005). These clones have different levels of resistance to isometamidium chloride and are genetically identical apart from the mutation(s) underlying the isometamidium chloride resistance phenotype. The susceptible clone (R₀) has a CD₅₀ (the curative dose that gives complete cure in 50% of the animals) in mice of 0.018 mg/kg. The resistant clones have a CD₅₀ of 1.8 mg/kg (R₁₀₀) and 3.6 mg/kg (R₂₀₀) for the low and high resistant clone, respectively.

8.2.3 Isometamidium chloride treatment of flies
All trypanocide-treated flies received a first bloodmeal containing isometamidium chloride 32 hours after eclosion (day 0). The drug was administered by feeding the experimental flies through a silicone membrane on defibrinated bovine blood that contained either 10 µg or 100 µg isometamidium chloride (Samorin®, Rhône-Mérieux, France)/ml blood. Sterile gamma-irradiated defibrinated bovine blood was obtained from the International Atomic Energy Agency (Vienna, Austria). To ensure that all experimental flies included in the experiment had ingested the trypanocide, only fully engorged flies were retained. After this first *in vitro* bloodmeal, all flies were fed on the ears of a healthy rabbit (*in vivo*), three times per week. The control fly groups were fed their first bloodmeal on isometamidium chloride-free defibrinated bovine blood.

8.2.4 Infection of flies
Different groups of isometamidium chloride-treated and control (n=50 flies per group) received a single infective bloodmeal either on days 3, 5, 10 or 20 after their first bloodmeal. For all these infective bloodmeals, flies were fed on anaesthetized NMRI mice that had an
infection with either *T. congolense* or *T. b. brucei* at a parasitaemia of at least 10^{8.4} parasites per milliliter of blood estimated according to the scale of Herbert and Lumsden (1976). Only fully engorged flies were retained. To determine the effect of isometamidium chloride on the fly’s infection rate with the resistant trypanosome strains, flies treated with 10 µg isometamidium chloride /ml blood received an infective bloodmeal with the resistant strains 3 days after the first feed.

To avoid re-infection of the flies with *T. b. brucei* or *T. congolense* during the *in vivo* maintenance, rabbits were replaced at weekly intervals. Thirty days after the initial infective bloodmeal, all surviving flies were dissected using the method described by Lloyd and Johnson (1924). Their midgut and mouthparts or salivary glands were examined for the presence of trypanosomes.

### 8.2.5 Data analysis

Statistical analyses were carried out in Stata 7 (StataCorp., 2001). Two types of analyses were used. A Poisson regression was used to analyse the data from the experiments determining the effect of dose and age on the *T. congolense* and *T. b. brucei* infection rate. Considering the number or zero infections, preference was given to a Poisson regression over a logistic regression. Hereby the flies' age, the isometamidium dose and the interaction between the two were used as explanatory variables. The number of flies dissected was entered as population exposed. A Poisson regression is appropriate for binomial data provided that the proportion of infection is low and that the population exposed is taken into account. The comparison of the infection rates of flies infected with resistant *T. congolense* strains was carried out using a logistic regression.

### 8.3 Results

Feeding teneral tsetse flies a first bloodmeal that contained isometamidium chloride significantly reduced the fly’s subsequent immature (midgut) and mature (mouthparts/ *T. congolense* or salivary glands/*T. brucei*) infection rate compared to the control flies (Tables 8.1 and 8.2). This effect is visible in the experimental flies that received the infective bloodmeal within 5 days after the first bloodmeal. In these series, only 6.8% (7/103) of the flies that received isometamidium chloride in the first bloodmeal (regardless of the dose) finally developed a mature infection of *T. congolense* in the mouthparts compared to 34.3% (24/70) for the control group (Table 8.1).
Table 8.1: Infection rates of male *G. m. morsitans* tsetse flies given a first bloodmeal on defibrinated bovine blood containing isometamidium chloride and subsequently infected with *T. congolense* (IL1180 clone R0) on day 3, day 5, day 10 and day 20 after their first feed.

<table>
<thead>
<tr>
<th>Dose rate isometamidium chloride (in µg/ml)</th>
<th>Day of infection</th>
<th>No. dissected</th>
<th>Proportion of infected flies</th>
<th>Maturation Rate&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Midgut (Immature)</td>
<td>Mouthparts (Mature)</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>20</td>
<td>0.20 (4)</td>
<td>0.10 (2)</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>19</td>
<td>0.16 (3)</td>
<td>0.11 (2)</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>21</td>
<td>0.00 (0)</td>
<td>0.00 (0)</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>29</td>
<td>0.03 (1)</td>
<td>0.03 (1)</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
<td>29</td>
<td>0.14 (4)</td>
<td>0.03 (1)</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>35</td>
<td>0.09 (3)</td>
<td>0.06 (2)</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>31</td>
<td>0.10 (3)</td>
<td>0.03 (1)</td>
</tr>
<tr>
<td>100</td>
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<td>17</td>
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<td>0</td>
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<td>37</td>
<td>0.27 (10)</td>
<td>0.22 (8)</td>
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<td>0</td>
<td>5</td>
<td>33</td>
<td>0.54 (18)</td>
<td>0.48 (16)</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>32</td>
<td>0.06 (2)</td>
<td>0.06 (2)</td>
</tr>
<tr>
<td>0</td>
<td>20</td>
<td>27</td>
<td>0.04 (1)</td>
<td>0.04 (1)</td>
</tr>
</tbody>
</table>

Flies were dissected 30 days postinfection

<sup>a</sup>Maturation rate = n mature infections / n midgut infections.

Moreover, none of the flies (0/105) that were administered isometamidium chloride and subsequently infected on day 3 or 5 with *T. b. brucei* developed a metacyclic infection in the salivary glands, whereas 22.7% (15/66) of the control flies were found positive upon dissection (Table 8.2). The level of significance of the observed differences between the infection rates (*T. congolense* and *T. brucei*) of isometamidium chloride-treated flies (at 10 or 100 µg/ml blood) and the control flies are summarised in Table 8.3.
Table 8.2: Infection rates of male *G. m. morsitans* tsetse flies given a first bloodmeal on defibrinated bovine blood containing isometamidium chloride and subsequently infected with *T. brucei brucei* (AnTAR1 clone) on day 3, day 5, day 10 and day 20 after their first feed.

<table>
<thead>
<tr>
<th>Dose rate isometamidium chloride (in µg/ml)</th>
<th>Day of infection</th>
<th>No. dissected</th>
<th>Proportion of infected flies</th>
<th>Maturation rate&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Midgut (Immature)</td>
<td>Salivary glands (Mature)</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>20</td>
<td>0.15 (3)</td>
<td>0.00 (0)</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>20</td>
<td>0.15 (3)</td>
<td>0.00 (0)</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>21</td>
<td>0.14 (3)</td>
<td>0.00 (0)</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>22</td>
<td>0.04 (1)</td>
<td>0.00 (0)</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
<td>33</td>
<td>0.06 (2)</td>
<td>0.00 (0)</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>32</td>
<td>0.00 (0)</td>
<td>0.00 (0)</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>27</td>
<td>0.18 (5)</td>
<td>0.00 (0)</td>
</tr>
<tr>
<td>100</td>
<td>20</td>
<td>22</td>
<td>0.04 (1)</td>
<td>0.00 (0)</td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>32</td>
<td>0.22 (7)</td>
<td>0.16 (5)</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>34</td>
<td>0.44 (15)</td>
<td>0.29 (10)</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>28</td>
<td>0.18 (5)</td>
<td>0.07 (2)</td>
</tr>
<tr>
<td>0</td>
<td>20</td>
<td>29</td>
<td>0.07 (2)</td>
<td>0.00 (0)</td>
</tr>
</tbody>
</table>

Flies were dissected 30 days postinfection

<sup>a</sup>Maturation rate = n mature infections / n midgut infections.

The effects of isometamidium chloride on the mature *T. congolense* or *T. brucei* infection rate were not affected by the dose of isometamidium chloride (P = 0.311 and P = 0.626 *T. congolense* and *T. brucei*, respectively). In all fly groups (either treated with isometamidium chloride or untreated) the infection rates decreased significantly when flies were infected on day 10 or 20 post-emergence.

A single treatment with isometamidium chloride (10 µg/ml blood) also significantly (P<0.001) reduced the fly’s subsequent immature (or midgut) and mature (proboscis) infection rates when infected with *T. congolense* strains that are resistant to the drug (Table 8.4). In total, only 6.5 % (13/201) of the flies that were administered isometamidium chloride and infected with one of the two *T. congolense* isometamidium chloride-resistant strains
developed a mature infection in the proboscis compared to 33.5% (65/196) for the untreated flies infected with the fully susceptible trypanosome strains.

Table 8.3: Significance (P-value) of the difference between the immature and mature *T. congolense* or *T. b. brucei* infection rates of isometamidium chloride-treated and control flies.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Immature</th>
<th>Mature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>T. congolense</em></td>
<td><em>T. brucei</em></td>
</tr>
<tr>
<td>10</td>
<td>0.038</td>
<td>0.07</td>
</tr>
<tr>
<td>100</td>
<td>0.004</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Table 8.4: Infection rates of male *G. m. morsitans* tsetse flies given a first bloodmeal on defibrinated bovine blood containing 10 μg/ml isometamidium chloride and subsequently infected on day 3 with one of two drug-resistant *T. congolense* strains (IL1180 clones R100 or R200).

<table>
<thead>
<tr>
<th><em>T. congolense</em> strain&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Group</th>
<th>Number dissected</th>
<th>Proportion infected flies</th>
<th>Maturation Rate&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Midgut</td>
<td>Mouthparts</td>
</tr>
<tr>
<td>R&lt;sub&gt;100&lt;/sub&gt;</td>
<td>Treated</td>
<td>104</td>
<td>0.11 (12)</td>
<td>0.07 (7)</td>
</tr>
<tr>
<td>R&lt;sub&gt;100&lt;/sub&gt;</td>
<td>Control</td>
<td>100</td>
<td>0.43 (43)</td>
<td>0.43 (43)</td>
</tr>
<tr>
<td>R&lt;sub&gt;200&lt;/sub&gt;</td>
<td>Treated</td>
<td>97</td>
<td>0.07 (7)</td>
<td>0.06 (6)</td>
</tr>
<tr>
<td>R&lt;sub&gt;200&lt;/sub&gt;</td>
<td>Control</td>
<td>96</td>
<td>0.24 (23)</td>
<td>0.23 (22)</td>
</tr>
</tbody>
</table>

<sup>a</sup>*T. congolense* IL1180 R<sub>100</sub> : CD<sub>50</sub> = 1.8 mg/kg; and *T. congolense* IL1180 R<sub>200</sub> : CD<sub>50</sub> = 3.6 mg/kg

<sup>b</sup>Maturation rate = n mature infections / n midgut infections.

8.4 Discussion

These data show that a first bloodmeal on defibrinated bovine blood that contains isometamidium chloride significantly reduces the tsetse fly’s ability to become infected with *T. congolense* or *T. brucei*. The effect of isometamidium chloride administration on the trypanosome infection rate can be observed for a period of at least 5 days after the meal containing the drug after which the tsetse fly (treated as well as untreated) susceptibility to a trypanosome infection declines substantially. For suppression of the *T. congolense* and *T. b.*
*brucei* development in the fly, a dose of 10µg isometamidium chloride/ml blood sufficed to obtain this effect.

The underlying mechanism by which isometamidium chloride affects the fly’s susceptibility for trypanosome infection is not clear. One possibility would be that the active compound resides in the fly for some period having a direct toxic effect on the trypanosomes in the midgut. However, the observed highly reduced infection rates in the treated flies of the *T. congolense* isogenic lines that are resistant to the drug directly conflicts with this possibility. Another possibility is that the isometamidium chloride administered during the fly’s first bloodmeal affects the midgut microbial environment. It has been shown that isometamidium chloride has some bactericidal activity (Khafagi *et al.*, 2003). Affecting this microbial fauna in the tsetse fly’s midgut could result in less favourable conditions for the establishment and the growth of procyclic trypanosomes. This could offer an explanation for both the highly reduced establishment of immature infections as well as the reduced maturation rate that were observed in flies fed with isometamidium chloride. With respect to the latter, it has been shown previously that the maturation rate of established procyclic trypanosomes is positively correlated with their growth rate in the tsetse midgut (Dale *et al.*, 1995). Hence, a detrimental effect on the growth rate of the procyclic trypanosomes will significantly reduce the maturation rate of these trypanosomes. Further studies of the effect of isometamidium chloride on the tsetse midgut environment would be valuable to give more insights into the factors that determine the vectorial ability of tsetse flies for trypanosomes.

Although past observations clearly show an effect of the drug on established or establishing trypanosome infections, they substantially differ from our experimental setup where the “prophylactic” effect of a single bloodmeal containing isometamidium chloride has been evaluated. In practice and with respect to the release of sterile male flies to eradicate an isolated tsetse fly population, our results show that administering isometamidium chloride during the first bloodmeal (and before release) would significantly reduce the ability of these released males to transmit *T. congolense* or *T. brucei*. 
Chapter IX
General Discussion
9.1 Introduction

Tsetse-transmitted trypanosomiasis is one of the most important diseases afflicting human beings and hindering sustainable livestock development in rural sub-Saharan Africa. The complex epidemiology of trypanosomiasis is determined largely by the proportion of trypanosome-infected flies transmitting the parasite (Leak, 1999; Aksoy et al., 2003). The proportion of infected tsetse flies is affected by a range of intrinsic and extrinsic factors. A large proportion of tsetse flies are considered to be refractory to trypanosomal infections. Nevertheless, the complex interactions between the tsetse fly and trypanosomes are not well understood. A better understanding of those mechanisms is important since this may lead to innovative disease control measures by disrupting the trypanosome transmission cycle. The work described in this thesis aimed at obtaining a better understanding of how some tsetse-related factors affect the susceptibility of tsetse to a trypanosome infection and describe the importance of these changes in the epidemiology of animal and human trypanosomiasis.

This discussion contains the following further sections. In the second section, important and relevant findings on the age/prevalence relationship of trypanosomal infections in female Glossina morsitans morsitans from a field population in Eastern Province of Zambia are discussed in combination with the findings of a laboratory study on the age-specific susceptibility of tsetse to trypanosome infections. In the third section, we explored the effect of starvation on the tsetse fly’s susceptibility to trypanosome infection, the course of the development of trypanosomes in tsetse’s midgut and the possible effect on the tsetse’s immune response to bacterial and trypanosome challenge. In the fourth section, the ability of infected adult tsetse to acquire a secondary trypanosome infection is discussed. The fifth section explores the susceptibility of tsetse treated with a single dose of isometamidium chloride and the potential application of this treatment in the SIT operations. Finally, the conclusions drawn from the experimental sections and the implications, potential application(s) and future research prospects are discussed in the sixth section.

9.2 Age/prevalence relationship of trypanosomal infections

The age-specific susceptibility of tsetse flies to trypanosome infections plays a crucial role in all trypanosomiasis transmission models. Generally speaking, tsetse flies are considered to be most susceptible to infection before they have taken a first blood meal (Wijers, 1958;
Welburn and Maudlin, 1992). A large majority of infected flies are thus considered to have acquired the infection during the first bloodmeal, leading to a general concept that only teneral flies, and not adult flies contribute to the overall infection prevalence of a tsetse population.

In chapter 4, the age/prevalence relationship of trypanosomai infections of *G. m. morsitans* in eastern province Zambia was studied. Using a mathematical model, this relationship has been described to provide estimates of the developmental period of trypanosomes in tsetse as well as the age-specific susceptibility to infection. Whereas previous studies (Woolhouse et al., 1993, 1994) described this age/prevalence relationship for mature or metacyclic infections only, our study added another dimension to the analysis by also investigating the age-prevalence relationship of immature or midgut infections.

Previous data on prevalence of trypanosomal infections in *G. m. morsitans* from central and eastern Zambia showed a high prevalence of *vivax*-type infections (Clarke, 1969; Okiwelu, 1977). *Vivax*-type infections were also predominant in *G. pallidipes* from the Luangwa Valley of eastern Zambia (Woolhouse et al., 1994). Contrary to these earlier findings, results of our study show that *Trypanosoma congolense* is the most prevalent in the study area. This is in accordance with the high bovine trypanosomiasis prevalence caused by *T. congolense* on the eastern plateau as is the case in other parts of eastern Zambia (Machila et al., 2001) and most other countries of southern Africa (Sigauque et al., 2000; Van den Bossche et al., 2000, 2001, 2006).

The results also show that the developmental period of *congolense*-type and *vivax*-type infections correspond well with those observed earlier by Elce (1971) and Davies (1977). More importantly, the age/prevalence curves of immature and *congolense*-type infections suggest that wild mature tsetse also acquire new trypanosomal infections. Indeed these data were confirmed with our laboratory study on the age specific susceptibility of tsetse (Chapter 5). The fact that adult flies acquire trypanosome infections that mature is in conflict with laboratory evidence (Distelmans, 1982; Mwangelwa et al., 1987; Welburn and Maudlin, 1992; Dale et al., 1995). Nevertheless, Harley (1967) and Woolhouse et al. (1993, 1994) also found increasing prevalence of *T. congolense* infections with age in several species of female tsetse flies. The observed relationship between age and the prevalence of *T. congolense* infections in *G. m. morsitans* on the plateau of eastern Zambia suggests that either the development period of *T. congolense* in tsetse is very variable or that adult tsetse flies still acquire trypanosome infections. The latter hypothesis was studied further in the following chapters of the thesis.
9.3 Effect of starvation on susceptibility of tsetse to trypanosome infections

Despite the “tsetse infection paradigm” that only teneral flies are highly susceptible to trypanosome infections, our field data and those of other researchers have confirmed that susceptibility to trypanosome infection seems not only limited to newly emerged tsetse flies. This is an important observation that may have repercussion for the epidemiology of HAT where the proportion of trypanosome infected tsetse flies is typically low. Indeed, in such a case, any factor that results in an increase in the susceptibility of the fly has serious repercussion on the epidemiology of the disease. Some indirect evidence exists suggesting that under nutritional stress or as a result of starvation, the susceptibility of teneral and adult tsetse to trypanosome infections increases (Wijers, 1958; Gingrich, 1982). The effect of starvation on the modulation of the vectorial ability of tsetse to transmit *T. congolesense* and *T. brucei brucei* was investigated in more detail (Chapter 5).

The outcome of those investigations shows that the nutritional status of a tsetse fly at the time of the infective bloodmeal affects its susceptibility to infection with *T. congolesense* as well as *T. brucei*. Indeed we observed that an extreme period of starvation (up to 4 days for teneral flies and 7 days for adult flies) lowers the development barrier for a trypanosome infection especially at the midgut level. This was further confirmed by the reduced rate of trypanosome elimination during the early phase of trypanosome development in the tsetse fly midgut (Chapter 6). It is plausible that when a tsetse fly is subjected to nutritional stress causing a serious depletion of its energy reserves, the immune functions are down-regulated (Schmid-Hempel, 2005). This may not be surprising considering the important role played by the fat body in the production of immunopeptides. This favourable environment will facilitate the growth of procyclic trypanosomes in the tsetse’s midgut and result in an increased mature infection rate. This hypothesis of the effect of starvation on the tsetse’s immune response is supported by the preliminary results of experiments that show a down-regulation of immunity genes in starved flies in response to especially bacterial challenge (Chapter 6). However, the immune response was relatively low in response to *T. brucei* challenge. This could be expected as immune response in tsetse and other insects is pathogen specific (Lehane *et al.*, 2004). Indeed immune response to trypanosome challenge was observed to be lower than that of *E. coli* by others (Hao *et al.*, 2001). Starvation did not seem to have any influence on expression of procyclins, EP and GPEET as results were similar in starved and non starved tsetse flies (Chapter 6).
The laboratory findings of the effect of starvation on the vector competence of tsetse flies may have important repercussions for our understanding of the complex epidemiology of animal and human trypanosomiasis. First, the proportion of infected flies in a tsetse population may not only be determined by the proportion of newly emerging flies that take up an infective meal but also by the environmental factors such as ambient temperature, that may reduce the tsetse’s energy reserves (Van den Bossche and Hargrove, 1999) and increase its susceptibility to infection. It is therefore possible that under field conditions and in certain periods of the year when nutritional stress is highest, the overall infection rate of a tsetse population is determined also by the proportion of non-teneral flies that acquire a (mature) trypanosome infection. Second, our findings may have repercussions for the distribution and prevalence of infections with trypanosome that are difficult to transmit such as *T. b. rhodesiense* (Welburn *et al.*, 1995). Indeed, since our results suggest that local conditions can enhance the susceptibility of teneral and non-teneral flies to infection, the distribution of those parasites may be limited to areas where the conditions for transmission and fly susceptibility are the most favourable. Third, our findings may have repercussion for the control of tsetse using SIT where large populations of sterile male tsetse are released. Even though the released male tsetse flies have received one or more initial bloodmeals before release, our results suggest that they can become efficient vectors under particular environmental conditions.

**9.4 Susceptibility of adult trypanosome infected tsetse flies to secondary trypanosome infections**

Several studies have shown that in the field, a substantial proportion of infected tsetse flies carry mixed trypanosome infections (e.g. Lehane *et al.*, 2000; Jammoneau *et al.*, 2004). These mixed infections can be the result of tsetse feeding on one animal carrying a mixed trypanosome infection (Van den Bossche *et al.*, 2004) or of tsetse feeding subsequently on a number of different infected animals. The latter would suggest that adult tsetse flies with a mature or immature trypanosome infection are capable of acquiring an additional infection. Chapter 7 describes a number of studies assessing the vector competence of infected tsetse flies.

The results of those studies showed that tsetse can acquire a secondary infection and that a secondary infection has no effect on the existing established mature infection or vice versa. This finding is in agreement with findings of Allander and Schmid-Hempel (2000)
investigating the immune defence reaction of the bumble-bee (*Bombus terrestris* L.) to *Crithidia bombi*. They found that the immune response, in their case melanisation, was not necessary enhanced by a previous challenge. Infected tsetse flies are as susceptible to a trypanosome infection when compared to non-infected flies of the same age groups. Nevertheless, Hao *et al.*, (2001) showed that 20-day-old midgut infected tsetse flies have elevated expression levels of two important antimicrobial peptides, attacin and defensin, compared with tsetse flies that had cleared trypanosomes from their midguts. The reasons for this susceptibility despite the increased level of immunopeptide expression are not fully understood.

The heterogeneity in the susceptibility to trypanosome infections in tsetse and the apparent absence in competition between trypanosomes infecting a fly may provide an explanation for the apparent aggregation of infections in the susceptible part of a tsetse population and the large proportion of mixed infections observed in the field.

### 9.5 Susceptibility of tsetse flies treated with isometamidium chloride to trypanosome infections

Recent studies have shown that the release of sterile male tsetse flies can be an effective method to eradicate tsetse in isolated pockets thus resulting in a permanent solution to the tsetse transmitted trypanosomiasis problem (Vreysen *et al.*, 2000). One possible disadvantage of the release of large numbers of sterile male tsetse is the significant increase in the number of potential vectors of trypanosomes (Chapter 5 and 7) in the early stages of the control campaign. To avoid this problem, isometamidium chloride, a trypanocidal drug with prophylactic action is administered by offering an isometamidium chloride-treated bloodmeal before the tsetse flies are released. Though the effect of isometamidium chloride on trypanosomal infections in tsetse is well documented (Hawking, 1963; Agu, 1984, 1985, Jeffries and Jenni, 1987), the “prophylactic” effect of a trypanocide administered to the first bloodmeal on the ability of released tsetse to acquire a trypanosome infection has not been determined yet.

In this study the susceptibility of *G. m. morsitans* to an infection with susceptible or resistant *T. congolense* stains after a single dose of isometamidium chloride contained in the first meal was evaluated. The results (Chapter 8) show that a dose of 10 µg of isometamidium chloride/ml of blood sufficed to suppress the development of *T. congolense* and *T. b. brucei* strains for at least 5 days after the treated bloodmeal. One possible mechanism by which the
isometamidium chloride affects the susceptibility to trypanosome infection is by having a persistent direct toxic effect on the trypanosomes in the midgut. However, the observed highly reduced infection rates in treated flies infected with *T. congoense* strains that are resistant to isometamidium chloride rejects this hypothesis. It thus seems more likely that isometamidium chloride affects the endosymbionts in the midgut of the tsetse fly as a result of its bactericide activity (Khafagi *et al.*, 2003). This may result in conditions that are less favourable for the establishment and development of trypanosomes in the midgut.

### 9.6 Conclusions and future prospects

The outcome of the studies presented in this thesis has contributed to an improved understanding of the complex epidemiology of tsetse-transmitted trypanosomiasis by giving answers to previously unanswered questions on the relationship and factors that determine the adult tsetse fly’s capacity to develop and transmit the trypanosome.

We conclude from the study that non-teneral tsetse are able to acquire a trypanosome infection and can contribute significantly to the proportion of infected tsetse in a population. Our findings are thus in conflict with the “tsetse infection paradigm” that only newly emerged tsetse acquire trypanosome infections. The study has also provided a further insight on the acquisition of mixed infections by tsetse flies and offered an explanation for the presence of high proportions of mixed trypanosome infections in the field populations of tsetse flies. Furthermore, the study has demonstrated that the physiological status of tsetse flies such as the level of starvation significantly affects the susceptibility of tsetse to trypanosome infections. In addition, it has also been demonstrated that a single dose of isometamidium chloride given to teneral male at the first bloodmeal significantly reduced the potential of adult tsetse to acquire a trypanosome infection. This observation has important repercussions for SIT operations.

Future research should be directed at confirming these laboratory findings under field conditions. In this respect, the effect of climatic changes or other factors that are known to induce nutritional stress in the tsetse population on the susceptibility of tsetse to trypanosomes and the subsequent infection status of the tsetse population needs to be determined. Since the studies presented in this thesis concentrated on determining the tsetse’s susceptibility to *T. congoense* and *T. b. brucei* further studies could use human infective trypanosome species i.e. *T. b. rhodesiense* and *T. b. gambiense* to confirm our findings.
Additional work, on the role of antimicrobial peptides in nutritionally stressed tsetse needs to be conducted to elucidate the role of the starved tsetse’s immune response to a trypanosome infection.

Finally, on the basis of the experimental work and the field observations and by adjusting trypanosome transmission parameters, mathematical models could be used to quantify the repercussions of the observed changes in the susceptibility or vector competence on the transmission of trypanosomes in human or animal populations.
References


and their epidemiological significance in a sleeping sickness focus of Cote d'Ivoire. Parasitology, 129, 693-702.


References


StataCorp. (2003) *STATA Statistical software: Release 8.0*. Stata-Corp, College Station, Texas, U.S.A.


Wijers, D. J. B. (1958) Factors that may influence the infection rate of *Glossina palpalis* with *Trypanosoma gambiense*. I. - The age of the fly at the time of the infected feed. *Annals of Tropical Medicine and Parasitology*, 52, 385-390.


SUMMARY

Tsetse-transmitted trypanosomiasis is one of the most important diseases hindering sustainable livestock development in sub-Saharan Africa. The complex epidemiology of the disease is determined largely by the proportion of trypanosome infected tsetse flies (Glossina spp.) transmitting the parasite in the tsetse population which in turn depends on a number of intrinsic and extrinsic factors. The work described in this thesis focused on obtaining a better understanding of how some of the tsetse-related factors affect the susceptibility of tsetse to trypanosome infections and discusses the implications for the epidemiology of animal and human trypanosomiasis.

In Chapter 1, important aspects of the relationship between tsetse flies, trypanosomes and its host are presented and the epidemiology trypanosomiasis and its control are reviewed. Chapter 2 reviews tsetse-trypanosome interactions with emphasis on the factors affecting susceptibility of tsetse to trypanosome infections and the epidemiological implications.

Chapter 3 states the objectives of the thesis. The general objectives were to study different aspects affecting the susceptibility of the tsetse fly, Glossina morsitans morsitans, to an infection with Trypanosoma congolense or T. brucei and evaluate their importance in the epidemiology of animal and human Trypanosomiasis.

In Chapter 4 the results of a study on the age/prevalence relationship of female G. m. morsitans in eastern province of Zambia are presented. Congolense-type infections were identified in 4.8 % of the flies, while vivax-type and immature type were identified in 1.8 % and 6.8 % of the flies respectively. The prevalence of all three types of infections increased with age. The per capita rate at which field flies acquire new trypanosome infections was found to be significantly higher for immature compared to mature infections. These observations strongly suggest that tsetse acquire new midgut infections at any age and that maturation of the infection is not limited to those obtained during the first blood meal.

In Chapter 5 experimental investigations confirmed earlier findings presented in chapter 4 that teneral flies are more likely to acquire and develop a mature infection but that mature tsetse flies still remain susceptible though at a significantly lower rate. However, the nutritional status of the fly at the time of the infective feed affects the fly’s ability to acquire a T. congolense or T. brucei infection. Extreme periods of starvation in adult flies (up to seven days) produced infections comparable to teneral flies. Moreover, the maturation rate of T. brucei infections established in the midgut was significantly increased. We propose that under
natural conditions nutritional stress in adult tsetse flies contributes significantly to the epidemiology of tsetse-transmitted trypanosomiasis.

Chapter 6 presents results from investigations on the effect of starvation on the developmental process of trypanosomes in tsetse fly midguts by examining changes in density, localisation and surface coat of the trypanosomes. The expression of EP and GPEET procyclins seems to be independent of fly-related factors. Furthermore, a preliminary study on changes in the tsetse’s immune response after starvation is presented. Results of the studies showed that starvation reduced the rate of trypanosome elimination during the early phase of infection but seems to have no effect on colonisation of the midgut by trypanosomes and the expression of procyclins on the trypanosomes’ surface. Results of the study on immune response indicate that in tsetse flies expression of certain immunopeptides is pathogen specific and seems to be down-regulated in nutritionally stressed flies. However, further studies are required to confirm those results.

In Chapter 7, results from experiments to determine the ability of adult-trypanosome infected tsetse flies to acquire a secondary trypanosome infection showed that adult flies with an existing *T. brucei* or *T. congolense* infection remained at least as susceptible to a secondary infection when compared to non-infected flies of the same age. We conclude that a secondary infection with another trypanosome species has no effect on an already established mature infection and that previous exposure to an infective blood meal or presence of an immature infection has no effect on the development or maturation rate of the subsequent infection. Our data helps to explain the aggregation of infections in the susceptible population of a tsetse population and the large proportion of mixed infection observed in the field.

Chapter 8 present the findings of investigations on the effect of a single treatment of tsetse with isometamidium chloride during the first blood meal on the tsetse’s subsequent susceptibility to infection with *T. congolense* or *T. b. brucei*. Results show that a single treatment of flies with the trypanocide sufficed to reduce its susceptibility to a trypanosome infection. This was also the case for infections with *T. congolense* strains that were resistant to isometamidium chloride. We propose that the administration of isometamidium chloride to tsetse flies in the first blood meal is an effective means to reduce the vector competence of tsetse flies released during control campaigns using the sterile insect technique.

In the general discussion (Chapter 9), the most important results presented in the different chapters of the thesis are highlighting and their implications for the epidemiology of tsetse transmitted-trypanosomiasis are discussed.
SAMENVATTING

Door tseetsee vliegen (Glossina spp.) overgedragen trypanosomiasis is een van de belangrijkste veeziekten die duurzame ontwikkeling van de veestapel in sub-Sahara Afrika verhinderen. De ingewikkelde epidemiologie van de ziekte wordt hoofdzakelijk bepaald door het percentage tseetsees geïnfecteerd met trypanosomen in een populatie. Deze infectiegraad wordt bepaald door een aantal interne en externe factoren. Het onderzoek beschreven in deze thesis is gericht op enkele van die factoren die de gevoeligheid van de tseetsee vlieg voor een infectie met trypanosomen kunnen beïnvloeden en het effect van die factoren op de vectoriële capaciteit van de vliegen en derhalve op de epidemiologie van dierlijke en menselijke slaapziekte.

In het eerste hoofdstuk van de thesis wordt een algemene beschrijving gegeven van de epidemiologie en controle van door tseetsee vliegen overgedragen trypanosomiases. Bovendien worden belangrijke aspecten van de relatie tussen de vector (tseetsee vlieg), de parasiet (trypanosoom) en de zoogdieren-gastheer weergegeven.

Het tweede hoofdstuk geeft een algemeen overzicht van de bestaande literatuur over de bekende factoren die de gevoeligheid van tseetsee vliegen voor infecties met trypanosomen bepalen, met speciale aandacht voor externe en interne factoren. Vooral het toenemende belang van het immuunsysteem van de vlieg in het bepalen van het verloop van een trypanosoom infectie wordt uitvoerig beschreven. Tenslotte wordt het belang van deze diverse factoren voor de epidemiologie van dierlijke en menselijke trypanosomiasis besproken.

In het derde hoofdstuk worden de objectieven van de thesis beschreven. De algemene doelstelling is de bepaling van het effect van een verandering in de fysiologische status van tseetsee vliegen op hun gevoeligheid. Aan de hand van deze bevindingen wordt getracht om een verklaring te vinden voor de ouderdomsgebonden toename van de prevalentie van trypanosoom infecties in tseetsee vliegen in veldpopulaties. Bovendien wordt een manier beschreven om de gevoeligheid van tseetsee vliegen voor infecties met trypanosomen drastisch te verminderen.

Het vierde hoofdstuk geeft de resultaten weer van een studie, uitgevoerd in oostelijk Zambia, ter bepaling van de infectiegraad van vrouwelijke vliegen (Glossina morsitans morsitans) van verschillende leeftijden. In het studiegebied was 4.8% van de gevangen vliegen geïnfecteerd met Trypanosoma congolense. Infecties met Trypanosoma vivax en immature infections (infecties beperkt tot de darm) werden respectievelijk gevonden in 1.8% en 6.8% van de vliegpopulatie. In tegenstelling tot de verwachtingen, stegen deze percentages
voor al de verschillende infectietypes met toenemende leeftijd van de vliegen; doorgaans werd verondersteld dat het merendeel van de tseetsee vliegen zich infecteert met *T. congolense* tijdens het eerste bloedmaal. Als dusdanig kan de proportie van de met *T. congolense* geïnfecteerde vliegen slechts toenemen vanaf het ogenblik dat al die, tijdens het eerste bloedmaal opgenomen infecties zijn ontwikkeld (± 10-14 dagen). Bovendien stijgt de proportie vliegen met immature infecties nog sterker met toenemende leeftijd. Deze veldobservaties suggereren dat, in tegenstelling tot de gangbare veronderstelling, een groot gedeelte van de tseetsee populatie zich pas infecteert na het eerste bloedmaal. In de volgende hoofdstukken zal experimenteel nagegaan worden of dit laatste werkelijk mogelijk is en welke factoren de gevoeligheid van oudere vliegen kunnen beïnvloeden.

Het vijfde hoofdstuk beschrijft hoe de leeftijd-specifieke gevoeligheid van mannelijke tseetsee vliegen (*G. m. morsitans*) voor infecties met *T. congolense* of *Trypanosoma brucei brucei* wordt bepaald. Aan de hand van experimentele infecties worden vliegen van verschillende leeftijden geïnfecteerd met één van beide trypanosoom species. De resultaten van de infectieproeven geven weer dat oudere vliegen zich nog kunnen infecteren maar dat hun gevoeligheid duidelijk minder is dan de gevoeligheid van jonge (tenerale) tseetsees. De gevoeligheid van oudere vliegen neemt echter significant toe naarmate men ze onderwerpt aan voedingsstress. Oudere vliegen gedurende zeven dagen gevast vóór de infectie maakt ze even gevoelig als jonge vliegen. Eveneens, indien men jonge (tenerale) vliegen voor drie dagen uithongert, neemt ook hun gevoeligheid toe. Deze observaties zijn belangrijk omdat ze een mogelijke verklaring bieden voor de geobserveerde toename in de proportie geïnfecteerde tseetsees met toenemende leeftijd. Tseetsee populaties zijn regelmatig onderworpen aan voedingsstress o.m. ten gevolge van hoge omgevingstemperaturen of een vermindering van de densiteit van de gastheren. Door die stress kunnen oudere vliegen gevoeliger worden voor infecties en een belangrijke bijdrage leveren tot de totale infectiegraad van de tseetsee populatie.

In hoofdstuk zes werd getracht de toegenomen gevoeligheid voor infectie van oude en jonge vliegen na het vasten te verklaren. Daartoe werd de ontwikkeling van trypanosomen bestudeerd in de darm van uitgehongerde en normaal gevoede jonge en oude vliegen. Bovendien werd, gebruik makend van een realtime PCR, de immuunrespons gemeten in deze vier experimentele groepen. Vasten had een effect op de vroege ontwikkelingsfase van trypanosomen in de middendarm van de vlieg maar had weinig invloed op de verdere kolonisatie en de expressie van de procycline mantel door de trypanosomen in de middendarm. Dit werd bepaald aan de hand van twee procyclines (GPEET and EP). De
overleving van trypanosomen gedurende de eerste vier dagen na infectie was beduidend lager in uitgehongerde vliegen dan in goed gevoede vliegen. De differentiatie in procyclische vormen was duidelijker in uitgehongerde oude vliegen. De expressie van enkele immunopeptide genen (attacin I, attacin II, defensin and cecropin) was meer uitgesproken in elk van de experimentele groepen nadat deze geïnfecteerd werden met *E. coli*. Hoewel de gegevens onvoldoende zijn om tot definitieve conclusies te trekken aangaande het verband tussen verhongeren en immunosuppressie, duiden de preliminaire resultaten op zulk een verband.

In het zevende hoofdstuk wordt nagegaan in hoeverre geïnfecteerde tseetsee vliegen nog gevoelig zijn voor een bijkomende infectie met een andere trypanosoom species. De bevindingen van deze studie zouden een verklaring kunnen geven voor het grote aantal gemengde trypanosoom infecties die gevonden worden in veldpopulaties van tseetsees. Eveneens werd gezocht of deze menginfecties een bijkomende verklaring kunnen bieden voor de toename in het percentage geïnfecteerde vliegen met toenemende leeftijd (zie hoofdstuk vier). Vliegen (*G. m. morsitans*) werden eerst experimenteel geïnfecteerd met *T. congolense* en twintig dagen later opnieuw met *T. b. brucei* en vice versa. Dertig dagen na de tweede infectie werd de infectiegraad bepaald van alle vliegen. De resultaten van de dissecties toonden aan dat geïnfecteerde (*T. congolense* of *T. b. brucei*) tseetsees even gevoelig zijn voor een tweede infectie met een andere trypanosoom species. Er werd geen interferentie waargenomen tussen de bestaande infectie en de ontwikkeling van de nieuwe infectie. De resultaten van dit experiment helpen de aggregatie van trypanosoom infecties in een deel van de tseetsee populatie te verklaren en geven aanvullende bewijzen voor de gevoeligheid van volwassen tseetsee vliegen voor een infectie met trypanosomen.

In hoofdstuk acht wordt nagegaan in welke mate de gevoeligheid van tseetsee vliegen voor een trypanosoom infectie kan beïnvloed worden. Dit is vooral van belang in controle operaties waarbij grote hoeveelheden mannelijke tseetsee vliegen, gesteriliseerd door irradiatie, vrijgelaten worden om te paren met fertiele veldvliegen. De resultaten van de experimenten weergegeven in de voorafgaande hoofdstukken tonen duidelijk aan dat dergelijke steriele vliegen, die al een eerste bloedmaal gekregen hebben vóór hun vrijlating, toch nog potentiële vectoren blijven en in sommige omstandigheden zelfs op zeer efficiënte wijze. Experimenteel biedt een éénmalige toediening, via het eerste bloedmaal, van het trypanocide, isometamidium chloride (10 µg/ml bloed), voldoende bescherming tegen infecties met *T. congolense* of *T. b. brucei*. De gevoeligheid was ook verminderd voor *T.*
congolense stammen die resistentie hadden ontwikkeld tegen isometamidium chloride, wat erop wijst dat de bescherming waarschijnlijk niet het gevolg is van een accumulatie van het product in the middendarm van de vlieg maar mogelijks op een aantasting van de bacteriële fauna van de middendarm waardoor ontwikkeling van trypanosomen verhinderd wordt.

Het laatste hoofdstuk geeft een uitvoerige bespreking van de resultaten in hun geheel. De bevindingen van de verschillende studies beschreven in dit werk tonen duidelijk de nood aan tot herziening van het alom aanvaarde “infectie paradigma” dat veronderstelt dat tseetsee vliegen zich hoofdzakelijk infecteren tijdens het eerste bloedmaal. Volwassen vliegen dragen duidelijk eveneens bij tot de infectiegraad van een tseetsee populatie en zijn onder specifieke omstandigheden even efficiënte vectoren als jonge of tenerale vliegen. Deze observaties zijn van groot belang voor het gebruik van de “Sterile Insect Technique (SIT)” als tseetsee-controle maar draagt tevens bij tot het begrijpen van de epidemiologie van deze trypanosoom infecties die moeilijk door tseetsee vliegen overgedragen worden, zoals de menselijke slaapziekte veroorzaakt door T. b. rhodesiense. Factoren die de gevoeligheid van tseetsee vliegen voor deze species bevorderen kunnen belangrijke gevolgen hebben voor de epidemiologie van menselijke slaapziekte en kunnen mogelijk de verspreiding van de ziekte verklaren. Verder onderzoek naar de overdraagbaarheid van deze parasieten wordt aanbevolen.
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List of Publications
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