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Abstract

Human African trypanosomiasis (HAT) or sleeping sickness is a disease caused by human-pathogenic protozoan parasites of the *Trypanosoma brucei* species. These parasites are transmitted by the tsetse fly vector in which parasites have to surmount several natural bottlenecks to eventually reach the insect salivary glands and to differentiate into a final stage that is infective for a new vertebrate host. During the development in tsetse flies, trypanosomes change their surface properties, traverse physical barriers and achieve colonization of various tissues while going through complex cellular proliferation and differentiation programs. The general tsetse fly physiology and immunology, the blood feeding machinery and symbiotic relationships are important parameters that affect the parasite transmissibility.

2.1 Introduction

Tsetse flies encompass 33 species and subspecies (Elsen et al. 1990; Leak 1999) that are all restricted to the African continent and are limited by several natural borders, such as the Sahara and Somali deserts in the North and Kalahari and Namibian deserts in the South (reviewed in Leak 1999; Despommier and Hotez 2006). Tsetse flies belong to the order of *Diptera* and based on their reproduction through adentropic viviparity (Leak 1999; Gooding and Krafur 2005), tsetse flies are categorized as members of the superfamily *Hippoboscidea*, the family of *Glossinidae* and genus of *Glossina*. Tsetse flies are characterized by a distinct proboscis, antenna with branched arista hairs and wings that fold at rest and that have a characteristic “hatchet” cell (Fig. 2.1). Three subgenera were identified

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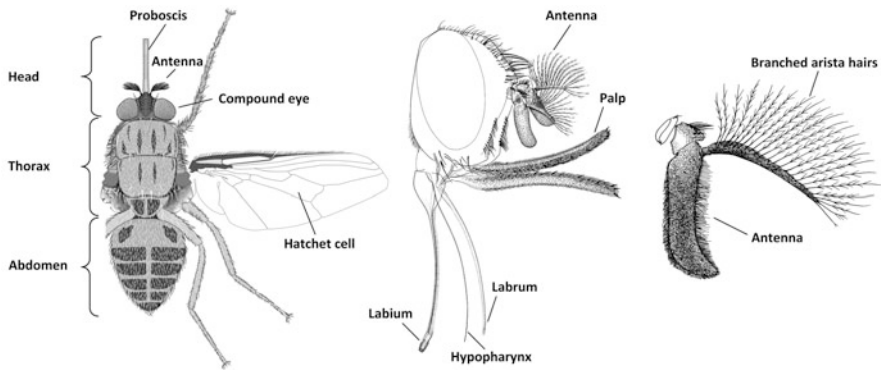


Fig. 2.1 Anatomy of the tsetse fly highlighting the characteristic wing pattern, the proboscis with a detail of the mouthparts and the antennal structure. Compiled figures reproduced with permission of ITM

within the *Glossina* genus: (1) *Morsitans*, (2) *Palpalis* and (3) *Fusca*. One *Glossina* species, *G. austeni*, previously classified under the *Morsitans* group is now under a distinct subgenus, *Machadomyia* (reviewed in Gooding and Krafur 2005). The current taxonomy is based on morphology, structural differences of the male and female genitalia and DNA sequence data of tsetse fly. The current taxonomy is also supported by 16S rDNA sequences of the maternally transmitted primary endosymbiont *Wigglesworthia glossinidia* (Chen et al. 1999).

Tsetse flies occur in a broad range of habitats distributed throughout the sub-Saharan continent [distribution maps are available on <http://ergodd.zoo.ox.ac.uk/tseweb/distributions.htm>, reviewed in Leak (1999) and Krafur (2009)]. Flies of the *Morsitans* group mainly occur in the East African savannah woodlands and lowland forests and in the narrow savannah belt next to the rain forest in West Africa. *G. austeni* of the *Machadomyia* subgenus occurs discontinuously along the East African coast from Kwazulu-Natal (South Africa) to Somalia. Tsetse flies of the *Palpalis* group are predominantly found in the lowland rainforests of Western Africa, from Guinea to Cameroon. They are considered as “riverine species” due to their particular occurrence along rivers and streams. *G. tachinoides* of the *Palpalis* group has been described to advance into Eastern Africa, probably as a result of agricultural development and human population growth. Except *G. longipennis* that discontinuously occurs in very dry habitats in East Africa, tsetse flies of the *Fusca* group live in moist West African forests and savannah.

All tsetse flies are obligate blood-feeding insects, implying that both male and female flies require a blood meal every 3–4 days (Leak 1999). They rely on a pool feeding strategy which involves the laceration of the skin with their proboscis and blood ingestion from a superficial lesion. Once the skin is pierced, the proboscis is often partially withdrawn before being thrust again at a slightly different angle to probe for suitable blood vessels and to enhance the blood pool formation (Lehane 2005). The expected lifespan of the adult tsetse fly stage is approximately 3–4

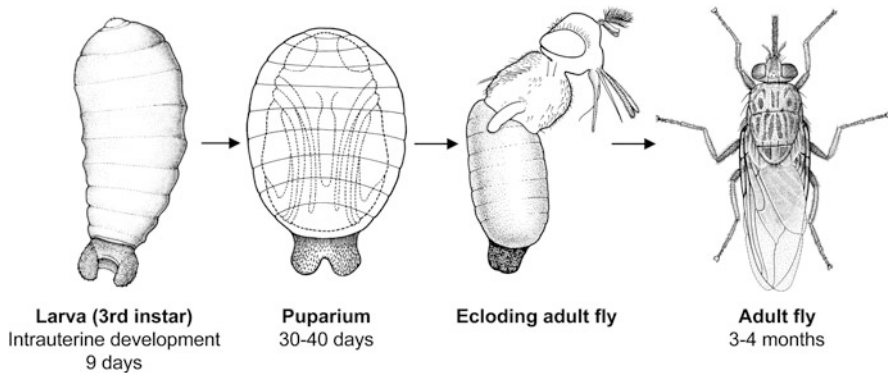


Fig. 2.2 The *Glossina* life cycle illustrating the development from an intrauterine larva into an adult fly. Compiled figures reproduced with permission of ITM

months. Female flies are viviparous (Leak 1999; Gooding and Krafur 2005) and generally mate once or twice (Bonomi et al. 2011), storing spermatozoites in spermatheca that allows the fertilization of one egg at the time. The egg embryonates in the female's reproductive tract and larvae develop inside the insect through feeding on material ("milk") secreted by the reproductive accessory glands. This secretion is rich in lipids and contains milk gland proteins (including lipocalin-like proteins and transferrin) as well the symbiotic micro-organisms (*Sodalis glossinidius* and *Wigglesworthia glossinidia*). Specific gene silencing of several of these proteins results in a reduced fecundity or delayed gestation and pupal abnormalities (lower masses and hatch rates) (Attardo et al. 2008; Benoit et al. 2012). Approximately 9 days after fertilization, a single mature larva is deposited on the ground and moves into the loose soil, where rapid pupation takes place within about 15 min. Depending on environmental parameters such as temperature and humidity, adult flies emerge after 30–40 days (Fig. 2.2). Due to this viviparity, tsetse flies produce only a limited number of offspring, with 8–10 larvae deposited at a 9–11 days interval in a well-managed laboratory colony. In nature, reproductive efficiency is estimated to be much lower, with an average over all seasons of two offspring that manage to reproduce per fly (Gooding and Krafur 2005).

2.2 Tsetse Flies as Invertebrate Hosts for Trypanosomes

Transmission of several pathogenic *Trypanosoma* species within the vertebrate host population primarily occurs through the bite of male and female tsetse flies. Only some specific species are important for trypanosome transmission to humans. Tsetse flies of the *Morsitans* group (*G. morsitans* ssp. and *G. pallidipes*) transmit *T. b. rhodesiense* infections in East Africa. In contrast, tsetse flies of the *Palpalis* group (*G. palpalis* ssp., *G. fuscipes* ssp. and *G. tachinoides*) are mainly responsible for the transmission of *T. b. gambiense* infections in West and Central Africa.

Infection rates of *T. brucei* ssp., the causative agents of HAT and *nagana* in cattle, in tsetse fly salivary glands are very low and in the range of 0.1 % based on a number of studies (Otieno and Darji 1979; Morlais et al. 1998; Msangi et al. 1998). These field data support observations of low tsetse fly infection efficiencies in laboratory conditions. Collectively, a high percentage of tsetse flies is refractory to trypanosome infection. Important factors influencing this phenomenon are believed to be the immune status of the fly at the time of parasite acquisition and the induction efficiency of innate immune responses.

2.3 The Tsetse Fly Immune System

The tsetse fly is a haven for micro-organisms and its alimentary tract continuously faces challenges with various micro-organisms including commensals, symbionts and opportunistic microbes or pathogens. The recognition, elimination, control or toleration of these organisms relies on complex and tightly regulated immune responses that balance between mounting anti-microbial effectors and controlling pathogenicity. Physical barriers including the cuticle, peritrophic matrix, epithelial layers and basal membranes are the first line of defence against micro-organisms. Based on extensive research in the *Drosophila* model system, a significant innate immune system was uncovered in insects including tsetse flies (reviewed in Lehane et al. 2004; Welburn and Maudlin 1999). Similar as for vertebrates, the insect innate immune system relies on both cell- and molecule-based (humoral) mechanisms. The cellular arm involves macrophage-like cells that can phagocytose the infecting micro-organism. The molecule arm involves pattern recognition molecules that recognize pathogen-associated molecular patterns (PAMPs), antimicrobial peptides (AMPs), lectins, proteases, reactive oxygen species (ROS) and nitric oxide (NO). In the induction of innate immune responses against fungi, Gram-positive and Gram-negative bacteria and parasites, three immunity pathways can be implicated: Toll, IMD and Jak/Stat. Involvement of cell-based trypanosome clearance has not yet been documented as trypanosomes do not enter the tsetse fly hemocoel, while there are a number of reports documenting the contribution of humoral anti-parasite responses.

In immune-challenged tsetse flies, the induction of three AMPs was detectable in the hemolymph: cecropin, attacin and defensin (Boulanger et al. 2002). The dipterecin AMP was found to be constitutively expressed in the fat body and gut tissue of *Glossina*, possibly reflecting the immune response against the Gram-negative endosymbionts (Hu and Aksoy 2006; Hao et al. 2001). Using RNA interference (RNAi), attacin driven through the IMD pathway was identified as an important effector in trypanosome midgut elimination (Hu and Aksoy 2006). As such, attacin expression could be a critical determinant of refractoriness/susceptibility of individual tsetse flies to trypanosome infection. The higher susceptibility of starved tsetse flies to trypanosome infection correlated to lower baseline and trypanosome-induced expression levels of the antimicrobial peptides attacin and cecropin in starved flies (Akoda et al. 2009). Furthermore, midgut extracts from

refractory flies display more erythrocyte agglutinating activity than those from susceptible ones, suggesting that lectins might be involved in parasite clearance (Maudlin and Welburn 1987; Ibrahim et al. 1984). An initial support for this hypothesis was that addition of the lectin-inhibitory sugars *N*-acetyl glucosamine and D-glucosamine to infective feeds increases midgut infection rates (Maudlin and Welburn 1987; Welburn et al. 1994). However, an alternative explanation is that glucosamine exerts a lectin-independent activity that could relate to the rate of blood digestion, parasite growth and interaction with reactive oxygen species (Peacock et al. 2006; MacLeod et al. 2007).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been suggested to play a role in the refractoriness of tsetse to trypanosome infection. ROS are oxygen-derived radical species that exert a cytotoxic activity by oxidizing lipids, proteins and nucleic acids. ROS are produced in insects by dual oxidase (DUOX), a transmembranal protein of the NADPH oxidase family. ROS can also induce NO production by nitric oxide synthase (NOS). Here, NO could represent an important innate immune effector and messenger. NO can exert a direct antimicrobial activity by causing DNA damage by nucleotide deamination and by inactivation of enzymes by S-nitrosylation. In addition, NO released in the gut can trigger AMP production in hemocytes and the fat body which could be an important aspect of inter-tissue immunological communication (Wu et al. 2012). Dietary supplementation of tsetse flies with antioxidants such as glutathione, L- and D-cysteine, *N*-acetyl-cysteine, ascorbic acid and uric acid in the infective blood meal leads to considerable increases in midgut trypanosome infections suggesting that antioxidants detoxify free radicals within the gut, thereby promoting trypanosome survival and establishment (MacLeod et al. 2007). In addition, hydrogen peroxide (H₂O₂) levels have been shown to be significantly increased in the midgut of tsetse following trypanosome challenge, suggesting that this activity might be involved in clearing trypanosomes early in the infection process (Hao et al. 2003). In tsetse flies, bacterial challenge results in a pronounced activation of NOS and release of NO in the proventriculus. However, ingestion of bloodstream trypanosome parasites only elicited an increase of ROS and feeding experiments with the NOS substrate L-arginine or the NOS inhibitor L-NAME did not modify trypanosome establishment rates in the tsetse fly midgut. However, L-NAME disturbed the procyclic midgut trypanosomes in their upstream maturation process suggesting an agonistic role of NO in this part of trypanosome development in the tsetse fly (MacLeod et al. 2007).

Comparison of trypanosome-susceptible tryptophan oxygenase-deficient (salmon eye colour mutant) versus wildtype flies, revealed a particular tsetse midgut protein rich in Glu/Pro (EP)-repeats (EP protein) to be differentially represented in the midgut proteome (Haddow et al. 2005). RNAi-mediated reduction in EP protein levels in the midgut significantly increased midgut infection rates (Haines et al. 2010). Concomitantly, starved flies that are sensitive to trypanosome infection have lower EP levels than the well-fed counterparts.

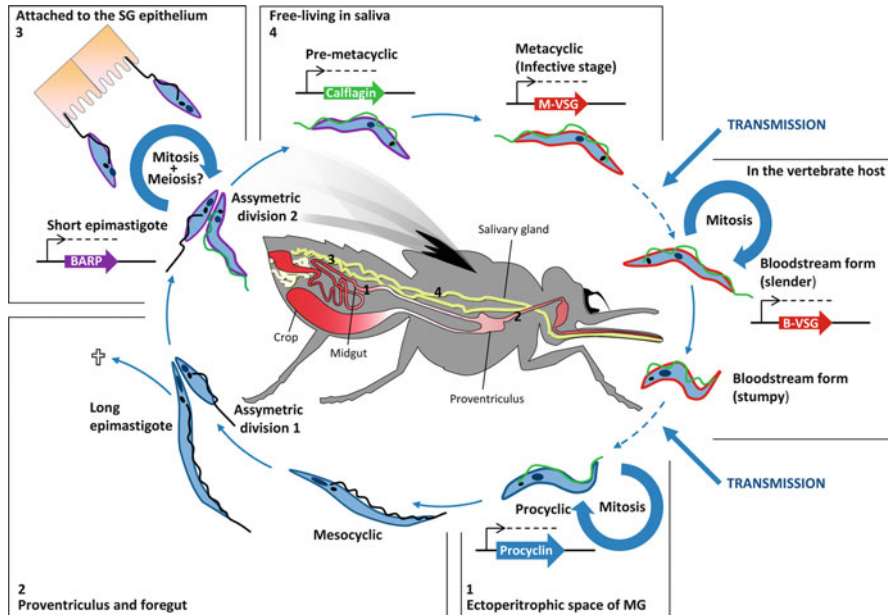


Fig. 2.3 Life cycle of the trypanosome in the vertebrate and invertebrate (tsetse fly) host: the complex cellular proliferation and differentiation events with indication of changes in surface properties, traversal of physical barriers and colonization of the various tissues. SG salivary gland, MG midgut, B-VSG and M-VSG bloodstream and metacyclic variant-specific glycoprotein, BARP *brucei* alanine rich protein. Tsetse fly figure reproduced with permission of ITM

Concerning the trypanosomal colonization of the salivary gland, no information on regulatory salivary components or immune responsiveness of this tissue is available yet.

2.4 The Trypanosome Journey in the Tsetse Fly

Tsetse flies can get infected through feeding on a parasitized host and subsequently accommodate the trypanosome during their entire adult lifespan (Fig. 2.3; reviewed in Dyer et al. 2013; Sharma et al. 2009). In the case of *T. brucei* ssp., engorged trypanosomes have to colonize the midgut and undergo differentiation into a proliferative, insect-adapted procyclic form. The first 3 days after the infective blood meal appear to be the most stringent for successful infection initiation. This requires the parasites to resist the digestive enzymes and innate immune responses present in the vector and to gain access of the ectoperitrophic space by penetrating the peritrophic matrix that lines up the gut epithelium. Tsetse flies have a type II peritrophic matrix that is composed of proteins, glycoproteins and chitin microfibrils in a proteoglycan matrix and that is produced by a specialized region of the anterior midgut (cardia). After a successful colonization of the tsetse midgut,

trypanosomes need to gain salivary gland access to ensure their transmission to a new host. This does not depend on entering the hemocoel but requires a directional migration upstream the alimentary tract, accompanied by differentiation of the procyclic to a mesocyclic form in the anterior midgut. Recently, “social motility” behaviour of procyclic trypanosomes was observed *in vitro* revealing the recruitment of parasites into multicellular communities accompanied by their polarized movement. It remains to be elucidated whether this striking phenomenon plays a role in tissue colonization, resistance to host responses and migration *in vivo* (Oberholzer et al. 2010). After migration from the anterior midgut to the foregut, oesophagus and the mouthparts, *T. brucei* parasites have to enter the lumen of the salivary gland as highly motile tetraploid (4N) epimastigote forms that undergo an asymmetrical cell division, yielding a short and a long epimastigote daughter cell (2N) (Van Den Abbeele et al. 1999). This represents a severe bottleneck in the completion of the trypanosome life cycle as it is believed that only very few parasites (<5) manage to reach the salivary gland (Oberle et al. 2010). The short epimastigote further replicates and attaches to the epithelium through intricate membrane and cytoskeletal connections between the parasite flagellum and the epithelial cell membrane (Tetley and Vickerman 1985). The short epimastigote gives rise to free VSG-coated metacyclic forms by a second asymmetric division and accompanied by a complex cascade of cellular and metabolic changes (Rotureau et al. 2012). These forms are uniquely adapted to survival in the mammalian bloodstream and are injected into a new host through the bite of the infected tsetse fly. Upon transmission to the vertebrate host, trypanosomes will transform into actively proliferating (long slender) forms to allow a systemic colonization of the host. Through a mechanism of quorum-sensing, trypanosomes in the bloodstream become arrested in the G1/G0 stage, undergo morphological changes into a short stumpy form and exhibit mitochondrial biogenesis (Vassella et al. 1997). These changes are considered to pre-adapt the trypanosome to uptake, survival and subsequent differentiation in the tsetse fly. During the complex life cycle of the parasite in the insect and vertebrate host, trypanosomes undergo several metabolic changes for the energy acquisition from different available sources and modify mechanisms for the uptake of host nutrients. In the fly, trypanosomes utilize amino acids (e.g. proline) as primary energy sources, while trypanosomes in the vertebrate hosts metabolize glucose via glycolysis in a unique organelle, the glycosome (Vickerman et al. 1988; Visser et al. 1981).

2.5 Colonization of the Tsetse Fly Midgut

When *T. brucei* short stumpy forms are ingested, they end up in the hostile environment of the tsetse fly alimentary tract. The midgut and especially the proventriculus are environments with a strongly alkaline pH varying between 9.0 and 10.6 in the proventriculus and 7.9–9.5 in the midgut. In addition, the posterior part of the midgut exhibits high proteolytic activity due to the presence of trypsin, chymotrypsin and other proteases and peptidases. Midgut trypsin was suggested to

induce differentiation of short stumpy trypanosomes into procyclic forms as has been illustrated with a transgenic procycline-reporter strain of *T. brucei* (Sbicego et al. 1999). However, no in vivo evidence for this could be obtained from artificial feeding experiments with trypsin and specific inhibitors and an antibody (Welburn and Maudlin 1999). Comparison of trypanosome lines with differential ability to transform into procyclics, resulted in the identification of two homologues of the carboxylate transporter family, PAD1 and PAD2, as the transducers of the citrate and cis-aconitate differentiation trigger. PAD1 expression is readily upregulated in stumpy trypanosomes, while PAD2 is thermoregulated and responds to temperatures compatible with parasite uptake in the insect (Dean et al. 2009).

Upon differentiation of the blood stream trypanosomes into procyclic forms, the variant-specific surface glycoprotein (VSG) coat is shed and replaced by another set of GPI-anchored proteins, the procyclins. Based upon the presence of specific internal amino acid repeats, procyclins are subdivided into two classes: the EP procyclins with Glu-Pro repeats and the GPEET procyclins containing Gly-Pro-Glu-Glu-Thr repeats (reviewed in Roditi et al. 1998; Roditi and Liniger 2002). During the initial midgut colonization, both procyclins are expressed simultaneously and both are susceptible for N-terminal cleavage by proteolytic enzymes present in the tsetse midgut (Acosta-Serrano et al. 2001). Coinciding with the penetration of the peritrophic matrix, GPEET expression is repressed (Vassella et al. 2000), while EP expression persists until the epimastigote stage in the salivary glands. Major disruption of the surface architecture in trypanosomes deficient in GPI anchoring of proteins severely impaired their ability to colonize tsetse fly midguts suggesting the importance of the procyclins in midgut establishment (Guther et al. 2006; Lillico et al. 2003). EP procyclin but not GPEET procyclin deletion mutants exhibited a reduced ability to colonize the midgut (Ruepp et al. 1997). Strikingly, procyclin null mutants were still able to complete their life cycle, but it should be noted that they achieved much lower densities in the salivary gland and seemed to only weakly adhere to the gland epithelium (Vassella et al. 2009). In addition, this mutant suffered a reduced fitness as compared to wildtype parasites during co-infection experiments. A membrane-spanning surface phosphoprotein, PSSA-2, that is present in different insect trypanosome stages, plays an important role in completion of trypanosome life cycle in the tsetse fly. This was exemplified with a null-mutant that established well in the midgut but was seriously compromised in colonizing the salivary glands (Fragoso et al. 2009).

Recently, a master regulator of the parasite maturation process, RNA-binding protein 6 (RBP6), has been identified and overexpression in procyclic trypanosomes is sufficient to reproduce in vitro all developmental stages that occur in the tsetse fly up to metacyclics (Kolev et al. 2013). Although the in vivo trigger is unknown, differential parasite transcriptome analyses detected the *RBP6* induction in the proventriculus. Another set of hypothetical RNA-binding proteins, Alba proteins (Alba3/4), are likely to contribute to the negative regulation of trypanosome differentiation in the midgut. This was elucidated from cellular differentiation of cultured procyclic trypanosomes observed after RNAi and an impaired in vivo differentiation by Alba overexpression (Subota et al. 2011).

2.6 Colonization the Tsetse Fly Salivary Gland

In the final phase, short epimastigotes resulting from an asymmetric division attach to the salivary gland epithelium and transform into metacyclic trypanosomes that express metacyclic VSG (M-VSG). Attachment of the epimastigote to the epithelium of the salivary gland is achieved by the insertion of the parasite's flagellum between the microvilli of the epithelial lining. Through this interaction, the immature parasite is prevented from being ejected with the tsetse's saliva thereby ensuring the permanent infection status of the tsetse fly. Detailed ultrastructural studies describe this *T. brucei*–tsetse junctional complex as 'hemi-desmosome'-like attachment plaques adhering the flagellar outgrowths of the epimastigote parasite to the microvilli at the apical surface of the insect epithelial cells (Tetley and Vickerman 1985). Recently, epimastigotes were demonstrated to undergo two distinct modes of proliferation occurring simultaneously in the salivary glands. Rapid colonization of the glands relies on a cycle that produces two equivalent cells that are attached to the epithelium. The second mode is more frequent at later stages of infection and involves an asymmetric division that produces a pre-metacyclic daughter cell that further matures into the infective metacyclic form (Rotureau et al. 2012). The cells that result from this asymmetric division seem to pre-adapt for infection of the vertebrate host as they express calflagins, calcium-binding proteins, that are highly present on bloodstream trypanosomes but absent in epimastigotes (Rotureau et al. 2012). A protein that is specifically expressed at the surface of the epimastigote trypanosome stage in the tsetse salivary glands is the GPI-anchored *brucei* alanine-rich protein (BARP) (Urwyler et al. 2007). Although the function is not known, it is unlikely that this protein is playing an active role in the parasite attachment with the salivary gland epithelium since it is covering the whole surface of the parasite, whereas the tight trypanosome binding to the epithelial cells is restricted to only the tip of the parasite flagellum.

The epimastigote stage is also of particular interest in terms of genetic exchange between trypanosomes. Coinfection of tsetse flies with parasites that exclusively express either a green or red fluorescent protein (GFP or RFP) resulted in the observation of short epimastigote hybrids as soon as they invaded the salivary glands and before attachment to the gland epithelium. Hybrids appeared to be diploid, triploid or tetraploid (Gibson et al. 2008). Meiosis is likely part of this process as the expression of three different homologs of meiosis-specific genes has been documented to occur in the short free-living epimastigotes. However, haploid stages could not yet be found despite the presence of clearly identifiable meiotic trypanosomes during the salivary gland colonization (Peacock et al. 2011).

Upon differentiation into metacyclic forms, an M-VSG coat is expressed that is individually homogenous as each trypanosome only transcribes a single *M-VSG* gene (Tetley et al. 1987). Different from the bloodstream VSG expression, transcription occurs from a monocistronic unit where the VSG gene is not preceded by expression site-associated genes (ESAGs). In the infective trypanosome dose, up to 27 different M-VSG coats are expressed (Turner et al. 1988), enhancing the likelihood of successful re-infection of a previously infected host. Switching from

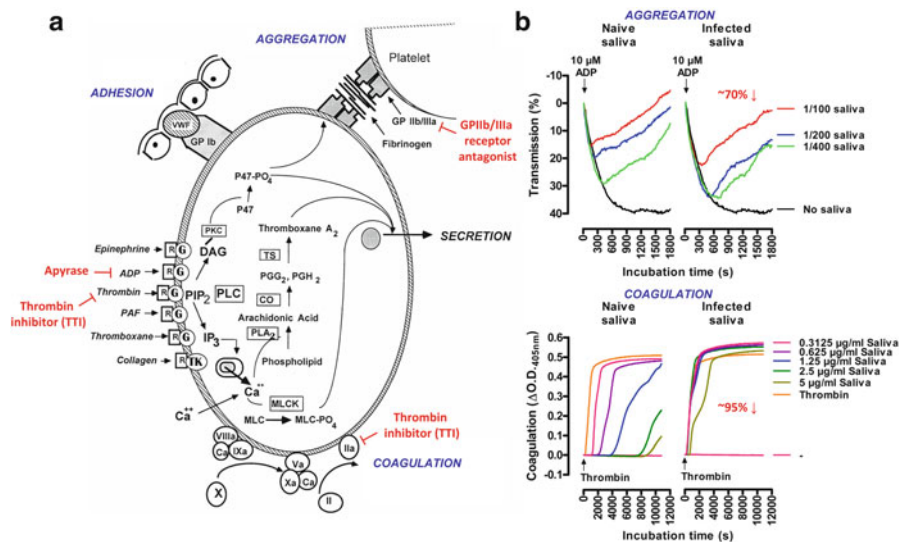


Fig. 2.4 (a) Overview of the anti-platelet and anti-coagulant activities documented for tsetse flies. Figure modified from (Rao 1998) (b) Effects of trypanosome infection on the ability of different saliva concentrations to inhibit platelet aggregation and plasma coagulation

M-VSG to bloodstream VSG (B-VSG) expression occurs within a few days following infection of a mammalian host.

2.7 Tsetse Fly Saliva to the Benefit of Trypanosome Transmission

Tsetse flies rely on a broad repertoire of physiologically active saliva components to efficiently acquire a blood meal (Fig. 2.4a). Anti-hemostatic components primarily interfere with host responses such as vasoconstriction (Elsen et al. 1990; Leak 1999), primary hemostasis through the adherence and aggregation of thrombocytes (Despommier and Hotez 2006; Gooding and Krafusur 2005) and a secondary hemostatic cascade mainly relying on serine proteases such as thrombin (Chen et al. 1999; Krafusur 2009). A component that underwent thorough functional analysis is the tsetse thrombin inhibitor (TTI, 3.5 kDa), characterized as an extremely potent inhibitor of thrombinase activity (Cappello et al. 1996). Additionally, an inhibitor (11–13 kDa) of thrombin's serine proteolytic and esterolytic properties has been detected (Parker and Mant 1979) and an apyrase with fibrinogen receptor (GPIIb/IIIa) antagonistic properties has been shown to inhibit platelet aggregation and to even cause thrombocyte disaggregation (Mant and Parker 1981; Caljon et al. 2010). These components are likely to play a crucial role in the blood-feeding process by ensuring persistent blood flux at the feeding site and preserving mouthpart, crop and digestive function by avoiding clot formation.

Comparison of these two major anti-haemostatic activities, i.e. anti-platelet aggregation and anti-coagulation activity, in infected versus non-infected tsetse flies, revealed a significant suppression as a result of the trypanosome-infection status (Van Den Abbeele et al. 2010). This effect was mainly related to the parasite-induced reduction in salivary gland gene transcription, resulting in a strong decrease in protein content and related biological activities. Apyrase activity that relates to inhibition of platelet aggregation was reduced by approximately 70 %. Strikingly, the anti-thrombin activity and inhibition of thrombin-induced coagulation was even more severely hampered as a result of the trypanosome infection. While naïve tsetse saliva strongly inhibited human thrombin activity and thrombin-induced plasma coagulation, activity in saliva from *T. brucei*-infected flies was reduced to less than 5 %. As a result, salivary gland *Trypanosoma brucei*-infected flies display a significantly prolonged overall feeding time due to an inefficient probing phase, thereby enhancing the likelihood of interrupted feeding and infecting multiple hosts during a single blood meal cycle.

By performing experimental infections using purified *T. brucei* parasites and harvested tsetse fly saliva, another important contribution of saliva to trypanosome transmission was demonstrated. Co-injection of parasites with salivary compounds can significantly enhance the early onset of a trypanosome infection (Caljon et al. 2006a). This effect was correlated with a reduced inflammatory gene transcription (IL-6 and IL-12 mRNA levels) at the skin site where the infection was initiated. Although it was not formally demonstrated, this immune modulatory effect could have significant effects on the minimal infective dose and thereby contribute to high infection success rates upon exposure to a trypanosome-infected fly in the field. Indeed, it was experimentally demonstrated in mice that a single fly can infect up to seven hosts in one blood-feeding cycle (Jenni et al. 1980). By analysing the immunogenicity profile, it became clear that tsetse fly saliva has a strong propensity to induce Th2 cellular responses and to moreover display the ability to suppress B and T cell responses against heterologous antigen (Caljon et al. 2006b). Nevertheless, the tsetse fly–host interaction results in the elicitation of saliva-specific IgG responses that are mainly reactive against the highly abundant 43–45 kDa Tsal family of nucleic acid-binding proteins (Caljon et al. 2006b, 2012). Also IgE responses could be detected, mainly against TAg5 that we have documented to be a potent allergen with high similarity to vespid toxin allergens and the propensity to induce anaphylactic reactions (Caljon et al. 2009). Interestingly, repeated exposure to tsetse fly bites and acquired immunity to tsetse fly saliva did not inhibit infection rates but rather enhanced the parasitemia onset in mice (Caljon et al. 2006a).

2.8 The Tsetse–Trypanosome–Symbiont Tripartite

Next to being a vector for trypanosomes, tsetse flies harbour several endosymbiotic organisms (reviewed in Aksoy 2000, 2003; Rio et al. 2004). Two Gram-negative symbionts, found in all tsetse flies, are members of the *Enterobacteriaceae*: the anciently associated obligate mutualist [primary (P) symbiont] *Wigglesworthia*

glossinidia and the more recently established commensal [secondary (S)] *Sodalis glossinidius*. A third facultative endosymbiont that occurs in some natural tsetse populations is an α -Proteobacterium (Gram-negative) of the genus *Wolbachia* (O'Neill et al. 1993). Given the viviparous reproductive biology of *Glossina* species, all three endosymbionts are maternally transmitted. *Wigglesworthia* and *Sodalis* are transmitted to the intrauterine developing larvae, while *Wolbachia* parasitizes developing oocytes in the ovary.

Wigglesworthia is found intracellularly within an organ immediately adjacent to tsetse's midgut called the bacteriome, as well as extracellularly within maternal milk gland secretions (Attardo et al. 2008). This enterobacterium is believed to supplement tsetse's nutritionally restricted blood diet with vitamins (Akman et al. 2002), and without *Wigglesworthia*, females are reproductively sterile (Pais et al. 2008). Recent studies also indicate an immunologic role for *Wigglesworthia* in tsetse. It was shown that when *Wigglesworthia* is absent from tsetse during the maturation of immature larval stages, subsequent adults are characterized by an underdeveloped cellular immune system and exhibit unusual susceptibility for trypanosome infections (Weiss et al. 2011).

S. glossinidius displays a wide tissue tropism and is harboured both intra- and extracellularly in the tsetse fly midgut, muscle, fat body, milk glands and salivary glands (Cheng and Aksoy 1999). In contrast to *Wigglesworthia*, the functional contributions for *Sodalis* are less well known. *Sodalis* appears to be present in all lab-colony flies, whereas a varying prevalence (0–75 %) has been observed in wild tsetse flies (Geiger et al. 2009; Farikou et al. 2010). However, experimental elimination of *Sodalis* resulted in a reduction of host longevity (Dale and Welburn 2001) suggesting a mutualistic function for this symbiosis in tsetse. *Sodalis* represents a bacterium in the early/intermediate state of the transition towards symbiosis evidenced by the absence of phylogenetic congruence in the evolution of *Sodalis* and tsetse (Chen et al. 1999) and a significantly reduced coding capacity of its genome (Toh et al. 2006). Moreover, *Sodalis* still has the capacity to grow in vitro in cell-free media (Welburn et al. 1987), supporting a recent symbiotic interaction. Although its actual role in the ability of tsetse flies to acquire and transmit the parasite still remains controversial, *Sodalis* infections have been suggested to be associated with an enhanced susceptibility of the tsetse host towards trypanosomes, an effect that varies with the genotype of the symbiont (Dale and Welburn 2001; Farikou et al. 2010; Geiger et al. 2007).

Wolbachia infects tsetse germline tissues (Cheng et al. 2000) and is involved in cytoplasmic incompatibility, a phenomenon of early cell death during embryogenesis due to imprinting differences between the oocyte and the sperm cell. Female *Wolbachia*-infected flies have a reproductive advantage as they can successfully produce progeny with both a naive and infected individual.

Maintaining these long-term symbiotic relationships requires both the host and the symbiont to constrain adaptive interactions. Indeed, endosymbionts are maintained within their insect hosts in spite of the broad spectrum of defence mechanisms against microbial intruders. In tsetse, both host- and symbiont-mediated adaptations have contributed to tolerance to microbial fauna. The *S. glossinidius*

PhoP–PhoQ system, which controls various virulence mechanisms including resistance to antimicrobial peptides (AMPs) through lipopolysaccharide (LPS) modifications, has undergone sensory adaptations allowing *S. glossinidius* to constitutively express genes that facilitate resistance to host-derived AMPs (Pontes et al. 2011).

Homeostasis between *Wigglesworthia* and the tsetse fly is maintained through the peptidoglycan recognition protein LB (PGRP-LB), expressed at high levels in *Wigglesworthia*-associated tissues. Tsetse PGRP-LB scavenges immunogenic peptidoglycan released by *Wigglesworthia* thereby protecting the symbiont from host damage by preventing immune activation. Additionally, increased PGRP-LB levels correlate with refractoriness to trypanosome infection, suggesting a dual role for PGRP-LB in tsetse flies by mediating the immune response towards symbionts on the one hand and enhancing parasite resistance on the other hand (Wang et al. 2009; Weiss et al. 2011).

2.9 Going Forward

Although some research on tsetse fly immune responses and biotic and abiotic factors that affect trypanosome development has been documented in literature, the molecular basis of the trypanosome life cycle in the tsetse fly still represents a genuine black box. The successfulness of trypanosome development in its insect vector is the outcome of the parasite molecular communication and adaptive capabilities to the complex and variable tsetse fly micro-environments. Several trypanosome- and tsetse fly-derived factors and molecular events are suggested to control trypanosome infection within the tsetse fly. Survival in and colonization of the tsetse fly midgut and proventriculus are the first crucial bottlenecks for the trypanosome to overcome. Tsetse fly-derived factors that have been demonstrated to be involved during this part of parasite development are the immune deficiency (IMD) pathway-regulated antimicrobial peptide attacin (Hu and Aksoy 2006) and an immune-responsive midgut EP-protein (Haines et al. 2010). Oxidative stress has also been suggested to play an important role in trypanosome establishment and development (MacLeod et al. 2007). However, many questions regarding the molecular mechanisms underlying the innate immune response of the tsetse fly towards trypanosomes remain. Furthermore, the alimentary tract of the tsetse fly represents a site of multi-taxon interactions that includes not only the arthropod vector and the pathogen but also the gut microbiome. Recent identification of regulators [RBP6 and Alba3/4 proteins (Subota et al. 2011; Kolev et al. 2013)] of insect stage parasite differentiation might pave the way to the discovery of the specific triggers which could eventually contribute to new modes of intervention. Besides trypanosome cell biological research, this requires intensive research upon the tsetse fly vector, which is currently hampered by (1) the viviparous reproductive features of tsetse flies that preclude large mutagenesis screens and transgenic approaches similar to those applied in *Drosophila* and mosquito functional research, (2) the efficiency of protein silencing by RNA interference is target-

dependent and only moderate (40–75 %) in tissues such as the salivary glands and (3) no in vitro tsetse insect cell culture or tissue explants techniques have been established. The only way to obtain novel insights into this part of the parasite life cycle is to profoundly study the tsetse fly environment and to experimentally explore trypanosome development in vivo, i.e. within the tsetse fly. The recent tsetse fly genome sequencing under impulse of the WHO-TDR International *Glossina* Genome Initiative (IGGI) will further advance this research on this under studied disease vector.

Genetically modified disease vectors that are rendered resistant (refractory) to pathogen transmission could provide unique tools for developing or complementing new and existing control strategies. One of these strategies focuses on reducing vector competence by utilizing genetically modified insect symbionts to express proteins that interfere with pathogen transmission, an approach also known as paratransgenesis. A paratransgenic approach in tsetse flies is currently of high interest, since tsetse flies are not amenable to germ-line transformation. This technology to render tsetse flies resistant to trypanosome infection would strongly complement the current Sterile Insect Technique (SIT) that has been proven effective in eradicating tsetse in isolated pockets. Indeed, as SIT involves the massive release of sterilized males, the accompanied temporary increase in the number of potential vectors for trypanosomes could potentially contribute to an increase in disease transmission. Therefore, the release of trypanosome-refractory tsetse flies would render this approach less controversial especially in regions where HAT occurs (Van Den Abbeele et al. 2013).

Therefore, a new line of research in the fight against African trypanosomiasis is focusing on the use of *Sodalis* as a delivery system for anti-trypanosomal components to control parasite development in the fly (Rotureau et al. 2012). *S. glossinidius* is ideally suited as a paratransgenic platform organism since it (1) resides in different tsetse tissues (midgut, hemolymph and salivary glands) that are in close proximity to pathogenic trypanosomes; (2) can be cultured and genetically modified in vitro; (3) can be re-introduced into the tsetse; (4) is maternally transmitted to the offspring and (5) is restricted to the tsetse host niche ensuring that this symbiont is a safe candidate for use in the paratransgenic strategy.

Very recently, an expression system that allows *Sodalis* to constitutively express and extracellularly release functional nanobodies was developed (De Vooght et al. 2012). These recombinant *S. glossinidius* strains were not affected in their growth, suggesting that they may be competitive with endogenous microbiota in the tsetse fly. These results are an important first step in the development of a *Sodalis* expression system that will allow the delivery of nanobodies to study or interfere with the complex trypanosome life cycle inside the tsetse fly.

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Trypanosomes and Trypanosomiasis

Magez, S.; Radwanska, M. (Eds.)

2014, X, 294 p. 27 illus., 20 illus. in color.,

ISBN: 978-3-7091-1556-5