Characterization of motility and erythrocyte adherence as virulence factors in African trypanosomes

Doctoral thesis for a doctoral degree at the

Julius-Maximilians-Universität Würzburg

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Nairobi 2015

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Summary

Pathogens causing African animal trypanosomiasis (AAT), the major livestock disease in sub-Saharan Africa, belong to the salivarian group of the African trypanosomes, which are transmitted by the bite of the tsetse fly (*Glossina* spec.). *T. vivax*, *T. congolense* and *T. brucei brucei* are major pathogens of cattle in particular, causing nagana, with dramatic socio-economic consequences for the affected regions. The parasites additionally have a huge reservoir of other livestock and wild animal hosts. *T. brucei*, the species which also includes the subspecies pathogenic to humans causing sleeping sickness, has been extensively studied as the cultivatable model trypanosome. But less is known about the other salivarian species, which are not routinely held in culture, if at all possible. A hallmark of trypanosomal lifestyle is the protozoan flagellates incessant motility, which enables them to populate an enormous range of habitats in very diverse hosts. We were now able to characterize, for the first time with high spatiotemporal resolution microscopy, the swimming behaviour and mechanism of the most relevant salivarian species isolated directly from blood. We show the influence of viscosity on the motility of bloodstream form (BSF) cells and simulate their movement between erythrocytes, giving a clear picture of how all analyzed species move under varying environmental conditions. We show that although the basic mechanism of flagellar motility applies to all analyzed species, there are clear morphological differences that produce different reactions to the physical environment. We could define specific conditions for highly increased swimming persistence and speed for compared to the behaviour in standard culture. These results have important implications for the parasites survival strategies in the host, e.g. regarding the capacity for antibody clearance. Although we show all species to effectively remove antibodies from the cell surface, *T. congolense* differed markedly in its motility behaviour, which gives rise to interesting questions about this species behaviour in the bloodstream. Most of the *T. congolense* parasites (and to a lesser extent *T. vivax*) adhere to sheep erythrocytes. Further *in vitro* studies showed that *T. congolense* and *T. vivax* adhered to rabbit, goat, pig and cattle erythrocytes- but binding behaviour was absent in murine blood. Notably, both *T. brucei* and *T. evansi* lacked adherence to all studied host erythrocytes. Generally, attachment to blood cells caused reduction of swimming velocities. Judging from its cell architecture, as well as the motility studies in higher media viscosity and in micropillar arrays, *T. congolense* is not adapted to swim at high speeds in the mammalian bloodstream. Low swimming speeds could allow these purely intravascular parasites to remain bound to the host erythrocytes.

Zusammenfassung

Die wichtigste Viehseuche des subsaharischen Afrika, die afrikanische Trypanosomiasis (AAT), wird durch Pathogene ausgelöst, die zu einer Gruppe der afrikanischen Trypanosomen gehört, die durch den Stich der Tsetsefliege übertragen werden (Salivaria). *T. vivax*, *T. congolense* und *T. brucei brucei* sind die Haupt-Erreger in Rindern, wo sie Nagana verursachen, mit dramatischen sozio-ökonomischen Folgen für die betroffenen Regionen. Die Parasiten haben zusätzlich ein riesiges Reservoir an Zucht- und Wildtieren als Wirte zur Verfügung. *T. brucei*, die Spezies die auch die humanpathogenen Subspezies umfasst, die Erreger der Schlafkrankheit, ist eingehend als *das* kultivierbare Trypanosomenmodell untersucht worden, aber es ist weniger über die anderen Salivaria Spezies bekannt, die nicht routinemäßig in Kultur gehalten werden, wenn überhaupt die Möglichkeit besteht. Ein Kennzeichen des trypanosomalen Lebensstils ist die unablässige Motilität der protozooischen Flagellaten, die es ihnen ermöglicht eine riesige Bandbreite an Habitaten in sehr diversen Wirten zu besiedeln. Wir waren in der Lage, zum ersten Mal mit räumlich und zeitlich hochauflösender Mikroskopie, das Schwimmverhalten und den Schwimmmechanismus der wichtigsten Salivaria Spezies zu charakterisieren, die direkt aus dem Blut isoliert wurden. Wir zeigen wie Viskosität die Motilität der Blutstromform (BSF)-Zellen beeinflußt und simulieren deren Bewegung zwischen Erythrozyten. Durch diese Ergebnisse erhalten wir ein klares Bild davon, wie die analysierten Spezies sich unter variierenden experimentellen Bedingungen bewegen. Wir zeigen, dass obwohl der grundlegende Mechanismus der flagellaren Motilität bei allen Spezies gleich ist, es klare morphologische Unterschiede gibt, die verschiedene Reaktionen auf die physikalische Umgebung zur Folge haben. Wir konnten spezifische Konditionen für stark erhöhte Persistenz und Schwimmgeschwindigkeit, im Vergleich zum Verhalten in der Standardkultur, bei *T. vivax*, *T. evansi* and *T. brucei* definieren. Diese Ergebnisse haben wichtige Implikationen für die Überlebensstrategien im Wirt, z.B. bezüglich der Kapazität für die Antikörperentfernung. Obwohl wir zeigen konnten, dass alle Spezies effektiv gebundene Antikörper von ihrer Oberfläche entfernen können, unterscheidet sich *T. congolense* stark in seinem motilen Verhalten, was interessante Fragen über das Verhalten dieser Spezies im Blutstrom aufwirft. Die meisten *T. congolense* Parasiten (und in geringerem Ausmaß *T. vivax*) adhärieren an Erythrozyten des Schafs. Weitere *in vitro* Versuche zeigten, dass *T. congolense* und *T. vivax* auch an Erythrozyten von Kaninchen, Ziege, Schwein und Rind binden, aber nicht im Blut von Mäusen. Interessanterweise adhärierten weder *T. brucei* noch *T. evansi* an Erythrozyten irgendeiner Wirts-Spezies. Im Allgemeinen hat die Bindung an Erythrozyten eine Reduktion der Schwimmgeschwindigkeit zur Folge. Nach der Zellarchitektur und dem Verhalten in Medien höherer Viskosität und zwischen Micropillar-Strukturen zu urteilen, ist *T. congolense* nicht adaptiert, um mit hohen Geschwindigkeiten im Blutstrom von Säugern zu schwimmen. Niedrige Schwimmgeschwindigkeiten könnten diesem rein intravaskulären Parasiten erlauben an den Erythrozyten des Wirts haften zu bleiben.

1.0 Introduction

The greatest concentration of poverty presently occurs in sub-Saharan Africa (SSA). In the year 2010, the World Bank report showed that 48.5% of more than 500 million people in SSA lived on less than US$1.25 a day (**Fig. 1.1**). Many studies have pointed out Neglected Tropical Diseases (NTDs) as the major poverty-accelerating factor in SSA, mainly in the rural poor and some urban disadvantaged populations (Molyneux *et al*, 2005; reviewed in Hotez and Kamath, 2009).
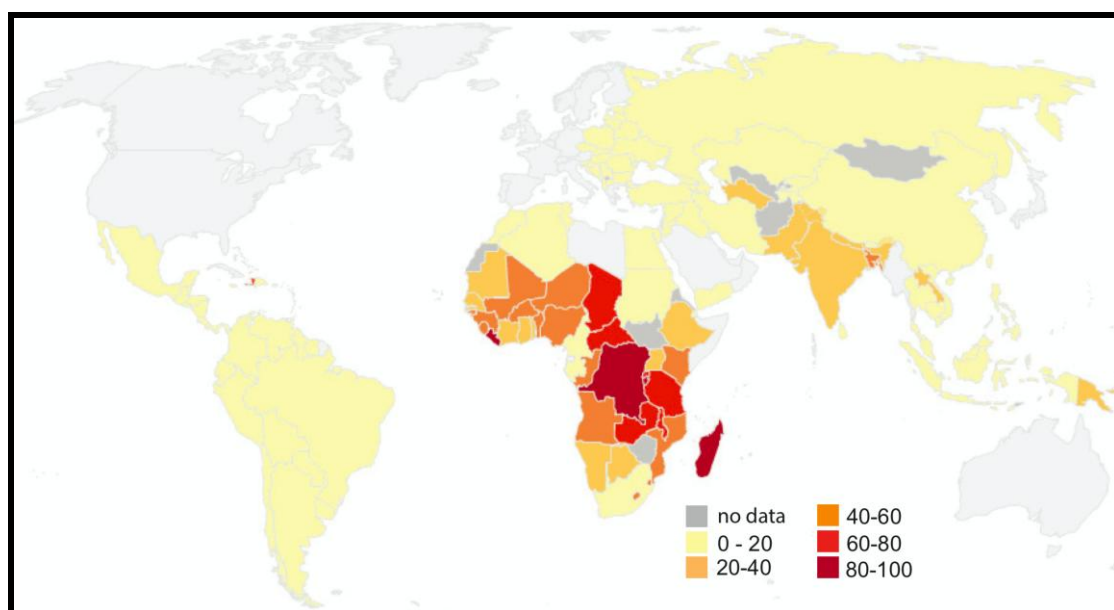
Figure 1.1: Map of world showing the relative percentage of human population per country that lived on less than $1.25 per day in the year 2012.
Source: The World Bank.

Agriculture, which is a backbone of most economies in Africa, must undergo major transformation to meet the challenges of food security and climate change (FAO, 2010). Thus, sustainable agricultural systems must be put in place in order to alleviate poverty. Livestock products such as milk and meat have been common in the diet of many people and especially in pastoralist communities in rural areas who entirely depend on domestic animals (such as sheep, goats, cattle and camels) for their income and subsistence. Unfortunately, pests, diseases and inadequate livestock nutrition, among other factors, challenge livestock rearing.

Tsetse-transmitted trypanosomiasis is arguably one of the greatest obstacles to increased livestock productivity in SSA. This problem led to establishment of a

Programme against African Trypanosomosis (PAAT) in 1997 by the Food and Agriculture Organization (FAO). The programme, facilitated by FAO, African Union (AU), International Atomic Energy Agency (IAEA), and World Health Organization (WHO) sought to ensure coordinated research and integrated approaches against tsetse fly and trypanosomiasis (T and T). In addition, the African Heads of State and Governments established the Pan African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC, 2000) during the African Union Summit held in Lome´, Togo, with an aim of rendering Africa free of T and T menace.

Today, the problem of T and T still persists. It is commendable that in 2009, the reported number of new cases of sleeping sickness dropped to below 10,000 (a decrease of 63% since year 2000; Simarro *et al.,* 2011) for the first time in five decades (WHO, 2010). Perhaps, due to much focus on the human sleeping sickness, there is lack of current statistics in SSA on the extent of the disease in livestock (nagana). In Kenya, several nagana hotspots have been reported and infections mostly occur in the regions infested with tsetse fly (Tarimo-Nesbitt *et al.,* 1999; Masiga *et al.,* 2002; Bett *et al.,* 2008; Thumbi *et al.,* 2010).

Recently, the Animal Health Group of *icipe* reported a prevalence of 33.9% of bovine trypanosomosis in Shimba Hills, Kwale County, Kenya (Mbahin *et al.,* 2013). Shimba Hills National Reserve is endowed with wild animals including buffaloes, elephants, warthogs, sable antelopes, waterbucks, bush pigs and monkeys. Human settlements with domestic livestock, mainly cattle, surround the park. Due to tsetse infestation in this region, farmers mostly rely on subsistence farming of food and cash crops, even though challenged by human-wildlife conflicts mainly caused by elephants.

The main options for dealing with animal trypanosomosis include; control of tsetse fly, use of trypanocides and rearing trypanotolerant cattle breeds. These disease control strategies have proved to be unsustainable (Holmes, 1997). Therefore, new technologies and strategies must be developed in order to address the existing limitations.

Our study focused on the African trypanosomes that are responsible for the animal (livestock) diseases. The basis of this study was to understand motility in relation to antibody clearance in African trypanosomes.

2.0 Literature Review

2.1 Trypanosomiasis

In sub-Saharan Africa, sleeping sickness and nagana are two important diseases (NTDs) caused by the African trypanosomes in man and his livestock, respectively. Tsetse flies (genus *Glossina*) cyclically transmit many African trypanosomes including *Trypanosoma brucei rhodesiense* and *T b. gambiense*, causative agents of human sleeping sickness, and *T. vivax, T. congolense* and *T b. brucei* that causes nagana (WHO, 2000).

2.2 Classification of African trypanosomes

Trypanosomes are classified as follows: **Sub-kingdom:** *Protozoa*, **Phylum:** *Sarcomastigophora*, **Order:** *Kinetoplastida*, **Family:** *Trypanosomatidae* and **Genus:** *Trypanosoma*. This genus has two main groups; Stercoraria and Salivaria (Hoare 1970, Hoare 1972, Baker *et al.,* 1978). Stercoraria contains genera in which the trypanosome completes its development in the hindgut of a triatomine vector and then transmitted through fecal contamination of wound, via skin abrasion or mucous membranes, on the mammalian host. *T. cruzi* that causes Chagas disease in South America belongs to the Stercoraria group. On the other hand, trypanosomes in the Salivaria group are transmitted through fly bites because the infective stages of the parasites are found in the mouthparts of infected insect vector. The key members of *Trypanosoma* that infect man and animals/livestock are summarized below (**Fig. 2.1**). The Salivaria group, containing major pathogens for African trypanosomiases, has four sub-genera that include; *Duttonella, Nannomonas, Trypanozoon* and *Pycnomonas* (Baker *et al.,* 1978).

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Genus                        Trypanosoma
                       Mammalian trypanosomes

Section        Stercoraria              Salivaria

Sub-genus      Duttonella      Nannomonas   Trypanozoon   Pycnomonas

Species    T. (D.) vivax   T. (D.) uniforme
           (pathogenic to cattle)
                    T. (N.) congolense    T. (N.) simiae
                    (pathogenic to cattle) (pathogenic to pigs)

              T. (T.) evansi    T. (T.) brucei    T. (T.) equiperdum
              (pathogenic to camels)             (pathogenic to horses)

                   T. b. brucei   T. b. gambiense   T. b. rhodesiense
                                  (pathogenic to humans) (pathogenic to humans)
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Figure 2.1: Classification of African trypanosomes (Levine *et al.,* 1980; Stevens *et al.,* 1998).

2.3 Transmission biology of African trypanosomes

Tsetse flies are haematophagus insects. Male and female flies carry trypanosomes and it is assumed that all species of *Glossina* can act as vectors (Aksoy, 2003). Bloodstream form trypanosomes ingested with the host bloodmeal, as the tsetse fly feeds, undergo rapid changes to adapt to the new environment in the fly; cooler temperatures, new food sources, hostile digestive enzymes and immune molecules (Aksoy, 2003). In *T.* brucei, a small fraction of gut infections succeed in developing into infective metacyclic forms lodged in the vector's salivary glands since the robust innate immune system of the fly eliminates most ingested midgut trypanosomes (Maudlin *et al.,* 1998). The initial establishment of *T. brucei* or *T. congolense* infection in the fly midgut involves transformation of bloodstream form trypomastigotes into procyclic trypomastigotes that multiply by binary fission. Subsequently, both species migrate from midgut towards the mouthparts of the fly through proventriculus or cardia. Notably, the infective metacyclic stages develop in the proboscis of the fly in *T. congolense*, unlike in *T. brucei* that occurs in the salivary glands (Peacock *et al.,* 2012). *T. vivax* lacks the midgut phase and thus completes its developmental stages in the proboscis of the fly (Vickerman *et al.,* 1988).

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Tsetse fly saliva provides a specific environment for trypanosome maturation and also becomes the fluid vehicle for the transfer of metacyclic trypomastigotes to the vertebrate host during blood feeding process (Vickerman, 1985). Once the trypanosomes in the saliva are inoculated into a host, they multiply for a few days at the site of infected tsetse fly bite, before invading bloodstream and lymphatics. They replicate in the extracellular environment in blood and express a thick surface coat composed of variant surface glycoproteins (VSGs) that undergo antigenic variation. Parasites multiply in the lymph nodes and bloodstream and later invade the brain and spinal cord (Blum *et al.,* 2001). Bloodstream form trypanosomes undergo differentiation from long slender to short stumpy forms that can then be passed from the bloodstream of an infected vertebrate host to tsetse fly as it feeds. It then undergoes another cycle of development lasting about 3-5 weeks, depending on the trypanosome species, before the infection in the fly is mature and the cycle continues over and over again (Vickerman et al, 1988).

Trypanosomes utilize glucose for ATP generation in the mitochondria. Since glucose is abundant in the mammalian blood, haematozoic trypomastigotes break it down only up to pyruvate stage. Pyruvate (end product) is then is excreted into blood; hence there is incomplete oxidation of glucose (Hoare 1972). The mitochondria is inactive in slender bloodstream forms of *T. brucei*, but gets activated in the stumpy forms so as to preadapt them to conditions of less glucose in the tsetse fly. These flagellates, therefore, develop highly complex mitochondria so as to utilize glucose that comes with the fly's bloodmeal. Importantly, the mitochondrion enlarges and starts synthesizing its enzymes for complete glucose breakdown leading to more energy generation.

2.4 African animal trypanosomiasis

African animal trypanosomiasis (AAT) is a serious livestock disease in sub-Saharan Africa. *T. vivax* and *T. congolense* are major pathogens of cattle; *T. simiae* causes high mortality in domestic pigs, whereas *T. b. brucei* affects all livestock (Mugasa *et al.,* 2008). In addition, *T. evansi* causes disease (Surra) in camels (Njiru *et al.,* 2010). AAT occurs mostly, but not exclusively, in regions infested with tsetse fly. For

instance, *T vivax* infections are common in South America where mechanical transmission of the parasite occurs through biting flies (Jones and Davila, 2001).

AAT is commonly characterized by low levels of parasitaemia which may persist for months depending on; the level of challenge, trypanosome species or strain, the breed of cattle and the level of nutrition, among others (Murray *et al.,* 1982). Infections by *T. congolense* or *T. vivax* are rather similar in terms of clinical symptoms. In both, the disease is characterized by progressive emaciation and weight loss, intermittent pyrexia and anemia (Morrison *et al.,* 1981). However, the virulence ranges from acute syndrome (observed in *T. vivax* infections) to a long chronic phase. Currently, anaemia is believed to occur through erythrophagocytosis initiated by trypanosome infection (Esievo *et al.,* 1982; Igbokwe and Mohammed, 1991).

2.5 Mechanisms for anaemia development during trypanosome infection

The cause of anaemia in the mammalian host during trypanosome infection has been investigated by many studies. Presently, it is believed to arise as a result of cleavage of sialic acids from the host erythrocytes by the trypanosomal sialidases, leading to erythrophagocytosis (Esievo *et al.,* 1982; Igbokwe and Mohammed, 1991). The rising levels of free sialic acids in the host serum supported the idea of sialic acids cleavage from the erythrocytes during trypanosome infection (Shehu *et al.,* 2006; Adamu *et al.,* 2009).

Sialic acids are negatively charged monosaccharides found in the cell membranes of higher animals and some microorganisms (Schauer, 2004). They play many roles including regulatory and protective functions (Schauer and Kamerling, 1997; Schauer, 2004). Sialidase cleaves the glycosidic bonds in sialylated glycoconjugates, hence exposing the underlying sugars (Gottschalk, 1957). Erythrocytes repel each other in the host's bloodstream, therefore preventing cell-cell agglutination, because of their negatively charged membranes caused by sialic acids (Schauer and Kamerling, 1997). In addition to sialidases, the roles of trans-sialidase in sialic acid (SIA) metabolism have been reported in many studies.

Trypanosomal (trans)-sialidases were reported in *T. brucei* (Engstler *et al.,* 1992), *T. vivax* (Esievo *et al.,* 1982)*, T. congolense* (Engstler *et al.,* 1995; Nok and Balogun, 2003) and *T. evansi* (Nok *et al.,* 2003). Bloodstream forms (BSF) of *T. congolense* and *T. vivax* show both sialidase (SA) and trans-sialidase (TS) activities (Coustou *et al.,* 2012; Guegan *et al.,* 2013). The activity levels of SA were comparable in *T. congolense* and *T. vivax*, but TS activity was about four times higher in *T. vivax.* In addition, SA and TS activities were more prevalent in insect stages of the parasite than in BSF (Guegan *et al.,* 2013). These elegant studies further showed that parasite infection in mouse was impaired when the activity of TS was decreased or during absence of the inactive *T. congolense* TS-Like2 protein (*Tco*TS-Like2). Interestingly, when the mouse was immunized with *Tco*TS, followed by challenge with *T. congolense*, parasitaemia development was affected which resulted in reduced parasite load, and this in turn led to reduced anaemia and consequently increased survival rates (Coustou *et al.,* 2012). Erythrophagocytosis of desialylated erythrocytes is the major cause of anaemia during *T. vivax* infection and, therefore, the concentration of TS in the host blood may be critical in anaemia progression (Guegan *et al.,* 2013). This was further demonstrated through inhibition of TS activity that led to decreased erythrophagocytosis and consequently resulted into decreased anaemia in mouse (Guegan *et al.,* 2013). It is now clear that only trypanosome species that show SA activity present severe anaemia: BSF-*T. brucei* did not show SA and TS activities. The presence of sialic acids (SIAs) in some life cycle stages of trypanosomes known to be incapable of synthesizing their own SIA was later explained to occur by the action of TS. The TS facilitates direct transfer of free SIA residues from one glycan to another without undergoing complex biosynthetic steps (Schauer, 2001; Schauer, 2004; Dyer *et al.,* 2013). The procyclic form-trypanosomes in the tsetse fly midgut acquire SIA, through TS, from fresh bloodmeal and this sialylation protects trypanosomes from complement lysis (Nagamune *et al.,* 2004). Trypanosome coats its surface with SIAs transferred from host cell membranes, such as erythrocytes, to protect itself from hosts' immune destruction (Engstler and Schauer, 1994; Schauer, 2004). On the other hand, desialylation of host erythrocyte unmasks the underlying sugar, which in turn exposes receptors for phagocytes that consequently bind and induce erythrophagocytosis in the infected vertebrate host; this eventually leads to anaemia (Esievo *et al.,* 1982, Bratosin *et al.,* 1998). Sequestration of aged red blood

cells in man (after about 120 days) follows the same procedures of desialylation and erythrophagocytosis (Bratosin *et al.,* 1998). Removal of 10% of the total erythrocyte SIA is enough to trigger elimination of the de-sialylated erythrocytes from the bloodstream (Aminoff *et al.,* 1976). Susceptibility to anaemia during infection depends on the host, among other factors. Comparison of the erythrocyte SIA content in healthy (uninfected) Zebu (trypano-susceptible cattle breed) and Ndama cattle (trypano-tolerant breed) showed the latter to have seven times more SIA content than the former (Esievo *et al.,* 1986). Although there was no difference in PCV (packed red cell volume) levels between both breeds, it is likely that Ndama will require much higher parasite load to achieve the same degree of anaemia in Zebu.

T. congolense binds to erythrocytes of some vertebrate hosts through surface SIAs on host erythrocytes (Banks, 1979). Destruction of RBC-adhered parasites by host's immune response concomitantly damages the bound erythrocytes (Banks, 1980). This process of RBC destruction formed the basis for the second proposed mechanism of anaemia development in the mammalian hosts. Anaemia has also been reported to occur due to mechanical injury on erythrocytes caused by the lashing action of the powerful flagella (Vickerman and Tetley, 1978).

2.6 Economic challenges resulting from African trypanosomiasis

Human sleeping sickness (HAT) and nagana (animal African trypanosomiasis; AAT) form a major barrier to economic empowerment of many nations in sub-Saharan Africa. The most affected are rural populations (living in regions where transmission occurs) that depend on agriculture, fishing, animal husbandry, or hunting (Simarro *et al.,* 2008). Human infections reduce labour resources, while AAT often causes high herd mortality causing loss of draught power, in addition to reduction in livestock reproduction rates. Therefore, AAT limits availability of meat and milk products, leaving much of the human population malnourished. Lack of draught power compels the farmers to rely on manual tillage, which lowers food production. Economic losses resulting from reduced agricultural and cattle productions are estimated at US$ 4.75 billion and US$ 1.2 billion per year, respectively (Swallow, 2000; Simarro *et al.,* 2008). Obstruction of agricultural productivity by HAT and other neglected tropical diseases threatens Africa's economic development and this leads to increased poverty

levels, worsened by the high human population growth rates. Therefore, means of controlling these diseases are required urgently.

2.7 Trypanosomiasis control

Currently, there is no vaccine against trypanosomiasis because of the ability of African trypanosomes to change their surface proteins through antigenic variation (Mehlert *et al.,* 2002). Management of this disease currently relies on active surveillance, treatment of infected hosts and on vector control. Efficient tsetse fly control methods that have been adopted include; use of insecticides, odour-baited traps, and sterile insect technique. Unfortunately, control of these diseases in SSA has not been sustainable due to poor diagnostic capacity, lack of proper disease surveillance, and inadequate control measures (Holmes, 1997). The impact of some effective tools on vector control has not been sustainable due to their local nature and extensive dependence on community participation (Kuzoe and Schofield, 2004). Additionally, uncontrolled use of trypanocides in treatment of the infected hosts has led to many cases of drug resistance (Clausen *et al.,* 2010).

Considerable efforts have been made to control trypanosomes, but the complex interaction of the pathogenic parasite with the vertebrate host and insect vector hinders development of reliable intervention methods. Better understanding of the association between tsetse fly, trypanosome and the mammalian host is of paramount importance in identifying novel ways to disrupt the transmission cycle of this parasite in their hosts. This will help in identifying important factors that enable survival and development of trypanosomes throughout their life cycle. In turn, the survival mechanisms of this parasite in the hostile environment, especially, of the mammalian host could be exploited to break their life cycle hence their transmission.

2.8 The biology of African trypanosome

2.8.1 Trypanosome cell architecture

The uniflagellated protozoon *T. brucei* has an exceptional cellular architecture with one Golgi and a single lysosome; both of which are located within the posterior part of the cell (Overath and Engstler, 2004; **Fig. 2.2**). The flagellum is important for directional motility. The flagellar pocket, whick lacks pellicular microtubules, is an

invagination of the plasma membrane around the emerging flagellum and is the only know site for exocytosis and endocytosis (Overath *et al.,* 1997; Gull, 2003).
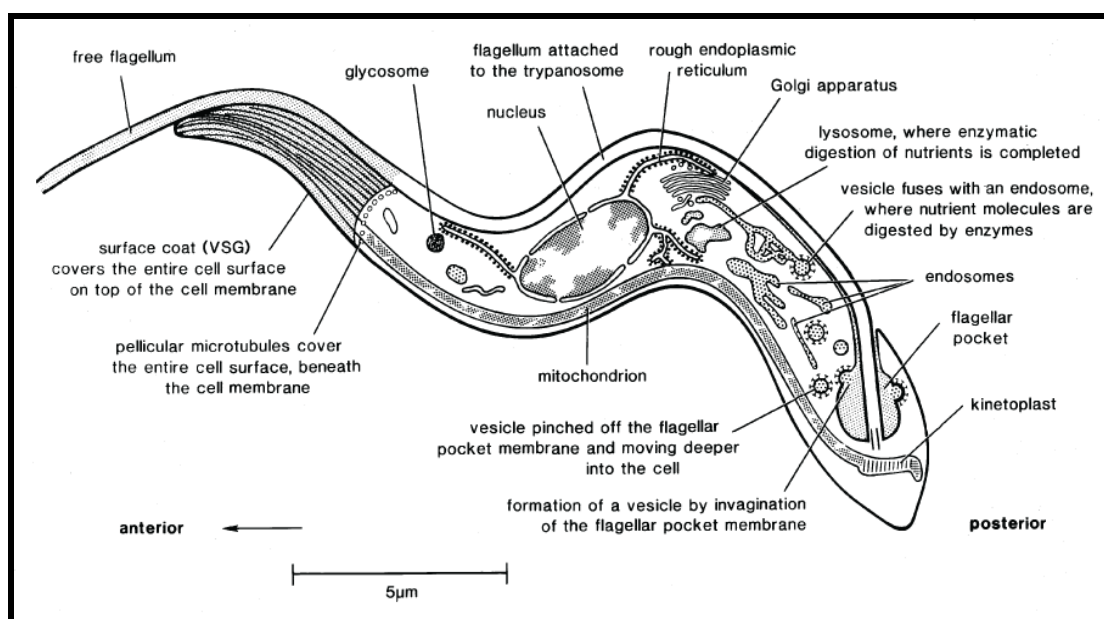
Figure 2.2: Illustration of the major organelles in a trypanosome (bloodstream-form). The arrow points to the anterior swimming direction. Adapted from Vickerman, 1970.

Flagellar pocket (FP) plays a great role in molecular trafficking and recycling of glycosylphosphatidylinositol (GPI)-anchored proteins such as procyclin and variable surface glycoproteins (VSG). Trafficking and recycling of procyclin and VSG from the cytoplasm via the flagellar pocket to the cell surface is one of the survival strategies of trypanosomes in their hosts (Engstler and Boshart, 2004; Bonhivers *et al.,* 2008).

2.9 Trypanosome survival strategies

Trypanosomes have a capacity to evade attack from the hostile immune system of the vertebrate host, hence form poor targets for vaccine development. The mammalian host responds against trypanosome infection by producing specific antibodies against a membrane-associated antigen of the trypanosome referred to as variable surface glycoprotein (VSG). The entire trypanosome surface is covered by VSGs making up to 95% or more of the total plasma membrane proteins (Overath and Engstler, 2004). Specific host antibodies may clear all trypanosomes sharing the same surface protein (for example VSG-1) by agglutination and lysis (Cross, 1975). However, a fraction of these parasites can switch their VSG type (e.g. from VSG-1 to VSG-2), thus

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presenting the host with a new type of antigen. These parasites with new antigenic signatures escape immune attack and continue to divide. This mechanism is referred to as antigenic variation and involves expression of a single VSG gene from a genomic repertoire of many hundreds through stochastic switching from the expression of one VSG gene to another (McCulloch, 2004). The second way of evading the host's immune system attack by the parasite is thought to occur through weakening the defense system of the mammalian host through suppression of important components of the immune response (Sternberg, 1998). Thirdly, rapid plasma membrane recycling and hydrodynamic protein sorting have been demonstrated to contribute to the removal of host-derived immunoglobulins from the trypanosome cell surface, a process named antibody clearance, which protects trypanosomes from complement lysis (Engstler *et al.,* 2007).

2.9.1 Antibody clearance

In *T. brucei,* surface immunoglobulin-VSG (Ig-VSG) immune complexes were sorted to the cell posterior for endocytosis through the flagellar pocket (Engstler *et al.,* 2007). Engstler and co-workers (in 2007) further demonstrated that movement of surface Ig-VSG complexes towards flagellar pocket depends on hydrodynamic drag forces generated through swimming. Conversely, reversed swimming in trypanosomes- achieved through knockdown of the dynein arm intermediate chain- resulted in accumulation of antibody-VSG complexes at the anterior pole away from the FP, proving that antibody sorting occurs purely as a result of physical forces. In addition, immotile trypanosomes, modified through down-regulation by RNA*i* of FLA1 protein, did not survive antibody challenge, a clear indication that directional cellular motility and swimming speeds- that generate hydrodynamic drag forces- are very important in endocytosis of host-derived antibodies. However, antibody removal was found to be effective in protecting the parasites against lysis at low to moderate antibody concentrations, but not sufficient at high antibody titers (McLintock *et al.,* 1993).

Since antibody clearance was only reported in *T. brucei* maintained in culture for long term*,* our immediate task was to investigate if the same mechanism existed in bloodstream forms of *T. vivax, T. congolense* and *T. evansi*. Because *T. vivax* naturally swims at higher speeds than *T. congolense,* the immediate questions

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included; why do trypanosomes swim at different speeds? Do all trypanosome species remove host-directed surface antibodies at the same rate? These questions formed the foundation of our studies in trypanosome motility and behaviour in different culture environments. Therefore, we sought to investigate the kinetics and modes of swimming in four trypanosome species (cultivated in mouse, rat, rabbit and sheep) by using high-speed video microscopy. Trypanosome motility was investigated in detail at single cell level in order to provide reasons for varied motility. In addition, motility behaviour was simulated in the mammalian host by changing the viscosity of the culture environment and by studying movement of the parasites in the micropillars with diameter and spacing of blood cells in the bloodstream. The next study was focused on comparative motility behaviour of trypanosome species in mammalian hosts in order to relate it to differences in virulence patterns. For instance, species varied in respect to erythrocyte adherence behaviour in mammalian blood.

In summary, our findings show variations in motility patterns and speeds according to the trypanosome species and swimming environments. The body shapes (3D-morphometry) and erythrocyte-binding behaviour also varied in different species and contributed to differences in swimming speeds. These variations, which depended on the swimming environment, suggested potential involvement of motility and erythrocyte binding as virulence factors in trypanosomes and thus could be responsible for varying pathogenicities associated with various trypanosome species. Finally, endocytosis of surface-bound antibodies occurred at similar rate in all studied species.

3.0 Materials and Methods

3.1 Trypanosome species and strains

The following trypanosome species were used in the study: *T. congolense* IL 1180 (originally isolated from a lion in the Serengeti National Park, Tanzania [Geigy and Kauffmann, 1973]), *T. congolense* IL 3000 (Savannah-type, origin: Transmara, Kenya; isolated from infected cow) and *T. congolense* KETRI 3827; *T. vivax* IL 1392 and *T. vivax* IL 2136; *T. b. brucei* IL Tat 1.4 and *T. brucei* KETRI 2710; *T. evansi* KETRI 4009 and *T. evansi* KETRI 2479 (type B; Njiru *et al.,* 2006, 2010). *T. congolense* IL 1180, *T. congolense* IL 3000, *T. vivax* IL 1392, *T. vivax* IL 2136 and *T. b. brucei* IL Tat1.4 were obtained from the trypanosome bank at the International Livestock Research Institute (ILRI, Nairobi, Kenya), while *T. congolense* KETRI 3827, *T. brucei* KETRI 2710, *T. evansi* KETRI 2479 and *T. evansi* KETRI 4009 were obtained from Kenya Agricultural and Livestock Research Organization-Biotechnology Research Institute (KALRO-BRI, Kikuyu, Kenya) trypanosome bank. Wild-type trypanosomes were isolated from seven infected rabbits that were used to support tsetse fly colony obtained from Shimba Hills (South coast, Kenya).

3.2 Diagnosis of trypanosome infection in cattle

Field study was conducted in different locations around Shimba Hills National Park **(Fig. 3.1)**. This region is infested by tsetse flies and is endemic for nagana. Wild animals in the park form a reservoir of trypanosomes. Cattle (n = 100) were restrained in crushes, screened for trypanosome infection using phase contrast and high-speed video microscopy. Blood was obtained from the ear vein using sodium-heparinized capillary tubes (80 IU/ml, Marienfeld, Germany) followed by detection of trypanosomes in the buffy coat (Paris *et al.,* 1982). About 5 ml of blood was collected from the jugular vein of infected cattle for passaging into 50 immunosuppressed mice (300 mg kg^{-1}, cyclophosphamide-Sigma Aldrich) and part of it (blood) was transported in liquid nitrogen to *icipe* for species identification by polymerase chain reaction (PCR).
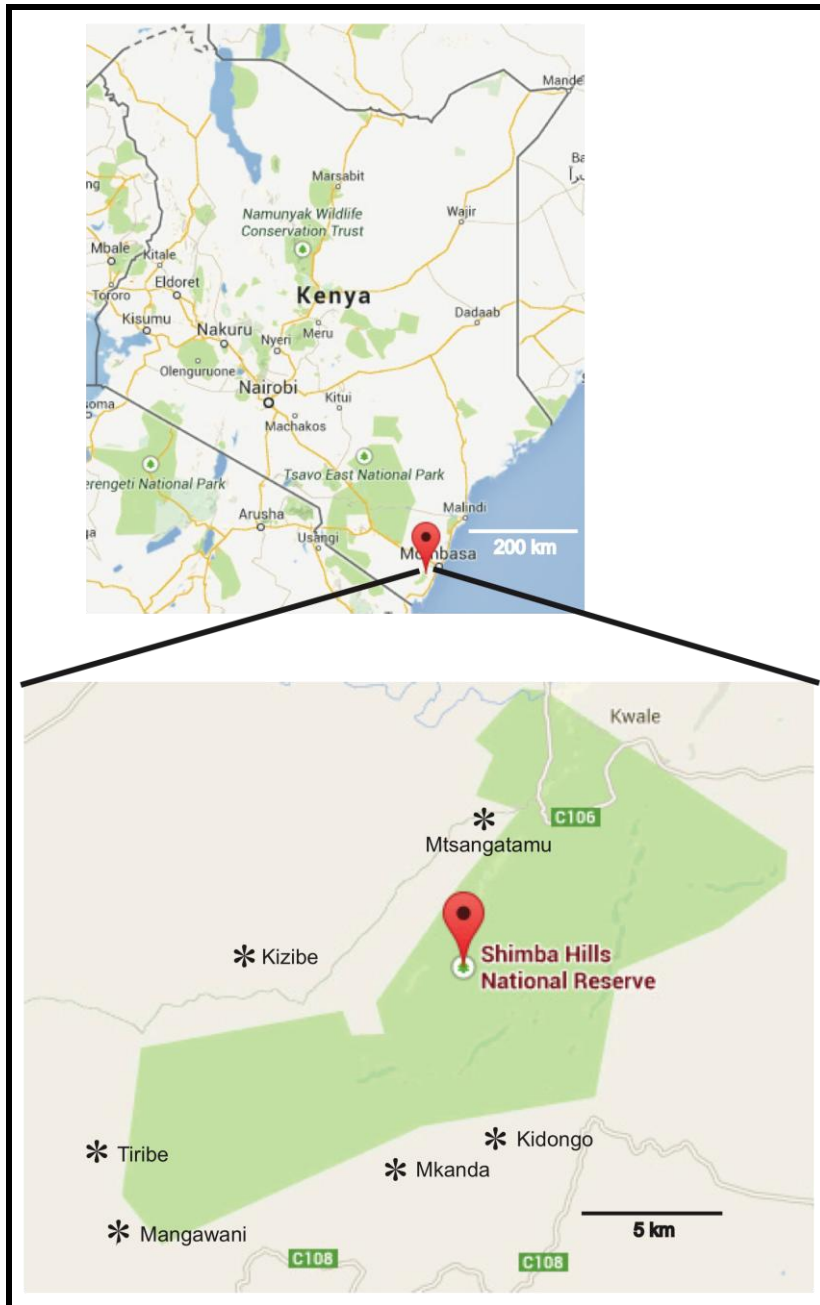
Figure 3.1: The cattle from the above sampling sites (marked with asterisk *) around Shimba Hills National Reserve were screened for trypanosomiasis. High-speed video microscopy was employed to capture and record trypanosomes in the infected cow blood. Swimming patterns and trypanosome motility behaviour was investigated from the recorded movies.

3.2.1 Trypanosome species identification

Trypanosomes species were identified through PCR-amplification of ITS1 gene using the following specific primers: *ITS1_CF:* 5' CCGGAAGTTCACCGATATTG 3' and *ITS1_BR:* 5' TTGCTGCGTTCTTCAACGAA 3' (Njiru *et al.,* 2005). Phusion® DNA polymerase (Finnzymes, Espoo, Finland) was used in amplification under the

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following thermocyclic conditions: 98°C for 2 min, 35 cycles of 98°C for 10 sec, 64°C for 30 sec, and 72°C for 30 sec, followed by final elongation for 7 min at 72°C. Negative control which lacked only the DNA template was included. The reaction was done in ARKTIK Thermal cycler (Thermo Fischer Scientific, Finland). PCR products were electrophoresed through ethidium bromide-stained 1% agarose gel for 1hr at 80 V (Bio-Rad model 200/2.0 power supply and wide mini sub cell GT horizontal electrophoresis system, Bio-Rad laboratories, Inc., USA). Gel visualization, editing and documentation were accomplished using KODAK Gel Logic 200 Imaging System (Raytest GmbH, Straubenhardt). After product size verification on the agarose gel, the DNA bands were excised under UV light followed by purification from the gel (QuickClean II Gel extraction Kit, GenScript Corp., Piscataway, NJ) and then sequenced (Macrogen, Korea).

3.3 Ethical statement

The permit to conduct small and large animal experiments at KALRO-BRI and *icipe* was granted and approved by the Kenya Veterinary Board and the KALRO-BRI Institute Animal Care and Usage Committee (IACUC, Ref: C/TR/4/325/125). All experiments were reviewed and carried out in conformity with the local and international standards of animal care and welfare.

3.4 Experimental infections and handling of animals

3.4.1 Rodent infection

Mice, rats and rabbits were reared at *icipe's* Animal Rearing and Quarantine Unit (ARQU) under clean conditions. Cages that housed laboratory rodents were washed and disinfected weekly and their beddings changed regularly. Food pellets and clean water was provided and the animals were allowed to feed *ad libitum*. Inbreeding was conducted to expand the colony when necessary.

Six to eight weeks old Swiss mice were immunosuppressed before infection with trypanosomes. Bloodstream forms of *T. congolense, T. vivax, T. b. brucei* and *T. evansi* were inoculated intraperitoneally. Similar experiments were performed in Sprague-Dawley rats and rabbits. A drop of blood from the tail (in rat and mouse) or ear vein (rabbit) was obtained daily for use in light microscopy to monitor parasitaemia levels. The infected mouse was anaesthetized using chloroform before

collection of blood, through cardiac puncture, in heparinized capillary tubes (80 IU/ml, Marienfeld, Germany). The experiments in mouse, rat and rabbit were carried out in triplicate. All experiments involving examination and recording of trypanosomes in infected wet blood films were conducted at room temperature, unless when specified otherwise.

3.4.2 Infection of sheep

Sheep were chosen in this study because they are more susceptible to trypanosome infection than goats (Irungu *et al.,* 2002). Eight black head Persian sheep were acquired from a trypanosome-free region in Kenya, ear tagged and kept at the KALRO-BRI barn. Clinical examination showed absence of ectoparasites such as ticks and mites. In addition, trypanosomes were not detected from the buffy coat blood films examination as well as in PCR using species-specific primers. However, worms identified in some sheep through fecal analysis (by floatation method) which necessitated deworming of all sheep with 10% suspension of albendazole (Valbazen, Basle, Switzerland) at 7.5 mg kg^{-1}. Further treatment was done using 1% (w/v) deltamethrin pour-on solution (Cooper, Nairobi, Kenya) for protection against ticks and 20% (w/v) tetracycline at 20 mg kg^{-1} to treat any possible but undetected bacterial infection. The sheep (n=8) were divided into two groups of equal number, control and test. They were provided with clean water, salt-lick and allowed to feed *ad libitum*. Baseline data, including packed red cell volume (PCV) and body weight measurements, was obtained before, during infection and after treatment.

T. congolense KETRI 3827 and a rodent-adapted *T. vivax* IL 1392 were used for comparative sheep and mice infection studies. These two species were initially chosen because of their importance in AAT (Goossens *et al.,* 1998; Masiga *et al.,* 2002). Each trypanosome stabilate was expanded in immunosuppressed donor Swiss white mouse (300 mg kg^{-1} cyclophosphamide) and harvested at peak parasitaemia for sheep infection. Infected mouse blood was diluted in phosphate buffered saline with glucose (PSG buffer; 0.15 M sodium phosphate, 0.1 M NaCl and 10% glucose, pH 7.4; filter-sterilized) and a final concentration of 10^6 trypanosomes was delivered intravenously. Two milliliters of this solution was injected intravenously into each sheep. Blood was obtained from ear vein using heparinized capillary tubes and PCV levels were

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monitored to determine the state of anaemia. Parasitaemia was also recorded daily for two months post-infection. Swimming trypanosomes in sheep wet blood films recorded using video microscopy. For comparative studies on motility behaviour, the same isolate was investigated in different host blood. When the experiments were concluded, all infected sheep were treated with a double dose of diminazene aceturate (Veriben, Libourne, France) at 7 mg kg^{-1}. Treatment of *T. vivax* and *T. congolense*-infected sheep was done after 11- and 24-days post-infection, respectively. Additional dose of isometamidium chloride at 0.5 mg kg^{-1} (Samorin, Rhone Merieux, France) was given when the parasites appeared to be resistant to Veriben.

3.4.3 Purification of trypanosomes from host blood

Trypanosomes were routinely purified from blood by differential centrifugation (200$\times g$, 5 min and 4°C). Wash step was repeated three times to remove most of the host erythrocytes. Trypanosomes in the supernatant were concentrated (1400$\times g$, 5 min, 4°C) and resuspended in ice-cold Trypanosome Dilution Buffer, TDB (5 mM KCl, 80 mM NaCl, 1 mM MgSO$_4$, 20 mM Na$_2$HPO$_4$, 2 mM NaH$_2$PO$_4$, 20 mM glucose, pH 7.6, filter-sterilized) to attain concentration of about 10^7 to 10^8 parasites/ml.

3.5 Fluorescent staining of trypanosomes

3.5.1 Antibody removal assays

Trypanosomes (1×10^8 cells/ml) were purified from murine blood, resuspended in 0.5 ml of fresh ice-cold TDB and surface-stained with 2 mM Sulfo-NHS-SS-biotin (Sigma-Aldrich, USA) for 15 min, light protected on ice, strictly at 0°C. The parasites were washed twice (1400$\times g$, 10 min at 0°C) with 1 ml ice-cold TDB to remove any unbound biotin. Subsequently, the cells were incubated on ice for 30 min in presence of 10 µg/ml mouse monoclonal anti-Biotin IgG antibody conjugated to fluorescent dye, CF™488A with absorption wavelength of 490 nm. Trypanosomes were washed twice in 1 ml ice-cold TDB, resuspended in about 0.8 ml final volume that was equally distributed into eight sterile pre-cooled (to 0°C) 1.5 ml Eppendorf tubes. Meanwhile, TDB and 4% (w/v) paraformaldehyde, PFA, in HEPES (with 0.05% v/v glutaraldehyde) was pre-warmed to 37°C. Endocytosis of the surface VSG$_{\text{Anti-biotin-IgG-CF488A}}$ was followed by incubating parasites in a water bath at 37°C for specific time

intervals (for instance: 0 sec, 10 sec, 20 sec, 40 sec, 1 min, 3 min, 5 min, 10 min). By taking one time-point at a time, 0.4 ml of pre-warmed TDB was added to the 1st tube containing 0.1 ml of labeled trypanosomes and then fixed immediately with equal volume of 4% PFA. This treatment represented the control at t = 0 sec and ideally at this step, VSG$_{Anti-biotin-IgG-CF488A}$ is expected to remain on the trypanosome surface. The same procedure was repeated for all the remaining time-points. After fixation step with PFA, the tube contents were incubated for 1 hour at 37°C followed by overnight incubation at +4°C. Thereafter, fixed trypanosomes were washed in 1 ml 1× PBS (pH 7.4). Individual cells were analyzed using fluorescence microscopy to check for endocytosis of VSG$_{Anti-biotin-IgG-CF488A}$. Kinetoplast and nucleus were visualized using diamidino-2-phenylindole (DAPI; Sigma-Aldrich, absorption wavelength = 375 nm) in order to allow for selection of individual trypanosomes with one nucleus and one kinetoplast (1K1N, marking cells at the same state of division). LED image stacks were also captured in each case.

3.5.2 Morphology studies

Live parasites were surface-labeled with 3 mM AMCA-Sulfo-NHS (Thermo scientific, Pierce, Rockford; 100mM stocks of AMCA were prepared in dimethyl sulfoxide). AMCA-Sulfo-NHS is an amine reactive fluorophore for labeling proteins. Trypanosomes were incubated in total darkness for 20 min, strictly on ice, followed by three wash steps- 1400×g, 10 min at 0°C- to remove any unbound dye. Trypanosomes were resuspended in 0.5 ml ice-cold TDB and then fixed in equal volume of 4% PFA (in HEPES) containing 0.25% (v/v) glutaraldehyde. Fixed cells were initially incubated at 37°C for 1 hour, followed by overnight incubation at 4°C. Subsequently, the trypanosomes were washed in 1× PBS (pH 7.4), ready for imaging. Fluorescent dyes such as AMCA-Sulfo-NHS that bind to VSGs on the trypanosome surface enable measurement of flagellum morphology. Therefore, we compared the 3D structure of flagellum in *T. congolense, T. vivax, T. evansi* and *T. brucei.*

3.6 *In vitro* studies to investigate trypanosome-erythrocyte binding

3.6.1 Isolation of host erythrocytes

Fresh blood was transferred into a clean 1.5 ml tube containing 0.5 ml of fresh TDB to make a total volume of 1 ml. In order to isolate host erythrocytes free from serum

sialidases and any free sialic acid residues, the above mixture was centrifuged at $400 \times g$ for 5 min at 4°C. This wash step was repeated three times before resuspension in 0.5 ml of TDB. Unless otherwise specified, rabbit erythrocytes were used in most adherence assays because rabbits are easy to handle during bleeding procedures than most large animals and much cheaper to maintain the colony.

3.6.2 Adherence assays

The number of erythrocytes and trypanosomes was determined in order to calculate trypanosome/RBC ratios for adherence studies. Actively swimming parasites (*see* section 3.4.3 for trypanosome isolation) were mixed with purified erythrocytes at a ratio of 1:1. The optimal temperature for adherence was determined by incubating the trypanosome-RBC mixture for 30 min under the following temperature conditions: 4 - 8°C (mixture incubated on ice for 30 min followed by scoring of adherence in the cold room maintained at 4-8°C), room temperature (18 - 26 °C) and 37°C. Erythrocyte suspensions and purified trypanosomes were kept on ice for not more than 2 hrs before use.

3.6.2.1 Factors influencing trypanosome-erythrocyte adherence

Erythrocytes isolated from mouse, rat, rabbit, sheep, goat and pig blood were used to investigate the influence of host erythrocytes on adherence capacities in different trypanosome species and strains. In another experiment, whole goat blood was used directly without washes in TDB in order to investigate the degree of agglutination in presence of whole blood components.

In order to determine if dead trypanosomes can bind RBCs and whether fixed RBCs adhere to live trypanosomes, cells were fixed in equal volume of pre-warmed 4% paraformaldehyde (PFA) in HEPES (with 0.05% glutaraldehyde) followed by three wash steps in TDB. Treated/untreated trypanosomes were mixed with fixed/unfixed RBCs and incubated at 37°C for 30 min followed by scoring of adherence using light microscopy.

The effect of the following heat-inactivated sera (56°C, 30 min) on parasite adherence was investigated: fetal bovine serum (FBS), horse serum, young goat serum,

trypanosome-infected cattle serum (obtained from a cow infected with wild-type trypanosomes), serum plus^{TM} (SAFC Biosciences, Hampshire, UK), and pig serum.

The adherence roles of the following enzymatic and sugar treatments were also investigated: neuraminidase (Type V from *Clostridium perfringens*, Sigma-Aldrich), trypsin (Type I from bovine pancreas), alpha-chymotrypsin (Type I-S from bovine pancreas); diminazene aceturate; sugars (lactose, maltose, sucrose).

A neuraminidase treatment was done to determine whether trypanosomes bind specifically to sialic acids on erythrocyte surface as previously reported. Lyophilized neuraminidase (Sigma-Aldrich) was dissolved in 1 ml phosphate buffer or TDB and 0.03% BSA (New England Biolabs) was added to the enzyme preparation. Rabbit erythrocytes were isolated and divided into two parts: one part was treated with 0.2U of neuraminidase and the remaining one served as control (untreated). Erythrocytes were incubated at 37°C for 30 min followed by three washes in TDB before mixing with the parasites. Neuraminidase-treated and untreated rabbit erythrocytes were tested for adherence against *T. congolense* and *T. vivax*. In the next experiment, trypanosomes were treated with neuraminidase and mixed with untreated RBCs to investigate adherence.

A similar approach was applied for studying the effects of trypsin, chymotrypsin, diminazene aceturate, lactose, maltose and sucrose on trypanosome-erythrocyte adherence. Excess trypsin inhibitor (from Glycine max, soybean, Sigma-Aldrich) was used to stop trypsin activity after treatment.

3.7 Microscopy

A drop of infected blood (from sheep, rabbit, mouse and rat) was placed on a clean microscopic slide and covered with 22×22 mm coverslip for examination by microscopy. Parasitaemia was monitored daily by visualization at ×400 magnification (*mg*) under phase contrast microscopy (Laborlux D, Leitz Wetzlar, Germany) and a rapid matching technique (Herbert and Lumsden, 1976) was used for quick estimation of parasitaemia based on the average number of trypanosomes per field of view at ×400 *mg*. Neubauer chamber was used to count trypanosomes whenever higher accuracy was required.

High temporal resolution movies for monitoring the details of trypanosome motility in blood were captured using automated iMIC microscope (Till Photonics, Gräfelfing, Germany) equipped with a high speed Phantom camera v5.2 (Vision Research, Wayne, NJ). Phantom Camera Control (PCC) software (Vision Research, Wayne, NJ) was used to control Phantom Camera, whereas the iMIC digital microscope platform was controlled by Live Acquisition software. Swimming trypanosomes were recorded under 60× and 100× objectives (Olympus) for studies on motility patterns, swimming speeds and erythrocyte binding. A frame-rate of 500 fps was sufficient for the analysis of fast flagellar beats in trypanosomes.

The main challenge of high-speed microscopy videos (500 - 1000 fps) was their large sizes of up to 16 GB/movie, recorded in just 16 seconds. Thus to reduce the size of the video, the following procedure was followed: The movie, initially saved in .CINE file format, was opened using Phantom camera control (PCC) software and then saved as a .TIFF 16,48 file. In FIJI (just ImageJ), under file menu, the image sequence of the movie was imported as a virtual stack and the required region was cropped out to reduce the size. Noise in the movie was removed using ImageJ by eliminating light flickering; bleach correction and removal of outliers. After conversion of 16-bit movie to 8-bit, the brightness/contrast was adjusted before saving the movie in TIFF and AVI formats. The movie in .AVI format was opened in XmediaRecorde (Sebastian Dörfler, Copyright© 2007-2015) and then saved in .WMV format (several file formats available). This format reduced the size of the movie considerably, while retaining good resolution.

Fluorescent microscopy was done using Pike camera (30 fps; Till Photonics, Gräfelfing, Germany) connected to iMIC microscope. 3D models of fixed cells were obtained from 150 image stacks (xyz) captured at intervals of 100 nm between any two successive images. High-resolution 3D image stacks were deconvolved and measured using morphometry software (Huygens® Essential Image processing software v4.3, Amira software v5.6.0).

Electron microscopy was used to acquire micrographs of trypanosomes bound to erythrocytes. Trypanosomes were mixed with rabbit erythrocytes and binding was allowed to occur. The control experiment was set up by exposing trypanosomes to

non-adherent mouse erythrocytes. The samples were initially fixed in 4% PFA (with 0.05% glutaraldehyde) followed by second fixation using 2% OsO4 (in 0.05 M cacodylate buffer). After several wash steps in water, the samples were dehydrated using increasing concentration of ethanol (50, 70, 90, and 100%) followed by treatment with propylenoxide. Subsequently, the samples were embedded in epon, followed by preparation of ultrathin cuts that were stained in Reynold's lead citrate, ready for microscopy.

3.8 Analysis of trypanosome motility

Individual trypanosomes (n = 900 parasites/species; parasitaemia was about 10^7 trypanosomes ml^{-1}) in fresh wet blood films were followed by microscopy, for 10-30 sec in order to determine their swimming patterns. The criteria applied to define swimming patterns in high-speed movies was according to Uppaluri *et al.,* (2011), Heddergott *et al.,* (2012) and Weiße *et al.,* (2012), with few modifications. Three swimming patterns were analyzed, namely; tumbling, intermediate and persistent swimming pattern. The swimming speeds reported here were generated automatically, from the recorded high-speed movies, using PCC software by defining the swimming distance covered by the trypanosomes. Therefore, a particular reference point was selected, i.e. at the posterior pole of the parasite, for measurement of speeds. The length and width of individual trypanosomes were also measured. Images of swimming parasite were opened using ImageJ (Rasband, National Institutes of Health, Maryland, USA) in order to adjust image quality, crop series in length and size and to enable saving of files in appropriate file format. Tumbling durations (total number of tumbling frames divided by the frame rate, fps) were measured for each swimming pattern (n=100 trypanosomes/pattern). Dead, non-motile or structurally abnormal parasites were excluded during scoring of adherence assays as well as in motility studies.

3.9 Simulation of trypanosome motility in the host bloodstream

3.9.1 Culture media viscosity

The viscosity of the wet blood films preparations containing trypanosomes differs from the bloodstream viscosity. Therefore, in order to simulate bloodstream viscosity, 0.4% (w/v) methylcellulose was added to the cell culture medium (Heddergott *et al.,*

2012). A range of methylcellulose concentrations in TDB or mouse blood, from 0.2 - 0.8% (w/v), was tested to investigate the effects of viscosity on trypanosome motion. Parasites were purified from blood by differential centrifugation and resuspended in TDB with a range of viscosity. In another similar experiment, methylcellulose was added directly to the infected blood so as to test the role of viscosity and presence of blood cells on parasite swimming. Trypanosome motility in specific concentration of methylcellulose was investigated by high-speed video microscopy in order to study effects on the swimming patterns and speeds of swimmers.

3.9.2 Micropillar arrays

Ordered micro-pillar arrays were used to mimic the diameter and spacing of blood cells in the bloodstream. Sixteen (16) polydimethyl siloxane (PDMS)-pillar arrays with defined diameters (8, 10 and 12 μm) and spacing (3, 4, 5 and 6 μm) were printed on thin glass slides. Thus, each pillar array was designed to have specific diameter and regular pillar-to-pillar spacing. Because blood cells in the bloodstream differ in their sizes (diameter), some pillar arrays consisted of mixed/heterogeneous pillars to mimic *in vivo* conditions, whereas other arrays were homogeneous. Heterogeneous pillars consisted of mixture of two pillar types with different diameters.

Trypanosomes were cultivated in mouse and thereafter; parasites were purified from blood components as previously described. Trypanosomes were resuspended in TDB and then introduced into the pillars.

Wetting of pillars was a crucial step because hydrophobic pillars thwarted parasite entry into spaces in between pillars. Thus, pillars were treated with a detergent known as extran (1 part of extran was diluted in 9 parts of distilled deionized water) in order to make them hydrophilic. A glass slide with all combinations of pillar arrays was placed in a clean petridish containing 10 ml of diluted extran. The petridish was placed on a shaker (at 100 rpm) for 30 min and then the pillars were washed thrice in distilled water. Every wash step was done for 30 min on a shaker. The fields of interest in pillar arrays were washed in TDB. About 10 μl of trypanosomes, diluted in TDB, were introduced into the pillar arrays and covered with 10 mm rounded coverslip ready for imaging. The optimal parasite number to introduce per field in the PDMS-pillar array was established to be about 32 trypanosomes/field at ×400 *mg*.

Too many parasites or carry-over mouse blood cells led to clogging of the spaces in between pillars hence obstructing swimming parasites. High-speed video microscopy (500 fps) was used to capture and record movies of swimming parasites in the pillars. Swimming patterns and speeds of trypanosomes were quantified under different parameters of pillar arrays. Wet pillars became hydrophilic only for a day and thus wetting procedure was repeated every time before imaging of parasites. After use, pillars were rinsed with water a few times and then air-dried.

The following questions formed the basis of our studies in methylcellulose and micro-pillars; do the speeds and patterns of swimming vary in relation to the previous observations on wet blood films? Do tumbling phases occur in defined environments?

3.10 Data analysis

DNA sequences (ITS region of trypanosome species) were edited using BioEdit (Hall, 1999) to remove ambiguous base calls. Consequently, a search to identify similar sequences was performed using BLAST algorithm of the NCBI GenBank (http://www.ncbi.nlm.nih.gov/).

Statistical analysis of the variations in trypanosome swimming patterns in mice and sheep blood and the differences in swimming speeds was performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) software. Analysis of variance (ANOVA) was executed after square root transformation in order to satisfy the distributional assumptions of ANOVA. After ANOVA, multiple comparisons procedure was performed based on Student-Newman-Keuls Test where $p<0.05$ was considered significant.

Quantitative adherence assays were conducted in duplicate, and counts of adherent (**A**) and freely swimming (**F**) trypanosomes were replicated three to six times (n = 600-1200 parasites). The data was used to compute means ± SE (standard error) or SD (standard deviation) and the proportions of **A** and **F** was compared in four trypanosome species/strains studied under different conditions of *temperature, erythrocytes types,* presence and absence of *serum* and *sugars*, among others. Differences between groups were analyzed using Student's t-test and $p<0.05$ was considered significant. Figures and still images were prepared using Adobe Illustrator CS6 and Adobe Photoshop CS6.

4.0 Results

4.1 Trypanosome motility

Swimming speed, swimming pattern and behaviour was analyzed in various culture environments. Speeds were analyzed because antibody clearance, one of the defense strategies of trypanosomes in the vertebrate host, depends on how fast individual parasites swim. In addition, trypanosome motility was studied in detail in order to elucidate mechanisms of swimming and reasons for variations in motility patterns and speeds. Three-dimensional morphology data was compared in four trypanosome species and related to their differences in motility. Thus, comparisons were done starting from species population level down to the individual cell so as to interpret the existing similarities and differences in motility.

The effects of varied swimming environments of the vertebrate hosts were investigated using parasites expanded in mouse, rat, rabbit, sheep and cattle. Laboratory rodents (mouse, rat and rabbit) were mostly used because they are easy to: handle, breed and infect with trypanosomes. On the other hand, larger animals such as sheep and cattle were included because they are natural hosts of African trypanosomes.

In order to simulate swimming in bloodstream, trypanosome motion was investigated under increased culture media viscosity and in PDMS-pillar arrays.

Our findings discussed below show striking motility differences among trypanosome species and importantly, through detailed analysis of swimming at cellular level, we demonstrate the reasons behind these variations.

4.1.1 Trypanosome motility in blood

We studied swimming in the bloodstream-forms of four trypanosome species in fresh wet blood films. *T. congolense* lacked free flagellum, which was present in *T. vivax, T. brucei* and *T. evansi.* In addition, *T. congolense* displayed sluggish wriggling motion and the rate of flagellum beat was relatively lower than in other species. In contrast, the flagellum of *T. vivax* was rapidly beating and the parasites displayed longer wavelengths that resulted in larger displacement and higher speeds. In *T. vivax,* the persistent swimmers were very active and traversed the whole field of view with fast speeds characterized by short tumbling durations in some cases. *T. brucei* were

also active swimmers. *T. evansi* was characterized by highly flexible flagellum and cell body that allowed the parasite to fold itself, thus resembling an elastic coil, especially at the anterior region (**Fig. 4.7: C5**). Conversely, its (*T. evansi*) pointed posterior region displayed rigid appearance during swimming. We observed that the tumblers in *T. evansi* were more active swimmers than the tumblers in *T. congolense*. In terms of size variations (lengthwise), *T. brucei* were smaller in size in comparison to *T. evansi*, but larger than *T. congolense* (**Table 4.2**).

4.1.2 Major swimming patterns in trypanosomes

The three main characterized swimming patterns in *T. brucei* include; tumbling, intermediate and persistent modes (Uppaluri *et al.,* 2011; Heddergott *et al.,* 2012; Weiße *et al.,* 2012). We defined tumblers as those trypanosomes that swam for shortest distance i.e. within radius of one cell length of ~20 µm for the entire recording duration of 16.28 sec (**Fig. 4.1 A**). Intermediate swimmers swam directionally for distances exceeding 20 µm followed by interruption due to tumbling phases in between directional swimming. Persistent swimmers, on the other hand, showed continuous directional swimming without tumbling for at least a distance of 150 µm across the field of view. Therefore, classification of these three patterns was based on distances travelled by the trypanosome.

4.1.2.1 Tumbling duration depends on the swimming pattern

Most trypanosomes were observed to exhibit tumbling motion at some point during swimming. Therefore, we sought to calculate the durations of tumbling phases under each swimming pattern. As expected, tumblers tumbled throughout the entire movie length of 16.28 sec. Intermediate swimmers exhibited average tumbling durations of 6 - 7 sec in both mouse and sheep blood. The average tumbling durations were further reduced to a maximum of 2 sec in persistent swimmers (**Table 4.1**).

In addition to the existing criteria for swimming pattern characterization based on distances travelled by the parasite, our findings on pattern-specific tumbling durations presented another potential way of identifying swimming patterns on the basis of their tumbling durations. The roles of persistent, intermediate and tumbling motions in relation to parasite survival and proliferation have not been understood yet. Importantly, whether these patterns truly existed under *in vivo* conditions remained

unclear. However, the tumbling motion pattern occurred in the micropillar arrays that were designed to simulate diameter and spacing of blood cells in the bloodstream. Nevertheless, the proportion of directional swimmers in *T. vivax, T. brucei* and *T. evansi* increased in the pillars. Only *T. congolense* maintained the status quo in its motility patterns and speeds in TDB, blood and pillars.

Table 4.1: Tumbling durations of trypanosomes in blood. Videos of swimming parasites were acquired at 500 fps followed by motion categorization into specific swimming pattern before generation of tumbling durations. The tumbling durations were computed by dividing the number of tumbling frames per frame rate. Data = mean ± SD.

Trypanosome species	Host blood	Average tumbling duration (sec)		
		Persistent	Intermediate	Tumbling
T. congolense KETRI 3827	Sheep	1.97 ± 1.20	6.84 ± 1.79	≥ 16.28 ± 0.00
	Mice	1.91 ± 0.52	7.49 ± 1.57	≥ 16.28 ± 0.00
T. vivax IL 1392	Sheep	1.57 ± 0.56	7.09 ± 1.22	≥ 16.28 ± 0.00
	Mice	1.70 ± 0.76	7.62 ± 0.56	≥ 16.28 ± 0.00

4.1.2.2 Proportions of swimming patterns is species-dependent

We compared the swimming patterns in four trypanosome species and found significant differences (**Fig. 4.1 B**). The most obvious pattern variation was the highest proportion of tumblers in *T. congolense* (78-92 % of the population tumbled). In contrast, the proportion of tumblers was lower in *T. vivax* (only 23% tumbled), and instead the directional swimmers formed majority (**Fig. 4.1 B, mouse**).

Subsequently, variation in swimming patterns was investigated in different mammalian hosts. In order to show that there were no fundamental changes between the wild-type trypanosomes when compared to the characterized laboratory strains, swimming patterns were investigated in cattle blood naturally infected with the wild-type parasites. Although, few parasites were analyzed due to very low levels of parasitaemia in cattle, we managed to identify the main swimming patterns observed in mouse blood. Similarly, sheep were infected with *T. congolense* and *T. vivax* in order to characterize their motility. The three main swimming patterns observed in mouse wet blood films were present in rat and sheep blood (**Fig. 4.1**). However, the relative proportions of motility patterns in mouse and sheep blood varied (**Fig. 4.1, compare mouse *versus* sheep**). Highest number of tumblers was recorded in *T. congolense* in both mouse and sheep wet blood films. In addition, the proportion of persistent swimmers in *T. vivax* increased by 28% in sheep relative to mouse blood. A similar trend of host-dependent variations in swimming patterns was also observed in

rat blood (**Fig. 4.1, rat**). Differences in blood viscosity, among other factors, between mouse, rat and sheep could affect parasites' swimming patterns and speeds. Individual Trypanosomes exhibited both intra- and inter-species variations in motility.
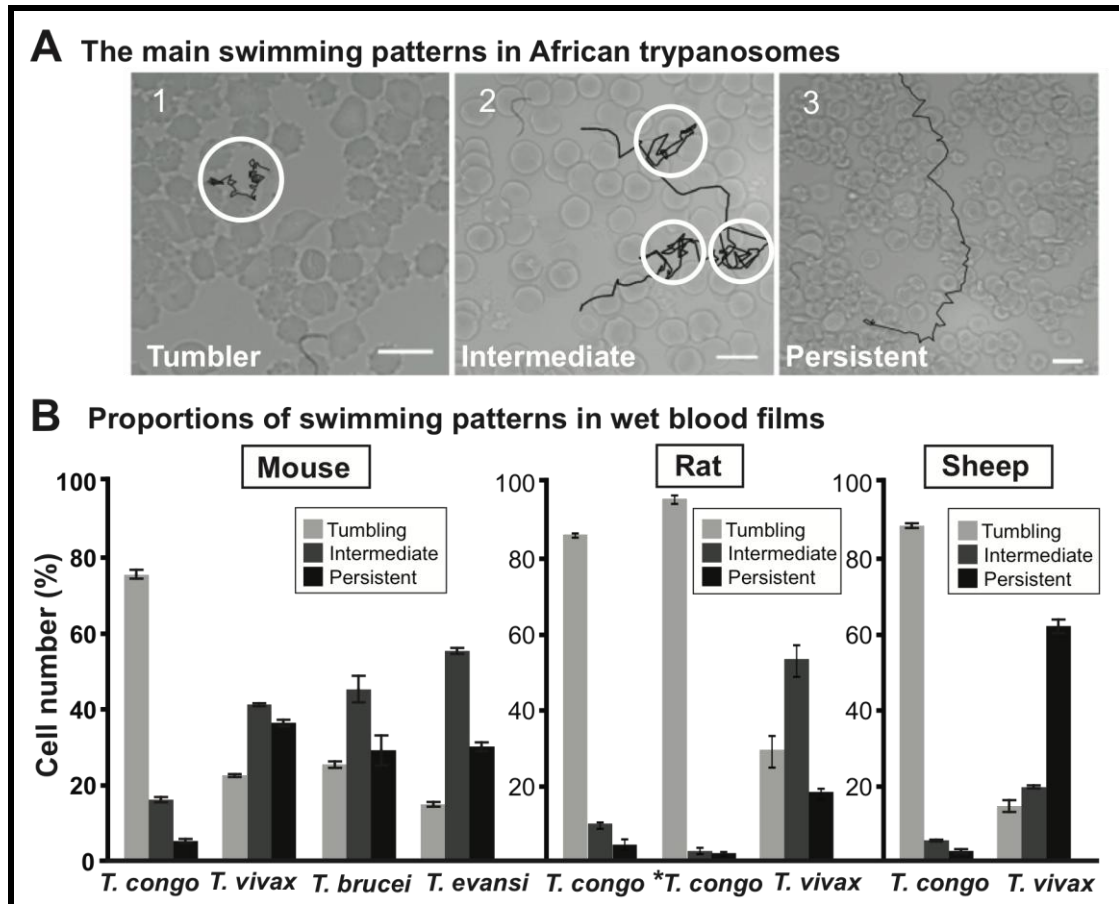

Figure 4.1: Swimming patterns in bloodstream-form trypanosomes in wet blood films.
(A) An illustration of the tumbling (panel 1), intermediate (panel 2) and persistent swimming patterns (panel 3) in mouse blood. Swimming trypanosomes were captured at 500 fps and swimming paths (shown above in black lines) traced to classify the motion pattern. The white circles in panel 1 and 2 represent tumbling mode. The scale bar is 10 µm.
(B) Comparison of the swimming patterns in mouse, rat and sheep blood (n=900 trypanosomes/species; parasitaemia score ≈10^7 trypanosomes/ml)**.** The findings from mouse blood indicated highest proportion of tumblers in *T. congolense* (78.11 ± 1.18%; **Fig. B**). In contrast, *T. vivax* IL 1392, *T. b. brucei* IL Tat 1.4 and *T. evansi* KETRI 2479 showed relatively higher proportions of intermediate and persistent swimmers. *T. congolense* was characterized by presence of tumbling majority in mouse, rat and sheep. The mean tumbling proportion of the same *T. congolense* KETRI 3827 isolate increased by 14% in sheep blood (**B,** sheep) when compared to mouse (**B,** mouse). A similar study in *T. vivax* showed higher percentage of persistent swimmers in sheep than mouse blood. *T. congolense* marked with asterisk* in **Fig. B** was the second strain *T. congolense* IL 1180, included for comparison of swimming patterns in rat blood. *T. congolense* KETRI 3827 was investigated in all hosts studied. Data = Mean ± SEM.
T. congo = T. congolense.

Trypanosomes show diverse swimming patterns that depend on the species and the swimming environment. Analysis of high-speed videos of swimming parasites in mouse wet blood films revealed higher proportion of persistent backward swimmers unique to *T. evansi* (discussed below).

4.1.2.3 Swimming direction

More than half of the population in *T. brucei, T. congolense* and *T. vivax* swimming by default in the forward direction were able to change swimming direction by swimming backwards over short distances before proceeding with forward movement. *T. evansi* was capable of continuous backward swimming (**Fig. 4.2 A, C**). Quantitative analysis of the *T. evansi* KETRI 4009 swimming directions showed that 56% of directional swimmers displayed persistent backward swimming (**Fig. 4.2 D**). The proportion of forward swimmers was 18%, while 19% of the parasites could alternate swimming in both directions (**Fig. 4.2 B**). The proportion of persistent backward swimmers was relatively lower in another strain of *T evansi* (KETRI 2479). Persistent backward swimmers were rarely observed in *T. evansi* when viscosity of the media was raised to match bloods' viscosity as well as in the micropillars. Generally, average backward swimming speeds were lower than forward swimming speeds.
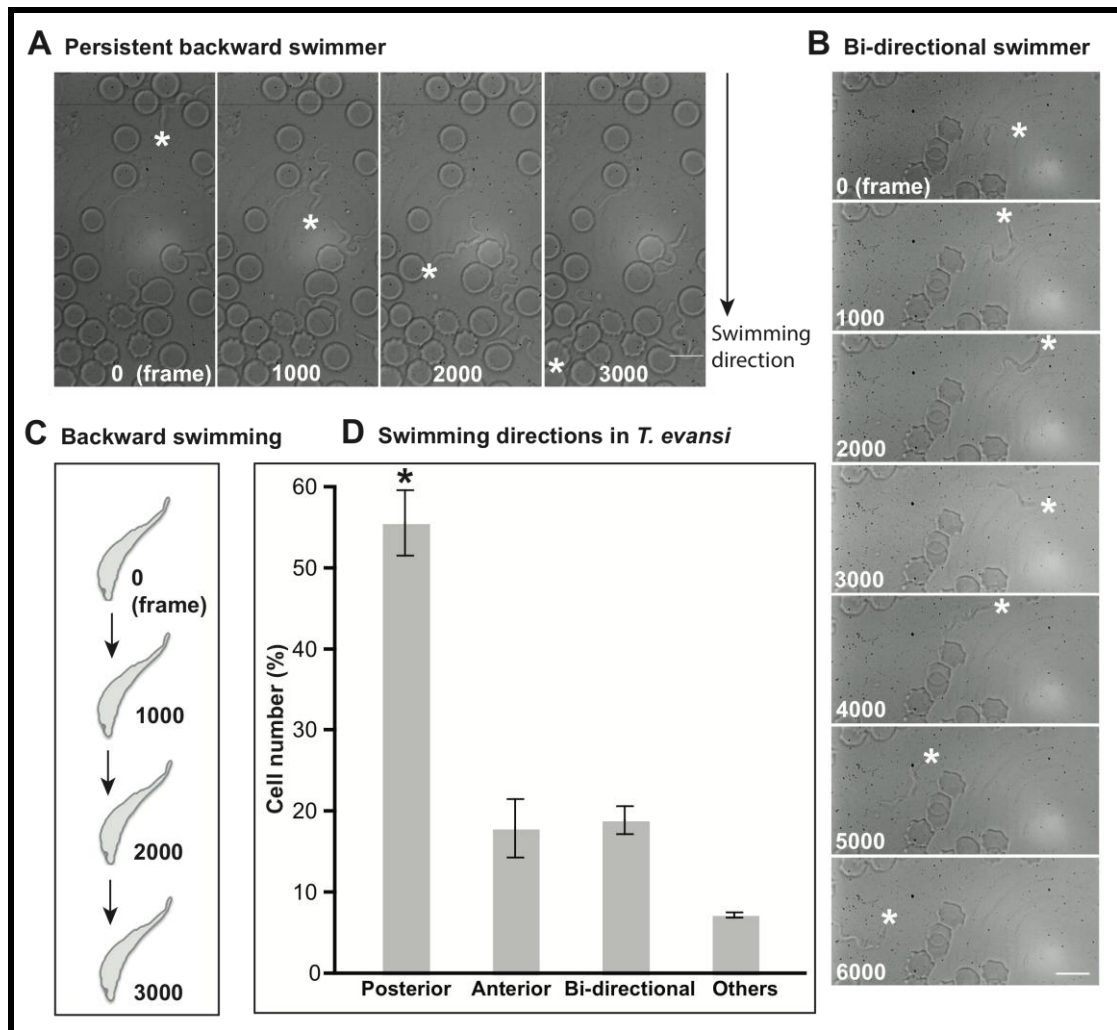
Figure 4.2: Analysis of the motility behaviour of *T. evansi* KETRI 4009 in mouse blood showed its ability to swim persistently in the forward or reverse direction. Swimming direction of the trypanosome is determined by the direction of flagellar waves (i.e. tip-to-base or base-to-tip waves) that travel through the cell body.

(A) Still images were selected from high-speed movie after every 1000 frames in order to show persistent backward swimmer.

(B) Still images of a bi-directional swimmer in *T. evansi* were taken at intervals of 1000 frames. Bi-directional swimmers are described as those parasites that swim forwards and then backwards or *vice versa*. In Fig. 4.2 B, backward swimming occurred from frame 0 - 2,000, while frames 3,000 - 6,000 showed forward swimming. The asterisk (*) shows posterior pole of the parasite. Scale bar (A and B) = 10 µm.

(C) Cartoon representation of the persistent backward swimmer illustrated in Fig. 4.2 A. The diagrams are not drawn to scale.

(D) Quantification of swimming directions in *T. evansi* (n = 300). More than 50% of directional swimmers swam persistently backwards in mouse blood (highlighted with asterisk). Continuous backward swimming was not observed in all other species studied. The fourth category named "others" was comprised of trypanosomes lacking definite swimming direction, such as non-motile parasites.

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4.1.3 Swimming speeds of trypanosomes

In order for trypanosomes to clear host-derived antibodies from their surfaces, they must swim directionally (i.e. forwards) at higher velocities (Engstler *et al.*, 2007). We initially compared the average velocities of four trypanosome species in mouse blood. Motility was investigated in blood so as to mimic conditions that are as close to the natural environment as possible. *T. vivax* was the fastest swimmer in mouse blood, with maximum speeds of more than 100 μm/s (**Table 4.2; Fig. 4.3 A, mouse**). In contrast, *T. congolense* was the slowest swimmer in mouse blood with average persistent speeds ranging from 7.86±3.1 to 9.56±5.0 μm/s. The mean population velocities were within same range of magnitude in all species studied, except in *T. vivax* whose average speed nearly doubled the mean speeds of the other species (**Table 4.2**).

Table 4.2: Average swimming speeds of trypanosomes (n=300 trypanosomes/species) in mouse blood. Swimming trypanosomes were categorized according to swimming patterns, followed by generation of speed. Average speeds were compared within patterns in all species and strains ($p<0.0001$). Data = Mean ± SD

Trypanosome	Length (μm)	Width (μm)	Average swimming speed (μm/s)			Mean population velocity (μm/s)
			Tumbling	Intermediate	Persistent	
T. congolense IL 1180	13.57 (10.6 - 18.2)	2.36 (1.82 - 3.11)	4.67 ± 2.2 (1.8 - 11.5)	8.73 ± 4.3 (2.7 - 24.7)	9.56 ± 5.0 (3.0 - 26.4)	7.20
T. congolense KETRI 3827	18.5 (13.7 - 24.5)	2.65 (1.93 - 3.7)	3.53 ± 2.6 (0.7 - 14.7)	6.00 ± 2.6 (2.4 - 13.9)	7.86 ± 3.1 (2.3 - 13.1)	6.12
T. vivax IL 1392	23.05 (22.8 - 23.3)	3.36 (2 - 3.18)	4.74 ± 3.3 (0.7 - 17.0)	11.10 ± 8.1 (2.3 - 38.5)	29.52 ± 19.3 (4.7 - 100.0)	15.20
T. b. brucei IL Tat 1.4	22.9 (20.1 - 26.1)	2.93 (1.95 - 3.58)	5.65 ± 6.9 (0.7 - 26.3)	10.46 ± 3.9 (2.8 - 22.5)	18.83 ± 4.8 (6 - 21.2)	10.67
T. evansi KETRI 2479	21.6 (19.4 - 24.3)	2.91 (2.1 - 3.7)	4.64 ± 2.4 (1.4 - 11.3)	9.14 ± 2.9 (4.4 - 15.6)	16.08 ± 5.42 (5.3 - 26)	8.06

The numbers enclosed in brackets represent minimum and maximum values in length, width and speed.

4.1.3.1 Swimming speed of trypanosome in sheep *versus* mouse blood

a) *T. vivax*

Significant speed differences occurred between three major swimming patterns in both mouse and sheep wet blood films ($p<0.0001$). There were no significant differences when the same swimming pattern in *T. vivax* was compared in sheep

versus mouse blood (p=0.5874; **Fig. 4.3 A**). For instance, the average persistent speed of *T. vivax* in mouse blood was 29.5 µm/s (n=100 trypanosomes), which was not significantly different from its mean persistent speed of 28.6 µm/s in sheep blood (n=510 trypanosomes). In general, speeds depended on the swimming pattern and the persistent swimming pattern achieved highest speeds, followed by the intermediate swimmers, while tumblers swam at lowest speeds.
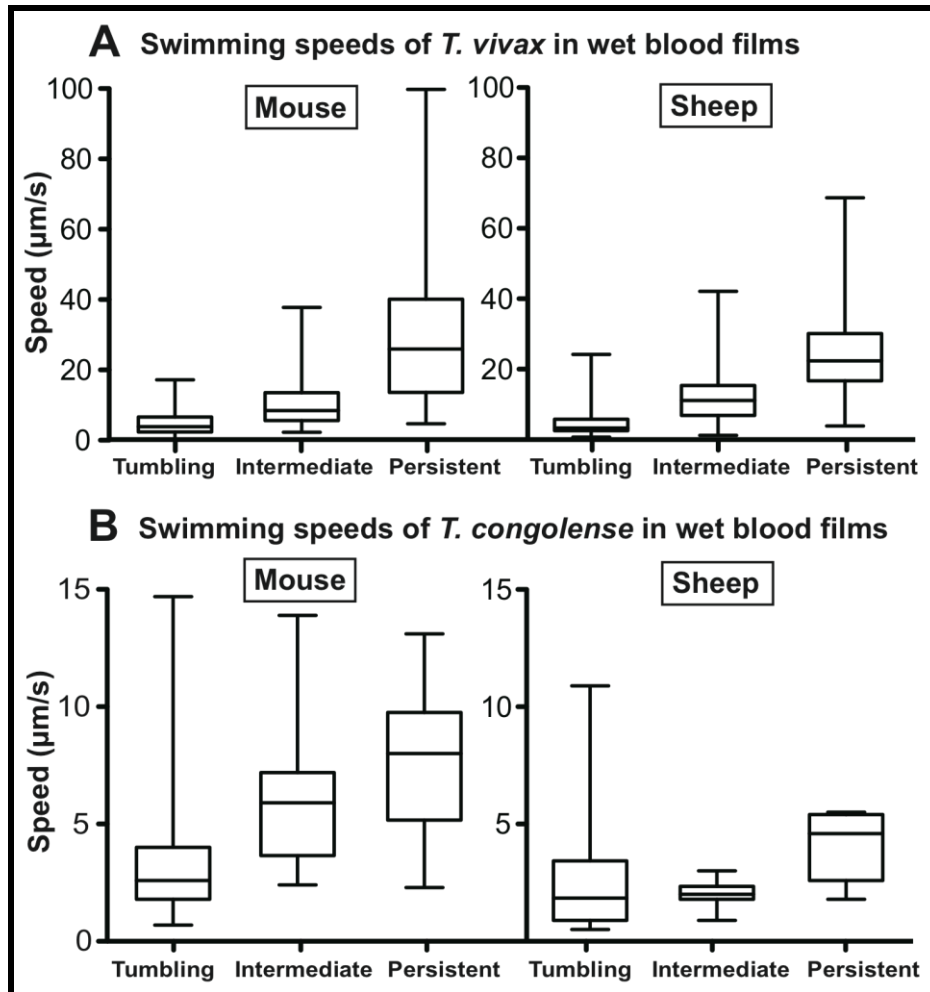

Figure 4.3: Comparative swimming speed of *T. congolense* KETRI 3827 and *T. vivax* IL 1392 in the blood of mammalian hosts (n=100 trypanosomes/swimming pattern). Mice and sheep were infected with trypanosomes and following the rise in parasitaemia, parasite motility was recorded using high-speed microscopy at 500 fps. Swimming speeds were computed and categorized according to the swimming patterns of individual swimmers, i.e. tumbling, intermediate or persistent pattern.
A: Shows no significant difference in the mean speeds of *T. vivax* swimmers in mouse and sheep wet blood films (p=0.5874).
B: The average swimming speeds of *T. congolense* were significantly higher in mouse blood than in sheep (p=0.0005).

b) *T. congolense*

In *T. congolense*, the average swimming speeds of the same swimming pattern in mouse versus sheep blood was significantly different- contrary to the case in *T. vivax* (*p*=0.0005; **Fig. 4.3B**). Importantly, *T. congolense* exhibited higher average speeds in mouse than in sheep blood (**Fig. 4.3B**).

In summary, trypanosome species showed varied proportions of swimming patterns and speeds in different mammalian hosts. These parasites adjusted their motility in different culture environments so as to ensure their survival, development and continuation of their life cycles.

4.2 Antibody clearance in African trypanosomes

4.2.1 Internalization of the surface VSG-antibody complexes

Qualitative analysis of surface antibody removal was studied in *T. brucei*, *T. congolense* and *T. vivax*. Initially at t = 0 sec, the trypanosome surface was uniformly covered with antibody conjugated to green fluorescent dye (**Fig. 4.4**). After 10 sec of incubation at 37°C, the VSG-IgG complexes accumulated at the flagellar pocket and at 20th sec, most of the fluorescing antibodies were internalized. At t = 40 sec, antibodies were further internalized and processing of the signal was shown by the increased number of internal vesicles (**Fig. 4.4,** see *T. brucei*). Consequently, processing of internalized VSG-IgG complexes continued during t = 1 to t = 5 min of incubation and after 10th min, the signal intensity had reduced in all species. In summary, *T. brucei, T. congolense* and *T. vivax* were capable of internalizing surface VSG-IgG complexes and surprisingly, a similar trend of endocytosis was followed in all species.

4.2.2 Hydrodynamic drag forces in swimming parasites

Trypanosomes must attain minimum speed threshold in order to clear surface antibodies (Engstler *et al.,* 2007). It is conceivable that the parasites swimming at higher velocities would experience stronger hydrodynamic drag forces. The drag force (F_d) can be calculated from Stokes equation by assuming that the cell body of the trypanosome is a sphere with defined radius (*r*): $F_d = 6\pi\eta r v$; where $\boldsymbol{\eta}$ is the dynamic viscosity of the medium and $\boldsymbol{v}$ is the swimming velocity. *T. vivax* ($r = 1.5$ μm) swimming in mouse blood ($\boldsymbol{\eta} = 4.5$ mPa s; Heddergott *et al.* 2012) at a velocity

of 100 µm/s will experience a viscous drag force of 12.72 piconewton (pN), whereas a parasite swimming at 0.7 µm/s (see *T. vivax* tumbler; **Table 4.2**) will experience a viscous drag force of only 0.09 pN. If, for example, the minimum speed threshold for antibody clearance in culture medium is 20 µm/s (= 2.54 pN), then it is tempting to conclude that most tumblers- characterized by lower swimming speeds- can hardly survive host's antibody challenge. However, this view is only speculative due to lack of information regarding, for instance, the role of microenvironment on the locomotion of trypanosomes. Highest swimming velocities were attained by most parasites in the PDMS-pillar arrays, while higher viscosity promoted higher speeds and increased proportion of directional swimmers. Only *T. congolense* followed a different path because all parasites tumbled in pillars and also exhibited lowest velocities. In addition, more than 80% of the parasites tumbled under increasing viscosities in TDB (**Table 4.4**).
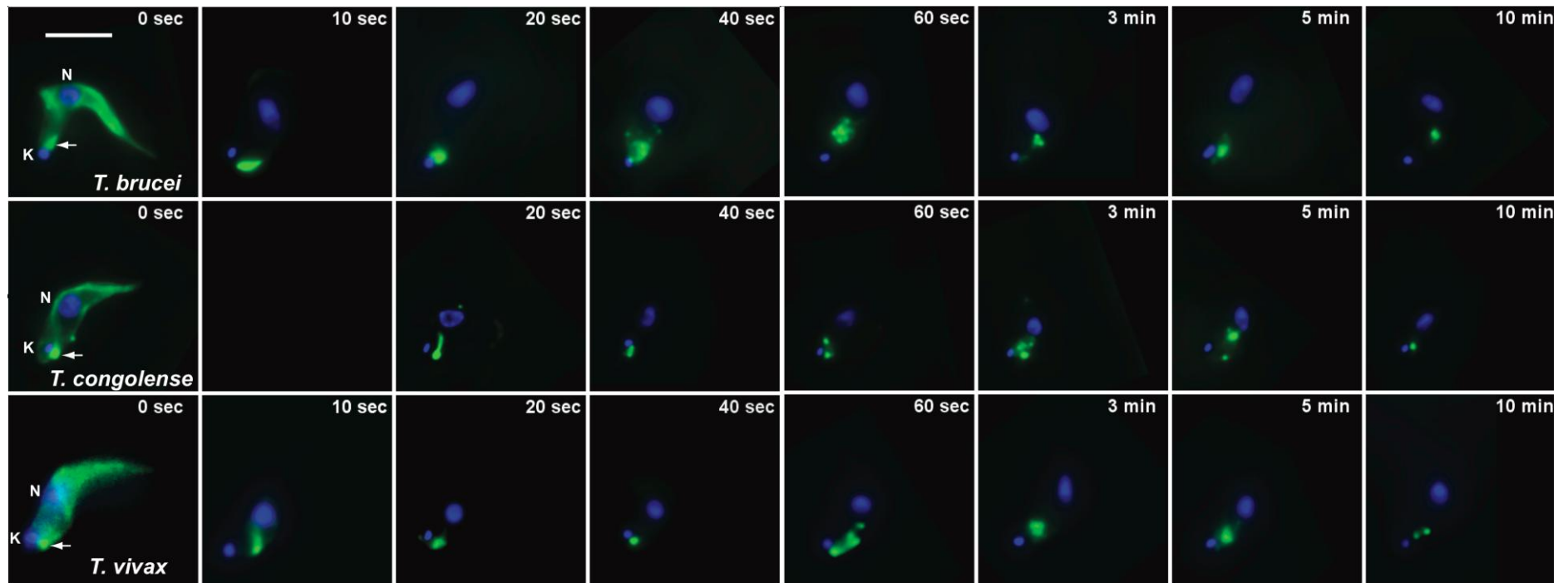
Figure 4.4: Antibody removal from the cell surface of bloodstream forms of *Trypanosoma brucei, T. congolense* and *T. vivax*. Trypanosomes were purified from mouse blood and surface-stained with 2 mM Sulfo-NHS-SS-biotin. Consequently, bound biotin was detected following incubation, for 30 min on ice, in 10 µg/ml mouse monoclonal anti-Biotin IgG conjugated to green-fluorescent dye, CF™488A. Endocytosis was followed at 37°C for 0-10 min and cells were immediately fixed in 4% paraformaldehyde at each time point. Blue-fluorescent dye, DAPI, was used to select trypanosomes at 1K1N stage (K= Kinetoplast, N= Nucleus). The above images of trypanosomes have the same orientation. The arrow points to the flagellar pocket. Scale bar= 10 µm.

4.3 The cellular basis for swimming behavior in trypanosomes

Trypanosomes can produce two kinds of flagella-driven waves; *tip-to-base (ttb)-* for forward propulsion of the cell and *base-to-tip (btt)* wave, which interrupts forward swimming. Parasites can generate continuous *btt* waves that results into continuous swimming in the reverse direction (Heddergott *et al.,* 2012). Interruption of *ttb* waves by *btt* waves has been thought to cause tumbling motion in swimming parasites (Branche *et al.,* 2006; Gadelha *et al.,* 2007).

4.3.1 Detailed analysis of trypanosome motility

Motility of individual parasites was investigated in order to understand the swimming behavior of trypanosomes and confirm that all other species move in the same way described for *T. brucei*. Detailed analysis of swimming has only been reported for cultured forms of *T. brucei* (Heddergott *et al.,* 2012). We, therefore, collected huge amount of high-resolution swimming data (more than 20 terabytes) in different culture environments. Recorded movies of motile trypanosomes facilitated better comparison of motility as swimming was resolved to single flagellar beats in different parasite species. Trypanosome motility was investigated in mouse blood so as to mimic natural conditions that are closer to the bloodstream environment in the mammalian host. Cellular motility was investigated in detail in order to reveal the causes of variations in trypanosome motility. Motility studies focused on specific details of swimming that included; beat-to-beat swimming speeds, flagella beat frequency and wavelength, direction of flagellar waves, interaction of swimming trypanosome with its surrounding environment, cell architecture in relation to motility, etc.

a) ***T. congolense*:** The speeds of *T. congolense* swimmer varied from one beat to the next depending on wave direction (*ttb* or *btt*) and how fast these waves travelled through the cell body. Slower and irregular waves produced asymmetric amplitudes of the anterior tip and slower motion in most cases. The distribution of the blood cells on the microscopic slide influenced the swimming speeds and the direction of waves (relate swimming data in **Fig. 4.5 A** to the snapshot in **Fig. 4.5a**). For instance, regions on the slide that were crowded with blood cells seemed to slow down parasites and in some cases, swimming was briefly halted

followed by reversed swimming that allowed for change of swimming direction. Maximum swimming speed of 34 µm/s was attained in mouse blood by *T. congolense* swimmer (**Fig. 4.5 A**). The average beat frequency for the analyzed *T. congolense* swimmer was 5.14 Hz (n = 27 beats; maximum frequency = 10.2 Hz).

b) ***T. vivax*:** Comparison of beat-to-beat speeds showed that the velocities of *T. vivax* swimmer varied along its swimming path. Depending on the interactions of swimming trypanosomes with the surrounding erythrocytes, speeds were either increased or decreased. Maximum speed of 126.06 µm/s was produced when the parasite rotated around the erythrocytes at 27th beat **(Fig. 4.5b; Fig. 4.5B)**. The anterior tip of the trypanosome poked the erythrocyte at 14th beat resulting into complete motion halt, speed = 0 µm/s (**Fig. 4.5B**). The average beat frequency in *T. vivax* swimmer was 28.66 Hz (n = 42 beats; maximum frequency = 35.71 Hz), while the average beat-to-beat speed was 87.5 µm/s.

c) ***T. evansi*:** The analyzed *T. evansi* swimmer occasionally caused temporary deformation of erythrocytes as it pressed against the blood cells using flagellar tip or cell body. Mechanical interaction between swimming trypanosomes and erythrocytes resulted in higher speeds- with highest attained speed being 66.39 µm/s. Increase in speed was attributed to the mechanical advantage arising from parasite's interaction with blood cells with correct diameter and spacing. However, the parasite appeared trapped between blood cells in some cases and this caused slower speeds **(Fig. 4.5C; Fig. 4.5c)**. In addition, interruption of forward speeds due to *base-to-tip* waves caused reduction in speeds.

The average beat frequency in *T. evansi* was 15.07 Hz (n = 29 beats; maximum frequency = 22.73 Hz), whereas average beat-to-beat speed was 24.46 µm/s.
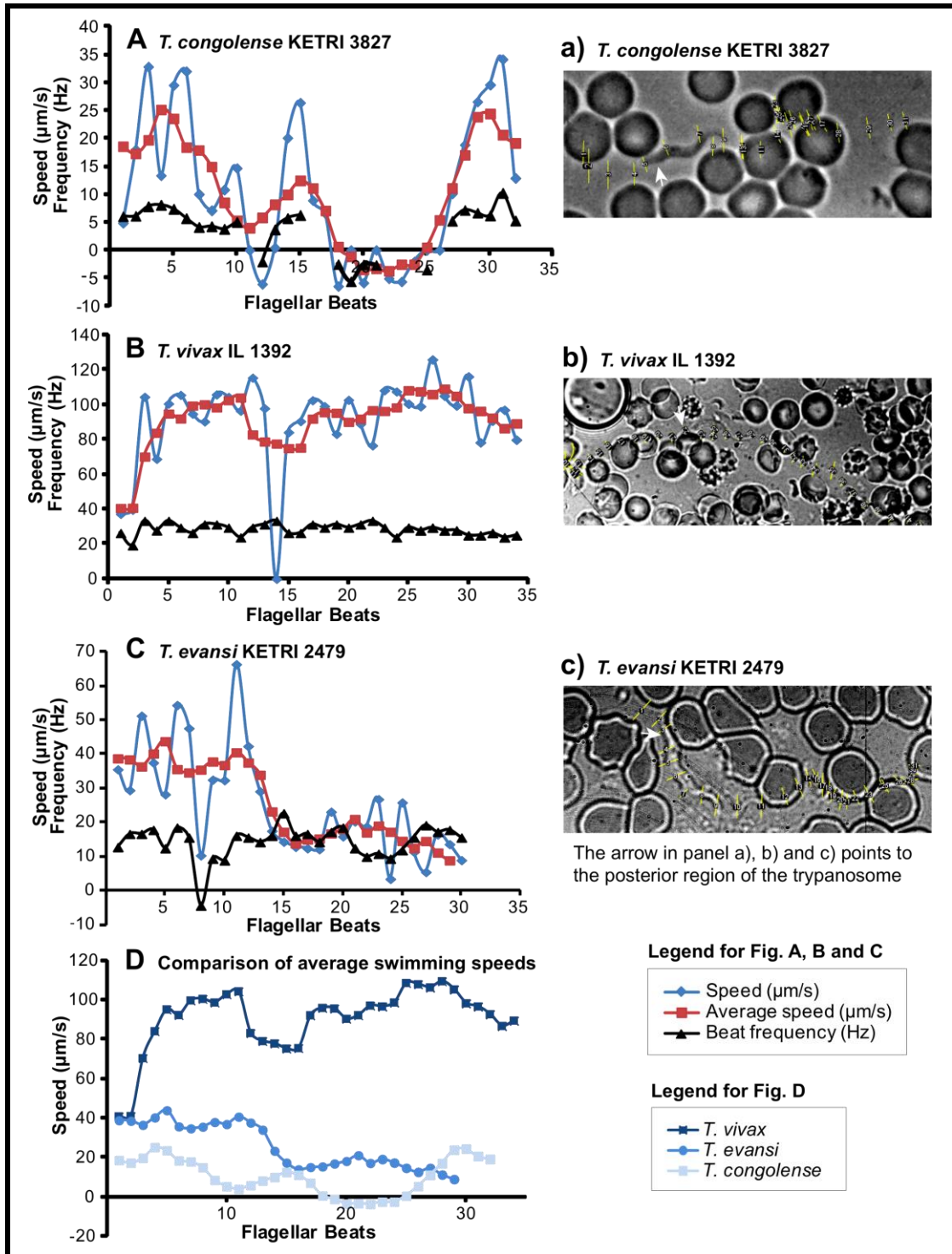
Figure 4.5: Detailed analysis of trypanosome motility in fresh mouse wet blood films
Trypanosomes exhibited huge variations in swimming speeds (A-D) and the slower swimmers in *T. congolense* were capable of achieving short bursts of higher speeds (Fig. A). **Panel a), b) and c)** showed snapshots of parasite's swimming path used to obtain information to characterize motility in individual cells. Representation of motility data, specifically beat frequency and resultant beat-to-beat swimming speed, were summarized in **Fig. A; B and C**.

4.3.1.1 Comparison of speeds between trypanosome species

Variations in trypanosome motility occurred at cellular level, between individual trypanosomes in the same population, as well as between species/strains. *T. vivax* was the fastest swimmer, followed by *T. evansi*, whereas *T. congolense* was the slowest. Characteristic beat frequencies were observed for each trypanosome species. For instance, highest frequencies were observed in *T. vivax* relative to *T. congolense*, which produced lowest beat frequencies (**Fig. 4.6 A, B, C, D, E**). However, parasites swimming at the same beat frequency were observed to swim with significantly different speeds (compare *T. vivax* in **Fig. 4.6 A and B**); hence swimming speeds mainly depend on trypanosome morphology and the swimming environment.

Analysis of the beat-to-beat swimming speeds along swimming path showed variation in speeds and this was contributed by factors such as; erythrocyte encounter, direction of flagellar beat along the cell body, trypanosome species, etc. Notably, the slowest swimmers in *T. congolense* occasionally attained swimming speeds of above 30 µm/s, but for short time scales (**Fig. 4.5 A**). We presumed that these short 'bursts' of higher speeds in *T. congolense* were enough to clear surface antibodies directed against the parasite by the vertebrate host.

4.3.1.2 Cell shape influences swimming velocities in trypanosomes

In *T. vivax* population, analysis of persistent swimmers revealed two morphotypes that swam at different speeds and were categorized as: the 'fastest' persistent and 'slower' persistent swimmers. 'Fastest' persistent swimmers were highly persistent fast swimmers that swam for relatively longer distances in wet blood films, as well as in the TDB and in PDMS-pillar arrays. They swam continuously for 12 - 60 sec and tumbled occasionally for about 2 sec in between incessant directional swimming.

On the other hand, 'slower' persistent swimmers represented larger proportion of persistent swimmers in *T. vivax* population relative to the 'fastest' swimmers that consisted of about 1% of total population in mouse blood.
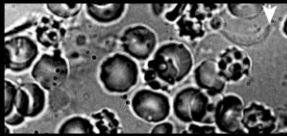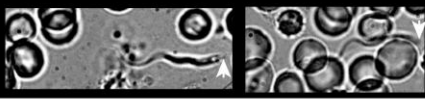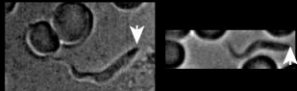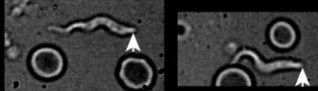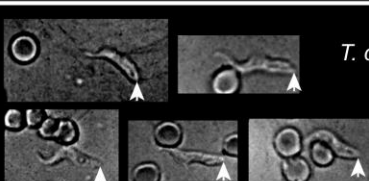
		Speed (µm/s)	Beat frequency (Hz)
A	*T. vivax* IL 1392 (mouse)	109	28.7
B	*T. vivax* IL 1392 (mouse)	42 - 48	21 - 28
C	*T. congolense* IL 1180 (mouse)	17 - 18	7 - 9.4
D	*T. congolense* KETRI 3827 (mouse)	21 - 24	5.6 - 6.1
E	*T. congolense* KETRI 3827 (sheep)	4.4 - 9.3	1.5 - 6.3

Figure 4.6: The role of trypanosome cell shape on trypanosome motility in blood.
T. vivax was the fastest swimmer with highest beat frequencies than *T. congolense*. Two
types of persistent swimmers were identified in *T. vivax* population based on their swimming
speed and body morphology (*see* panel **A**= 'fastest' persistent swimmer and **B**= 'slower'
persistent swimmer). Arrow points to the posterior region of trypanosome.

The 'fastest' persistent swimmers produced larger waves and reached swimming
velocities of over 100 µm/s (beat frequency = 28.7 Hz), whereas the 'slower'
persistent swimmers swam at relatively lower average speed of 29.52 ± 19.3 µm/s
(beat frequency = 21-28 Hz) in mouse blood.

The cell shape of the two morphotypes was distinctly different i.e. the cell body of
'fastest' swimmer appeared straighter and rigid during swimming, unlike in 'slower'
persistent swimmer with wavier and flexible body (Compare **Fig. 4.6 A** and **B**). The
morphology of **B8** in **Fig. 4.7** exhibited hallmarks of 'fastest' persistently swimming
waveforms.

Our findings above showed that speeds of individual parasites in a population varied
greatly and motility was influenced by the cell architecture. It is conceivable that the
force of the wave propagated through the cell body, depends on the cell shape- thus
influencing swimming velocities.

4.4 Three-dimensional analysis of cell architecture

4.4.1 Surface morphology

Trypanosomes are naturally asymmetrical in shape- with broad posterior region that becomes thinner towards anterior region. The length from posterior to anterior tip varied depending on the species/strains; *T. congolense* was the shortest parasite, about 13-18 μm in length. However, the width- being the widest point across the cell body- was fairly similar in all trypanosome species/strains (**Table 4.2).**

Three-dimensional surface morphology models of trypanosome species were compared in order to relate morphological differences to variations in motility. The trypanosome surface was stained with fluorescent AMCA-Sulfo-NHS dye that revealed both the outline of cell body and flagellum.

The shape of posterior tip was rounded in *T. congolense* and *T. vivax,* whereas *T. brucei* and *T. evansi* had a pointed outline (**Fig. 4.7 A-E**).

4.4.2 Flagellar morphology

Trypanosomes have single flagellum that is attached to the cell body, originating from the flagellar pocket at the posterior region and extends to the anterior end. Free flagellum, defined as the free or unattached part of the flagellum that extended beyond the anterior tip, was present in *T. vivax, T. evansi* and *T. brucei*, but absent in *T. congolense* (**Fig. 4.7 A, B, C** and **D**).

The flagellum of *T. congolense* was attached to the cell body and ran from the flagellar pocket to the anterior region following a straight path (**Fig. 4.7 A** and **4.7 E**). Straight attachment of the flagellum looked very similar in most cells. Like in other trypanosome species, *T. congolense* displayed rotational movement while swimming. It appeared like a turn of the flagellum was present in some swimmers and this turn was further anterior where the body became thinner and more flexible- this made it very difficult to quantify it in detail.

Both *T. vivax* IL 1392 and *T. evansi* KETRI 2479 had right-handed flagella (**Fig. 4.7 B, C**). Handedness, here, referred to the direction of the flagellum turn beginning from the flagellar pocket. Previous studies described the morphology of *T. brucei* MiTat 1.6 as an s-shaped cell body with a single left-handed flagellum wrapped around the posterior region of the cell body following a 180° turn (**Fig. 4.7 D**). In comparison, the bloodstream form *T. brucei* ILTat 1.4 was longer and thinner

morphotype whose flagellum followed a right handed turn of 180° around the cell body.
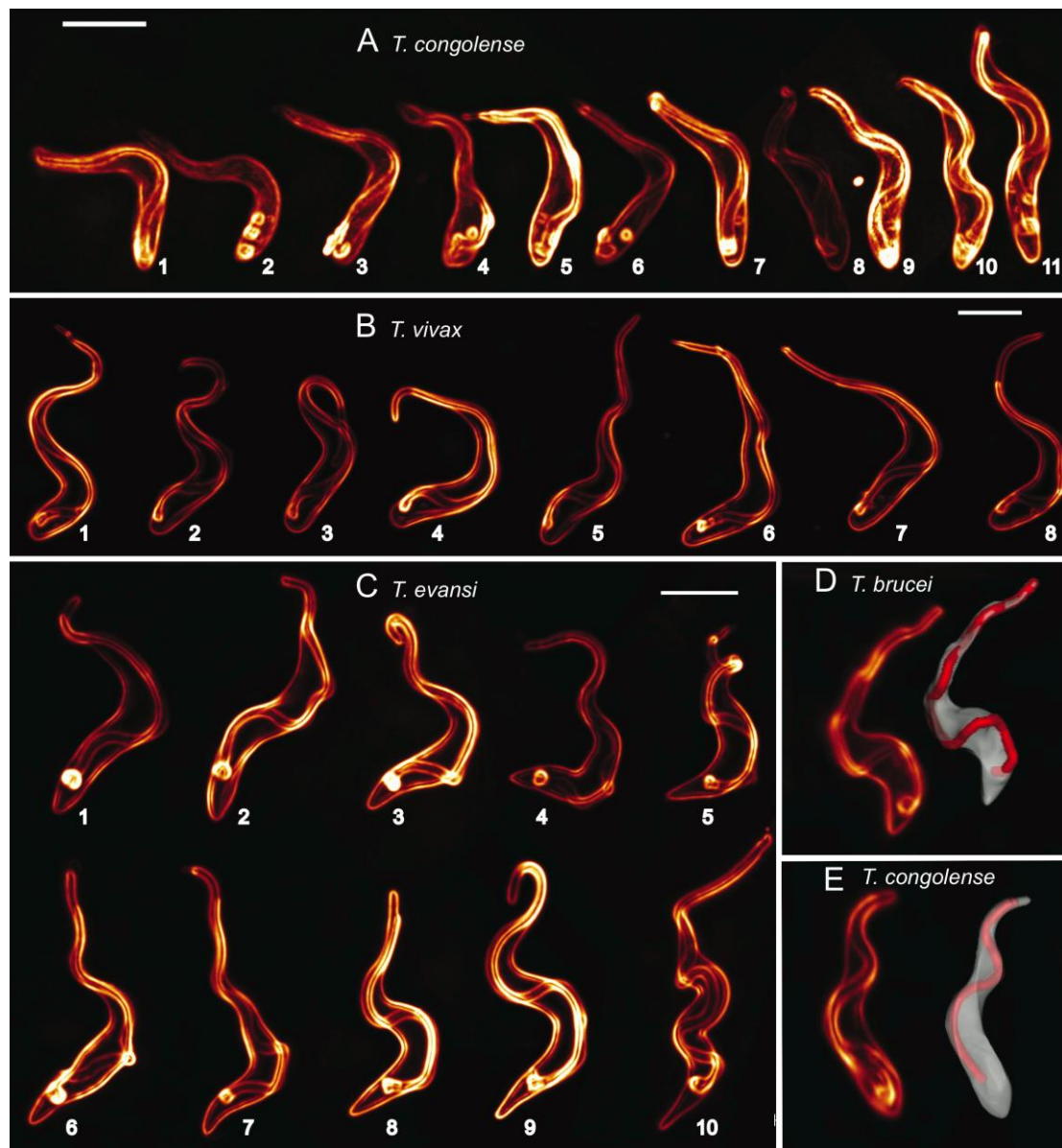

Figure 4.7: Morphology of trypanosome species. These models were generated by staining the surface of parasites with AMCA-sulfo-NHS followed by acquisition of 150 xyz stacks in 100nm steps. Free flagellum was lacking only in *T. congolense* **(A)**. Analysis of the flagellar morphology in three-dimensions revealed three types of flagella shapes; straight (see **A and E**), left-handed **(D)** and right-handed **(B and C)**. Scale bar = 5 μm. The shape of the posterior tip was either rounded **(A and B)** or pointed **(C and D)**.

Presumably, *T. congolense* waveforms with **'L'**-shape were not directional swimmers **(Fig. 4.7 A: 1-7)**. The surface morphology of the slightly larger *T. congolense* found to the right of the figure looked like the persistent swimmers waveforms (**Fig. 4.7 A: 9, 10 and 11**). In all cases, however, the trypanosomes rotated when swimming.

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Rotational movement occurred simply because of the asymmetrical nature of the cell shape.

4.5 Simulation of trypanosome motility in the host bloodstream

Our findings on trypanosome motility reported so far was mainly generated using high-speed video microscopy that relied on fresh sample preparations on a microscopic slide. Definitely, the environmental conditions on the slide preparations differed from those in the mammalian host- thus resulting in changes in motility. For instance, the viscosity of the slide preparation was obviously different from the bloodstream viscosity in the host. Media viscosity has been shown to influence trypanosome motility by Heddergott *et al.,* (2012).

Blood is constantly under flow in the bloodstream and is characterized by the presence of differently sized particles such as blood cells that together with other blood components define its viscosity. Therefore, we simulated bloodstream conditions by raising viscosity of TDB and infected blood using 0.4% (w/v) methylcellulose (Heddergott *et al.,* 2012).

In another separate experiment, trypanosome motility was simulated in the mammalian bloodstream by employing inert micropillar arrays whose diameter and spacing matched the parameters of blood cells. The diameter of blood cells in mouse blood ranged between 6.1 - 12.3 µm (average diameter = 8.35 µm; n = 30). Furthermore, since some distance separated the blood cells during blood flow, pillars were spaced to match the expectations in the bloodstream.

4.5.1 Viscosity of the culture media influences trypanosome motility
4.5.1.1 Effects on swimming speed

Higher viscosity in TDB and infected blood achieved by supplementing more than 0.2% (w/v) methylcellulose (MC) led to reduction of swimming speeds in *T. vivax* (**Fig. 4.8 E, F**). Highest mean speed of 52.83 ± 20.61 µm/s was achieved in *T. vivax* at 0.2% (w/v) MC in TDB, whereas maximum average speed of 38.96 ± 12.55 µm/s was achieved in mouse blood at the same concentration of 0.2% (w/v) MC. Similarly, a maximum speed of 75.6 µm/s was recorded in fresh wet blood film supplemented with 0.4% (w/v) MC (**Table 4.3**).

On the other hand, the mean speeds of *T. brucei* and *T. evansi* increased with the increased concentration of MC in TDB as well as in blood (**Fig. 4.8 A, B, C, D**). A maximum speed of 55.5 µm/s was recorded in *T. evansi* at 0.8% (w/v) MC in blood (**Table 4.3**). This behaviour was similar to that of *T. brucei*, with the highest average speed of 53 µm/s and maximum speed of 80 µm/s recorded in 0.6 % methylcellulose (**Fig. 4.8 B**).

Swimming speeds of *T. congolense* in buffer and in blood did not have major motility changes, even with increased viscosity (**Fig. 4.8 G, H**). Maximum speed in TDB was achieved at 0.2% (w/v) MC, whereas in wet blood films, the highest speed of 11.5 ± 4.94 µm/s was attained at 0.6% (w/v) MC in *T. congolense* (**Fig. 4.8 G, H; Table 4.3**).

Table 4.3: Maximum swimming velocity of trypanosomes in TDB and infected mouse blood. Trypanosome motility was simulated in the host bloodstream by raising media viscosity, using methylcellulose (% (w/v) MC), to match bloodstream viscosity. In addition, motility was investigated in the PDMS-pillar arrays.

Culture media	Maximum swimming speeds (µm/s)			
	T. vivax	*T. evansi*	*T. congolense*	*T. brucei*
TDB	95	13.2	11.7	33.3
TDB + MC	90 (0.2% MC)	60.6 (0.6% MC)	30.2 (0.2% MC)	69.2 (0.8% MC)
Infected blood	100	26	26.4	25.7
Infected blood + MC	75.6 (0.4% MC)	55.5 (0.8% MC)	27 (0.2% MC)	79.7 (0.6% MC)
PDMS-Pillars	205.2 (d= 8, Sp= 3)	101.8 (d= 8/10; Sp= 4)	11.5 *All tumbled	174.5 (d= 10, Sp= 4)

d = diameter of the pillar in µm ; Sp = spacing between pillars in µm.
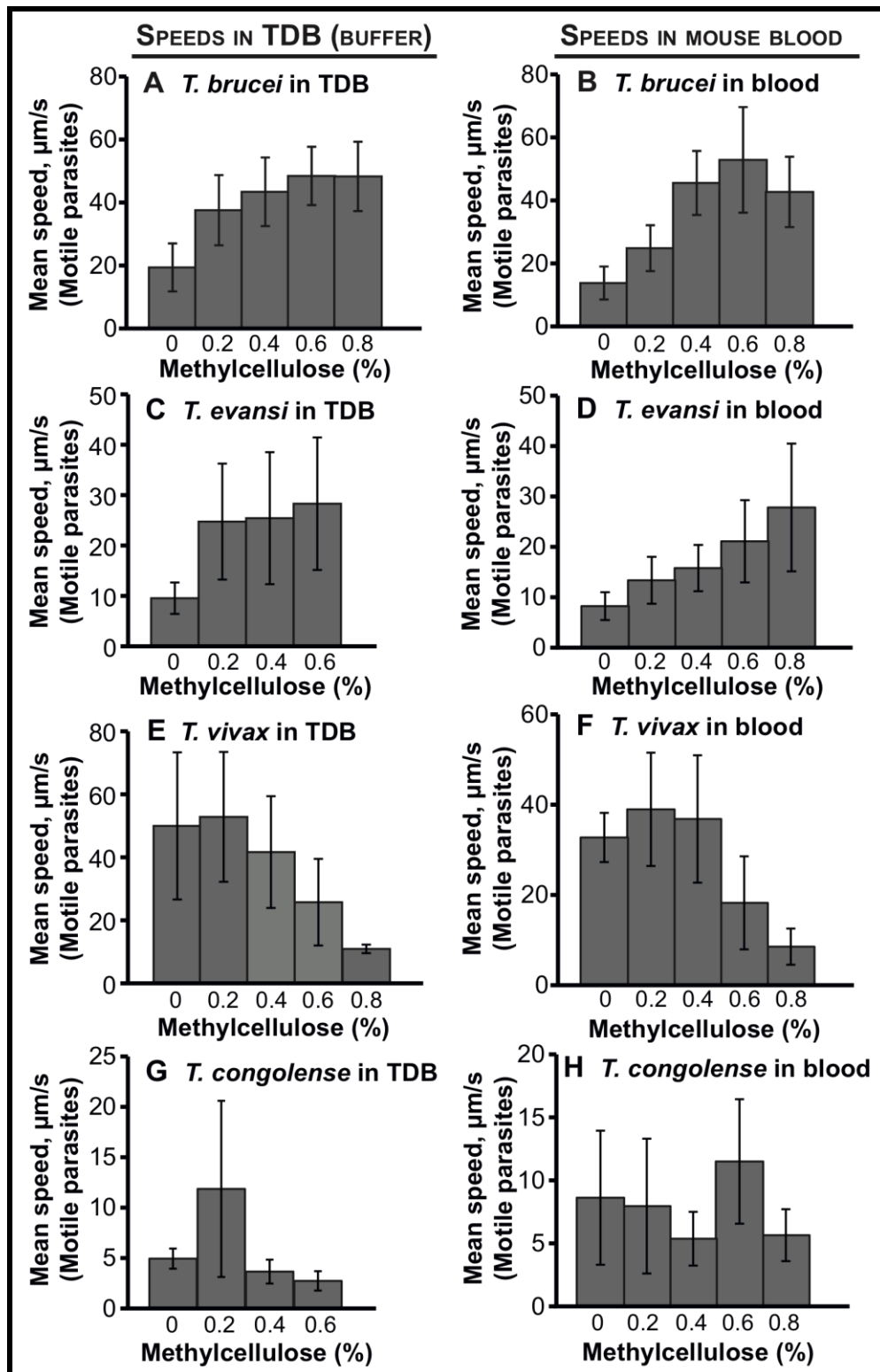
Figure 4.8: The influence of viscosity on trypanosome swimming speed. Experiments were performed in TDB (**A, C, E, G**) and in mouse blood (**B, D, F, H**). Increased viscosity led to reduction of speeds in *T. vivax,* both in TDB and in wet blood films **(E and F)**. In contrast, average speeds in both *T. brucei* and *T. evansi* steadily increased with increase in viscosity **(A, B, C and D)**. Persistent swimmers were absent in *T. congolense* and thus speeds of intermediate swimmers were measured. Highest average speed in *T. congolense* was achieved at 0.2% (w/v) MC in buffer **(E)**.

4.5.1.2 Culture viscosity affects the proportions of swimming patterns

Backward swimming occurred frequently, but for short distances, in most forward swimming parasites studied under conditions of increased viscosity. Backward swimming occurred when the trypanosome changed its swimming direction, as well as during encounter with obstacles.

Higher viscosity in TDB favoured intermediate swimming pattern in *T. vivax* and at 0.8% (w/v) MC, 99 ± 1.73% of swimmers followed intermediate swimming mode that was characterized by low mean speeds of 9.73 ± 1.48 µm/s (**Fig. 4.8 E; Table 4.4**). In addition, increased viscosity resulted in higher proportion of directional swimmers (i.e. both intermediate and persistent swimmers). Highest percentage of directional swimmers, comprising over 90% of the swimmers, in *T. vivax* was achieved in blood at 0.4% (w/v) MC. Persistent backward swimming in *T. vivax* was rarely observed in blood as only one continuous backward swimmer, with mean speed of 6.7 µm/s, was captured at 0.8% (w/v) MC.

In *T. evansi* most parasites swam directionally in TDB containing MC. An intermediate backward swimmer was observed at 0.6% (w/v) MC in TDB. Our initial findings of *T. evansi* motility in fresh wet blood films showed that more than half of the swimmers swam persistently in the reverse direction. In contrast, persistent backward swimming in *T. evansi* was hardly observed after addition of MC to the infected mouse blood.

Higher proportion of intermediate swimmers in *T. evansi* was recorded with increasing MC concentration in TDB and 0.6% (w/v) MC resulted in highest mean proportion of intermediate swimmers of 89 ± 2.65% (**Table 4.4**). Thus, the number of tumblers and persistent swimmers decreased with increased media viscosity. On the other hand, highest number of *T. evansi* swimmers (intermediate and persistent swimmers = 85%) in blood was recorded at 0.4% (w/v) MC.

Increased viscosity in TDB caused increased swimming activity in *T. congolense* and hence parasites swam for relatively longer distances than in control. However, the population consisted of tumbling majority (82 - 94% tumblers), few intermediate swimmers characterized by lower swimming speeds, and hardly any persistent

swimmer. Highest mean proportion of intermediate swimmers at 18 ± 5.29% was recorded at 0.2% (w/v) MC in TDB (**Table 4.4**).

Table 4.4: The effects of viscosity on the swimming patterns of trypanosomes (n=300 trypanosomes/pattern). Trypanosomes were isolated from mouse blood and resuspended in fresh TDB with or without methylcellulose. Swimming patterns and behaviour of trypanosomes was studied using light microscopy. Data = Mean ± SD.

Species	Pattern (Cell %)	Methylcellulose concentration (% w/v)				
		0	0.2	0.4	0.6	0.8
T vivax IL 2136	Tumbling	27.33±5.5	11.33±6.43	1.00±1.0	3.33±3.51	0.33±0.6
	Intermediate	32.67±6.0	48.33±4.73	49.33±3.5	89.33±2.5	99.00±1.7
	Persistent	40.00±2.0	43.33±11.2	49.67±4.5	7.33±1.2	0.67±1.2
T evansi KETRI 2479	Tumbling	75.33±4.5	68.33±2.08	31±2.7	9.67±2.9	44±10.5
	Intermediate	19.33±1.5	27.33±0.58	68±3.5	89±2.7	55.67±10.5
	Persistent	5.33±3.8	4.33±2.3	1±1	1.33±0.6	0.33±0.6
T congolense IL 1180	Tumbling	94±2.7	82±5.3	88±1.7	92±1.7	89.33±0.6
	Intermediate	6±2.7	18±5.3	12±1.7	8±1.7	10.67±0.6
	Persistent	0	0	0	0	0
T. brucei IL Tat 1.4	Tumbling	57.67±6.4	36.33±4.7	22.33±5.7	14.00±4.0	59.33±6.5
	Intermediate	8.67±7.0	18.67±6.0	13.33±7.6	57.00±5.6	29.67±1.5
	Persistent	33.67±8.3	45.00±3.6	64.33±12.7	29.00±8.7	11.00±5.6

Similarly, findings on *T. brucei* also showed highest proportion of persistent swimmers at 0.4% (w/v) MC in TDB (persistent = 64.33 ± 12.74%; **Table 4.4**). The average proportion of intermediate swimmers was increased to 57 ± 5.57% at 0.6% (w/v) MC in TDB. Forward and backward swimming, for short distances, was very common in *T. brucei* after addition of methylcellulose.

In summary, viscosity of culture medium influences the swimming speeds as well as the patterns of swimming in trypanosomes. In some cases, higher viscosity promoted faster swimming speeds, whereas in others, it slowed down the parasites. The proportion of swimming pattern exhibited by specific trypanosome species varied with the viscosity of the medium, and in most cases, higher viscosity caused increase in the proportions of intermediate and/or persistent patterns.

4.5.2 Trypanosome motility in the micropillar arrays

***T. congolense*:** All swimmers in *T. congolense* that entered into focus plane in the PDMS-pillars tumbled in all pillar arrays tested. Tumblers appeared to have increased swimming activity in the pillar array with d = 10 μm and Sp = 4 μm; however, this did not promote directional motion. All parasites lacked directional swimming and as a result, swam at low speeds. Analysis of high-speed movies of swimmers in the

pillars showed that *T. congolense* does not utilize pillars for generation of friction forces that permitted efficient swimming in other trypanosome species. In addition, swimming in the pillars did not influence the proportions of directional swimmers as observed in other species.

T. vivax: We analyzed motility of 'fastest' persistent *T. vivax* waveforms in the pillars and they displayed the same morphological distinctions previously reported in the mouse blood (**Fig. 4.6 A**). The cell body of fastest swimming waveforms interacted closely with the pillars for efficient swimming. Lower speeds were attained if the dynamic curvature of cell body failed to align properly with pillar surfaces. One possible explanation for lower speeds might be due to minimal interaction between the parasites' cell body and the pillars that produces smaller mechanical forces, which in turn resulted into lower speeds. In order to maneuver the narrow spacing in the pillars as well as to avoid collision with obstacles such as pillars and carry over blood cells, most trypanosomes were capable of swimming backwards for short distances. Most directional swimmers adopted unusual 'straighter' body conformation in order to maneuver through narrow pillar spacing of 3 μm and d=12 μm. In the narrow spacing, these trypanosomes achieved 'bursts' of high speeds over short swimming distances. Continuous backward swimming was observed in *T. vivax* at d= 12 μm and Sp= 6 μm. However, continuous backward swimming was uncommon in most pillar arrays.

A maximum speed of 205.2 μm/s was recorded in *T. vivax* swimmer in the homogeneous pillar array with d= 8 μm and Sp= 3 μm (**Fig. 4.9 A; Table 4.5**). This speed in the pillars was twice (2×) the maximum speed recorded for the same species in mouse blood. Highest average speeds of 80.29 ± 24.38 μm/s (d= 8/10 μm, Sp= 4 μm) and 78.01 ± 19.78 μm/s (d= 8/12 μm, Sp= 5 μm) were also recorded in the heterogeneous pillar arrays. Lowest mean speed of 9.82 ± 0.75 μm/s was achieved in pillar arrays with d= 12 μm and Sp= 6 μm (**Table 4.5**). Generally, *T. vivax* showed increased proportions of persistent and intermediate swimmers in the pillars.
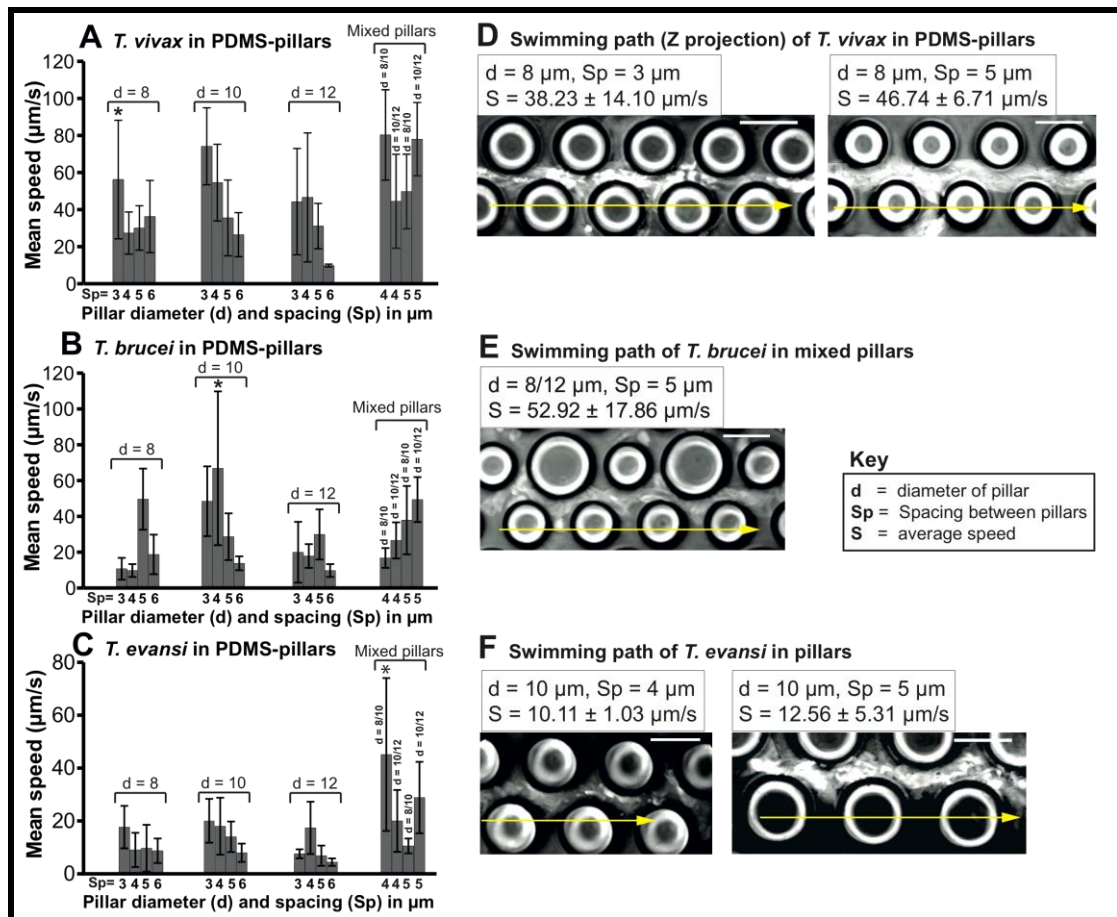
Figure 4.9: The role of PDMS-pillars on trypanosome swimming speeds. Pillars with defined diameter (d= 8, 10 and 12 μm) and spacing (Sp= 3, 4, 5 and 6 μm) were printed on thin microscope slides followed by motility studies in the pillar arrays. Swimming parasites were recorded by high-speed video microscopy at 500 fps. Maximum velocities of over 205.2 μm/s in *T. vivax* IL 2136 (d=8, Sp=3); 174.5 μm/s in *T. brucei* IL Tat 1.4 (d=10, Sp=4); and 101.8 μm/s in *T. evansi* KETRI 2479 (d=8/10, Sp=4) were achieved in pillars (shown above in **A, B and C** with an asterisk *). Interactions between the dynamic curvature of the cell body and the pillar array depended on the size of the pillars and spacing (swimming trajectories in **D, E and F**). For instance, in the heterogeneous pillars; smaller pillars produced smaller body curvatures than the larger ones (see **E** above). Scale bar = 10 μm. Yellow arrows point the direction of swimming.

T. brucei: Some *T. brucei* persistent swimmers were observed to swim without interacting with pillars whenever the spacing between pillars was wide enough, e.g. Sp= 6 μm. Generally, swimmers that lacked mechanical interaction with pillars attained relatively lower speeds. Highest mean speed of 66.83 ± 42.96 μm/s (mean ± SD; n=18) was achieved in the homogeneous pillars with d= 10 μm and Sp= 4 μm. Lowest average speed of 9.74 ± 3.60 μm/s was attained in PDMS-pillar array with d= 12 μm and Sp= 6 μm (**Table 4.5**). Nearly all trypanosomes were capable of reversing swimming direction in the pillars so as to maneuver in the spaces between obstacles and also to enable change of swimming direction. Finally, swimming in the PDMS-

pillars influenced swimming patterns as shown by the increased proportion of directional swimmers. Tumbling motion was exhibited in the pillars and the parasites appeared 'trapped' when the spacing between pillars was smaller (e.g. Sp= 3).

T. evansi: These swimmers did not readily enter into the PDMS-pillars, unlike in *T. vivax*, *T. brucei* and *T. congolense*. Most parasites that swam in the pillars soon moved out of focus after brief swimming duration. Speeds increased considerably whenever the swimming parasite sustained optimal contact with the pillars. Both flagellum and cell body are usually pressed against the surface of the pillars leading to generation of mechanical forces that are utilized for increased velocities. We observed that the anterior and posterior region, as well as the entire cell body of the trypanosome could interact with pillar surface during swimming, hence enabling efficient forward propulsion of the cell. Trypanosomes with straighter cell body generated larger wavelength that often matched the curvature of the pillars. This led to production of stronger mechanical interaction forces that promoted efficient motility. Maximum mean speed of 45.13 ± 28.91 µm/s (mean ± SD; n=9) was achieved by *T. evansi* swimmer in heterogeneous pillar arrays with d= 8/10 µm and Sp= 4 µm. Lowest average speed of 4.43 ± 1.40 µm/s was attained in the pillar array with d = 12 µm and Sp = 6 µm (**Table 4.5**).

Backward swimming for short distances, in the pillars, was very common in *T. evansi*. In addition, continuous backward swimming was observed under the following pillar arrays; (d = 8 µm, Sp = 6 µm) and (d = 12 µm, Sp = 3 µm). At d = 12 µm and Sp = 3 µm, trypanosomes seemed 'trapped' in the pillars because of larger pillar diameter and narrow spacing. Generally, the number of persistent and intermediate swimmers increased when parasites swam in the pillar arrays. Some parasites tumbled in the pillars and the tumblers lacked directional swimming in the pillars as they do in the culture media.

4.5.2.1 Pillar size and spacing affects trypanosome motility

Based on the trypanosome motility in several pillar arrays, we found that the homogeneous regularly spaced pillar array with d= 12 µm and Sp= 6 µm does not support efficient swimming in trypanosomes. *T. vivax, T. brucei* and *T. evansi* attained lowest speeds in this pillar array. *T. vivax* swimmer, known for its fastest

swimming speeds exceeding 200 µm/s in some pillar arrays, achieved maximum average speed of 9.82 ± 0.75 µm/s in this pillar array (d= 12 µm, Sp= 6 µm). Widest spacing of 6 µm allowed parasites to swim freely without interaction with pillars and in *T. brucei*, for example, most persistent swimmers showed either minimal interaction with pillars- or no interaction at all. Thus, wider spacing and larger pillar diameter made a poor parameter combination for generation of fast speeds (**Table 4.5**).

The best pillar parameters that generated maximum average speeds for both *T. vivax* and *T. evansi* was heterogeneous pillar array with d= 8/10 µm and Sp= 4 µm (**Table 4.5**). Highest mean speed in *T. brucei* was achieved at d= 10 µm and Sp= 4 µm. Heterogeneous pillar array with d= 8/12 µm and Sp= 5 µm worked best for all three species (**Fig. 4.9 E**).

The optimal spacing that produced highest swimming speeds in all trypanosome species was 4 µm (**Table 4.5**). The next spacing option that worked well was 5 µm. However, the pillar spacing of 6 µm did not produce consistent results in our studies and in most cases the resultant swimming speeds were lower in comparison to Sp= 4 or 5 µm.

The pillar diameter was important because it influenced trypanosome motility, particularly the swimming speeds. Higher speeds were obtained when pillar diameter of 8 µm and 10 µm were used, either individually (homogeneous) or when mixed (heterogeneous). Importantly, higher velocities were achieved in heterogeneous pillar array even when mixed with the largest pillars with d= 12 µm. Heterogeneous pillars enabled generation of highest mean speeds in both *T. vivax* and *T. evansi* (**Table 4.5**). Achievement of higher speeds in the heterogeneous pillar array than in homogeneous array suggested that trypanosomes motility was adapted to the situation in the mammalian bloodstream, which contains blood cells of different sizes.

Swimming trajectories (i.e. Z projections) produced by the swimmers in the pillar arrays produced regular waves (**Fig. 4.9 D, E, F**). Wider spacing of 5 µm resulted into increased width of the swimming path (compare the two swimming paths in **Fig. 4.9 D**). Thus, the trajectory path produced by the swimmers in the pillar arrays was related to the pillar diameter and pillar-to-pillar spacing.

Table 4.5: The effects of pillar size and spacing on the swimming speed. Highest speeds (µm/s) were recorded in *T. vivax* followed by *T. brucei*.

*Mean speed rank	*T. evansi*					*T. brucei*					*T. vivax*				
	Pillars (d, Sp)	n	Minimum Speed	Maximum Speed	Mean speed	Pillars (d, Sp)	n	Minimum Speed	Maximum Speed	Mean speed	Pillars (d, Sp)	n	Minimum Speed	Maximum Speed	Mean speed
1	8/10,4	9	3.8	101.8	45.13 ± 28.91	10,4	18	17.9	174.5	66.83±42.96	8/10,4	23	31.6	159.2	80.29 ± 24.38
2	8/12,5	18	3.1	53.1	28.86 ± 13.51	8,5	23	27.3	90.5	49.6±17.07	8/12,5	10	57.7	110.7	78.01 ± 19.78
3	10,12,4	13	5.2	51.7	20.02 ± 11.70	8/12,5	22	29.6	81.1	49.38±12.54	10,3	35	25.3	109.7	74.16 ± 20.77
4	10,3	7	9.8	29.5	20.07 ± 8.28	10,3	23	17.6	105.4	48.43±19.49	8,3	68	12	205.2	56.21 ± 31.99
5	10,4	7	8	35.3	18.00 ± 10.78	8/10,5	20	14.5	89.8	37.87±19.10	10,4	45	10.3	114.9	54.54 ± 20.69
6	8,3	10	6.6	28.2	17.67 ± 8.03	12,5	21	10.4	72.1	29.90±14.00	8/10,5	27	21.6	93.4	49.78 ± 20.13
7	12,4	13	3.9	32.3	17.38 ± 9.90	10,5	34	10.9	59.3	28.64±13.04	12,4	9	14.1	97.9	46.58 ± 34.81
8	10,5	12	9.3	31.2	14.01 ± 5.77	8,4	22	17.2	40.4	27.00±5.78	10/12,4	45	7.3	96.9	44.51 ± 25.35
9	8/10,5	12	4.2	14	10.55 ± 2.81	10/12,4	18	13.1	48.5	26.57±10.08	12,3	45	5.7	99.6	44.27 ± 28.7
10	8,5	12	4.5	36.6	9.69 ± 8.84	12,3	12	6.8	68.3	19.96±16.98	8,6	91	10.6	131.3	36.25 ± 19.47
11	8,4	12	4.3	26.8	9.05 ± 6.47	8,6	21	4.1	50.4	18.74±11.04	10,5	27	6	89.1	35.50 ± 20.46
12	8,6	4	4.1	14.8	8.73 ± 4.64	12,4	14	8.7	30.9	17.86±6.63	12,5	27	8.2	54.4	31.09 ± 12.24
13	10,6	9	4	15.1	7.97 ± 3.46	8/10,4	20	6.8	25.8	16.78±5.52	8,5	42	6.5	56.2	30.08 ± 11.99
14	12,3	4	5.9	9.6	7.58 ± 1.69	10,6	16	8.3	22.1	13.78±3.89	8,4	82	5	61.1	27.33 ± 11.37
15	12,5	9	2.4	12.2	6.84 ± 3.83	8,3	13	3.5	23.9	10.72±6.10	10,6	38	9.2	46.9	26.45 ± 11.89
16	12,6	10	2.6	6.8	4.43 ± 1.40	12,6	20	3.9	17.4	9.74±3.60	12,6	5	8.9	10.7	9.82 ± 0.75

*Mean speed rank = mean speeds ranked from the highest to lowest; *n* = number of analyzed speed values (µm/s); diameter- **d** and spacing- **Sp** in µm

4.6 *In vitro* binding of African trypanosomes to host erythrocytes

4.6.1 Factors affecting adherence

Binding capacities of *T. congolense* and *T. vivax* to host erythrocytes under *in vitro* conditions depended on several factors including; incubation temperature, sedimentation, whole blood components, trypanosome species/strains, host sera, host erythrocyte, among others. The effects of these factors are discussed below.

4.6.1.1 Time-course experiment to monitor trypanosome-erythrocyte adherence

Binding of trypanosomes to erythrocytes occurred immediately within seconds after mixing purified trypanosomes with rabbit erythrocytes. During swimming, adherent *T. vivax* was capable of releasing bound erythrocyte(s)- due to its fast swimming speeds. However, in *T. congolense*, most parasites remained bound to RBCs throughout the examination period of about 5 min.

In vitro binding assays showed time-dependent increase in erythrocyte binding to *T. congolense* and *T. vivax* (**Fig. 4.10 A**). Mean adherence % in four studied parasite species/strains increased exponentially between 10th and 120th min of incubation at room temperature in serum-free buffer. After 5 hrs of incubation, mean adherence percentages reached plateau phase in most parasites (**Fig. 4.10 A**).

Trypanosomes that were fixed in 4% PFA prior to adherence assay lost binding capacity to host erythrocytes. When fixed RBCs (washed after fixation) were tested for adherence ability to live trypanosomes, all parasites were fixed- unexpectedly- hence did not bind to the RBCs. In addition, fixing both trypanosomes and RBCs resulted in lack of binding.

The mean erythrocyte adherence % was consistently higher in *T. congolense* than *T. vivax* (**Fig. 4.10 A**). Centrifugation of trypanosome-erythrocyte mixtures prior to incubation at 37°C influenced mean adherence. For instance, mean adherence percentages increased in *T. congolense* IL1180 and *T. congolense* IL3000 by 16.7% and 31.3%, respectively. Therefore, during quantitation of mean adherence, centrifugation of trypanosome-erythrocyte mixtures was avoided.
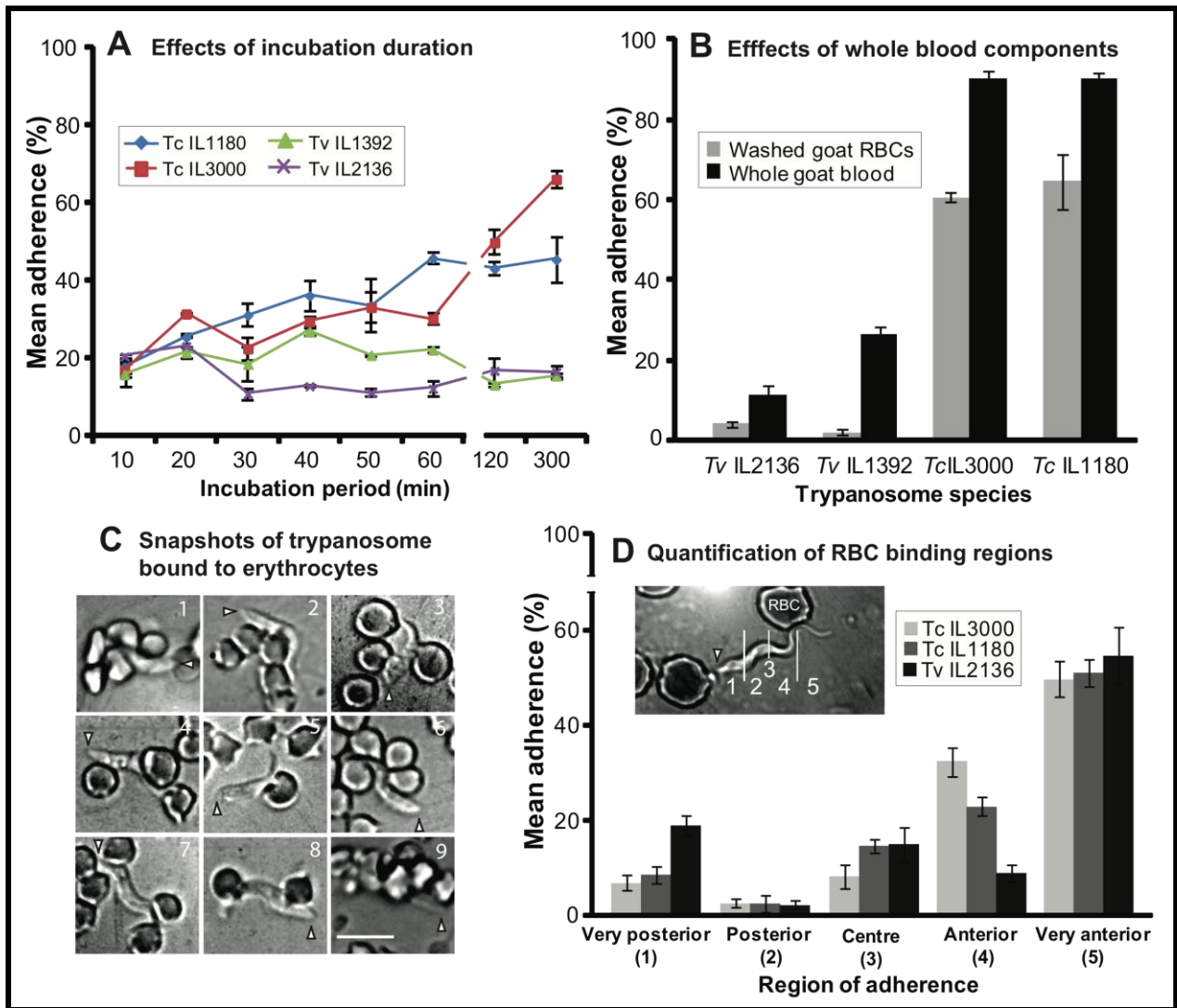
Figure 4.10: Factors influencing *in vitro* binding of trypanosomes to host erythrocytes.
(A) Time course experiment showing the effects of incubation duration on the binding capacities of trypanosomes to washed rabbit erythrocytes. The experiment was performed at room temperature (24°C). The mean adherence to erythrocytes was similar in all trypanosome species in the first 10 min. However, binding capacities in different species/strains increased with time and *T. congolense* showed higher mean adherence to erythrocytes than *T. vivax.* After 5hrs of incubation, the mean adherence in most parasites reached stationary phase. *T. brucei* and *T. evansi* were excluded in the experiment because they were non-adherent to the rabbit RBCs.
(B) Summary of experiments conducted with or without washing of goat erythrocytes (in TDB) to determine the role of whole blood components in trypanosome-RBC binding. Relatively higher mean adherence was achieved in the whole goat blood in all trypanosome species/strains.
(C) Representative snapshots of trypanosomes bound to erythrocytes. Arrowhead indicates posterior pole of the trypanosome. Scale bar = 5µm.
(D) Quantification of RBC-binding regions on the surface of trypanosomes. The cell body was divided into five parts (very posterior, posterior, centre, anterior and very anterior) to enable scoring of the RBC-binding region. Binding preference occurred on the anterior region of the parasite. Three replicates (n=300 parasites/replicate) were used in **A, B and D**. Data = mean ± SEM.

4.6.1.2 Effects of washing erythrocytes on trypanosome adherence capacity

The role of whole blood components during *in vitro* binding of the parasite to host RBCs was investigated by quantifying adherence in washed and unwashed goat erythrocytes (whole blood). Our data showed significantly higher mean adherence in the whole blood than in washed goat erythrocytes **(Fig. 4.10 B**, $p<0.05$). Trypanosomes adhered to more blood cells (i.e. 2 - 8 RBCs per parasite) when mixed with whole blood, unlike in the washed erythrocytes. This observation suggested that some factor(s) in the whole blood promoted adherence. Thus, we expect higher adherence in the mammalian bloodstream relative to the *in vitro* assays.

Higher mean adherence percentage in the whole blood was consistently observed in *T. vivax* IL 2136, *T. vivax* IL 1392, *T. congolense* IL 3000 and *T. congolense* IL 1180 with increased mean adherence percentages of: 7.33, 24.33, 29.66 and 25.33, respectively **(Fig. 4.10 B)**.

4.6.1.3 Erythrocyte-binding regions on the trypanosome surface

Most erythrocytes were bound to the very anterior region of the trypanosome, almost at the flagellar tip **(Fig. 4.10 C and D)**. The mean RBC-binding % at the very anterior region of the parasite was more than 50% in both *T. congolense* and *T. vivax*. The combined mean adherence percentage at the anterior region of the parasite (i.e. anterior *plus* very anterior region) was 82% in *T. congolense* IL 3000, 74% in *T. congolense* IL 1180 and 63.7% in *T. vivax* IL 2136 **(Fig. 4.10 D)**.

Analysis of high-speed videos of swimming parasites showed that the very posterior tip of the trypanosome hardly bound erythrocytes, but may move the red cells for short distances especially during backward swimming or when tumbling. Thus, in our categorization of the five regions on the parasite surface **(Fig. 4.10 C and D)**, it is important to clarify that the very posterior region does not necessarily mean the posterior tip of the trypanosome, but the region between the posterior tip and boundary of the posterior region. During swimming, erythrocytes rolled along surface of the cell body. Generally, an average of four RBCs were bound to *T. congolense* and occasionally, the entire parasite surface was covered by bound host erythrocytes **(Fig. 4.10 C, panel 9)**. Analysis of adherence region through electron microscopy showed presence of tight junction at the attachment zone between trypanosome and the bound erythrocyte (**Fig. 4.11**).
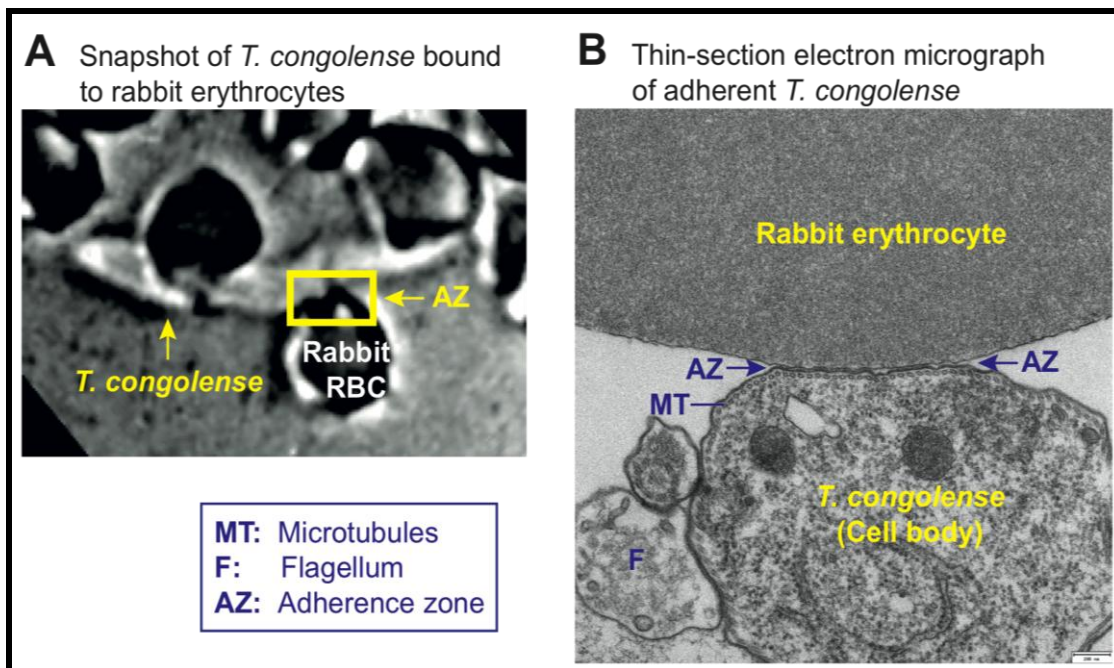
Figure 4.11: Microscopic analysis of attachment zone between individual trypanosome and the bound erythrocytes.
(A) Shows an image of *T. congolense* IL1180 that is bound to three rabbit erythrocytes. Erythrocytes were washed in TDB and trypanosomes were purified from infected mouse blood. Trypanosomes were mixed with washed RBCs followed by high-speed video microscopy (at 500 fps) to capture movies of adherent parasites.
(B) Electron micrograph showing a cross section of *T. congolense* IL1180 bound to rabbit erythrocyte. The two arrows point to the boundary of attachment region that shows a tight junction between the trypanosome and the bound erythrocyte.

4.6.1.4 Effects of incubation temperature and host sera on erythrocyte adherence

Trypanosome-RBC binding occurred at 4 - 8°C, room temperature (average = 24 °C) and at 37°C (**Table 4.6**). Studies in serum-free TDB showed higher adherence at 37°C, followed by incubation at RT. Binding was remarkably slowed down when trypanosome-RBCs mixture was incubated on ice (0 - 1°C) because the parasites became inactive and thus swam at much reduced speeds thus limiting the chances of interaction with the erythrocytes.

Adherence studies were also investigated in the absence and presence of host sera. Generally, most sera significantly increased mean adherence percentages ($p<0.05$) in *T. congolense* IL 1180 and *T. congolense* IL 3000 (**Fig. 4.12; Table 4.7**). In contrast, these sera significantly reduced mean adherence in *T. vivax* IL 2136 and *T. vivax* IL 1392 ($p>0.05$). *T. b. brucei* IL Tat 1.4, *T. brucei* KETRI 2710 and *T. evansi* KETRI 2479 did not bind rabbit erythrocytes even in the presence of host sera.

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Table 4.6: Trypanosome-erythrocyte mean adherence was studied at different incubation temperatures in serum-free TDB. Prior to scoring adherence, trypanosome/RBC mixture was incubated for 30 min at 37°C. Washed rabbit erythrocytes were used in all cases, unless when specified otherwise. Three replicates (n=300 parasites/replicate) were used. Data = mean ± SE.

Trypanosome	4-8°C	RT (24°C)	37°C
T. vivax IL 2136	16.67 ± 1.45	31.33 ± 5.53	28.17 ± 4.71
T. vivax IL 1392	12.33 ± 3.84	13.67 ± 0.67	13.67 ± 0.88
T. b. brucei IL Tat 1.4	0	0	0
T. brucei KETRI 2710	0	0	0
T. evansi KETRI 2479	0	0	0
T. congolense IL 1180	21.5 ± 2.32	31.8 ± 4.53	44 ± 4.17
T. congolense IL 3000	44 ± 2.1	54 ± 4.65	62.83 ± 3.37

0 (zero): means no adherence, RT= room temperature.

Increased mean adherence was recorded in *T. congolense* IL 1180 in presence of goat serum (47% increase), followed by FBS (41%), whereas in *T. vivax* IL 2136, highest decrease in mean adherence were observed in presence of trypanosome-infected cattle serum (-20.8% decrease), followed by the horse serum (-19.8%) (**Table 4.7**).
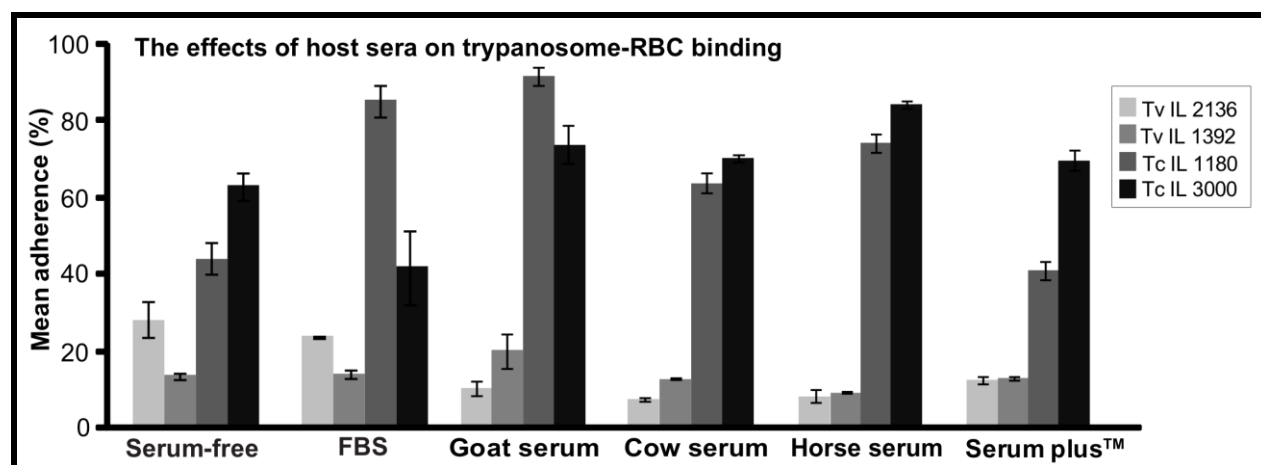

Figure 4.12: The role of host serum on trypanosome-erythrocyte binding capacity. Adherence assays were conducted using purified trypanosomes and washed rabbit erythrocytes. Relative to the control experiment in serum-free TDB, different host sera influenced mean adherence at 37°C. Each experiment was replicated at least three times (n=300 parasites/replicate). Data = mean ± SE. **Tv:** *T. vivax,* **Tc:** *T. congolense.*

Adherence assays of *T. congolense* IL 3000 in serum-free TDB resulted in an average binding of 4 RBCs/trypanosome. However, addition of 30% FBS caused adherent parasites to regroup. After 80 min of incubation at 37°C, adherent parasites formed large masses (an average group consisted of 12 trypanosomes bound to 10 RBCs). Other sera from goat, horse and serum plus™ did not cause the parasites to re-group into masses of swimming parasites.

Table 4.7: Mean percentage changes in trypanosome-erythrocyte binding capacities in presence and absence of 30% host sera. Prior to quantification of adherence that was scored either as adherent or freely swimmer, trypanosome/RBC mixture was incubated at 37°C for 30 min, with or without sera. At least three replicates were used (n=300 trypanosomes/replicate).

Percentage change in mean adherence ($\Delta_{\%Adherence}$) at 37°C was computed in each case by subtracting mean adherence in TDB with serum from mean adherence of the same parasite in TDB;
$\Delta_{\%Adherence} = (Mean\ A_{Serum} - Mean\ A_{TDB}) \times 100\%$

Trypanosome	Percentage change in mean adherence in presence of serum					
	FBS	**Horse**	**Goat**	**Cattle**	**Serum plus™**	**Pig**
T. vivax IL 2136	-4.5	-19.84	-17.84	-20.84	-15.84	ND
T. vivax IL 1392	0.33	-4.34	6.33	-1	-1	-4.67
T. b. brucei IL Tat 1.4	0	0	0	0	0	0
T. brucei KETRI 2710	0	0	0	0	0	0
T. evansi KETRI 2479	0	0	0	0	0	0
T. congolense IL 1180	41	28.67	47	19.33	-3.67	24.67
T. congolense IL 3000	-21	19.84	10.5	6.84	5.84	ND

0 (zero): means no adherence, ND: not determined.

The effect of host sera on parasite survival after overnight incubation was also investigated at different incubation temperatures. All parasites that were incubated in 30% host sera for 20 hr at 37°C died. However, parasites survived after an overnight incubation at 4°C and room temperature (24°C) in presence of the above six sera. In *T. b. brucei* IL Tat 1.4, highest mean survival rates of up to 98% were achieved at 4°C following overnight incubation. However, the mean survival rates at 24°C reduced by 11% after overnight incubation. All sera improved parasite survival with highest survival rates observed in FBS (4°C= 98%; 24°C= 87%) followed by young goat serum (4°C= 96%; 24°C = 56%). Pig serum poorly supported overnight cultures (4°C= 33%; 24°C= 20%). Most of the overnight cultures did not loose mouse infectivity, except those incubated at 37°C because parasites did not survive, perhaps due to exhaustion of energy source such as glucose in TDB.

Generally, our findings showed that host sera promoted adherence in *T. congolense* but not in *T. vivax* (**Table 4.7**). Changes in adherence proportions due to presence of serum varied in *T. congolense* strains, as well as in different trypanosome species. However, goat serum promoted adherence to erythrocytes in *T. congolense*. Goat serum is currently the most preferred sera for use during *in vitro* cultures of *T. congolense* probably because it aids the cells to attach to the culture flasks. Indeed, commercially available sera such as Serum plus™ play important roles in cell cultures because it contains proteins that promote cell attachment.

In summary, our findings may improve *in vitro* culture protocols for *T. congolense* (and probably *T. vivax*) by way of investigating the adherence roles of various treatments (such as; host sera, temperature, sugars).

4.6.1.5 Host erythrocytes

Trypanosome adherence assays were performed using purified erythrocytes from mouse, rat, rabbit, pig, sheep and goat blood. Our findings showed that mean trypanosome-RBC adherence proportions depended on the host erythrocyte **(Table 4.8)**. In *T. congolense* IL 1180, highest mean adherence percentage of 64.67 ± 6.84 was recorded in goat erythrocytes followed by 54.33 ± 3.18 in pig erythrocytes, while in *T. congolense* IL 3000 the highest mean adherence of 62.83 ± 3.37 was observed in rabbit erythrocytes followed by 60.67 ± 1.33 in goat erythrocytes. Similarly, highest mean adherence percentages (to rabbit erythrocytes) of 28.17 ± 4.71 and 13.67 ± 0.88 were recorded in *T. vivax* IL 2136 and *T. vivax* IL 1392, respectively. However, *T. vivax* recorded the lowest mean adherence percentage to goat erythrocytes, contrary to the case in *T. congolense* **(Table 4.8)**.

4.6.1.6 Trypanosome species and strains

The proportion of erythrocyte binding depended on trypanosome species and strains (**Fig. 4.13**). The following species were capable of binding some host erythrocytes: *T. vivax* IL 2136, *T. vivax* IL 1392, *T. congolense* IL 1180, *T. congolense* IL 3000 and *T. congolense* KETRI 3827. They adhered to erythrocytes sourced from rabbit, pig, goat, sheep and cattle blood, but lacked adherence to mouse and rat erythrocytes **(Table 4.8)**. Erythrocyte-adherence capacity was lacking in *T. b. brucei* IL Tat 1.4, *T. brucei* KETRI 2710 and *T. evansi* KETRI 2479 exposed to erythrocytes from mouse, rat, rabbit, pig and goat. Higher mean adherence rates were exhibited by *T. congolense* than *T. vivax* **(Table 4.6, 4.8, Fig. 4.13)**.
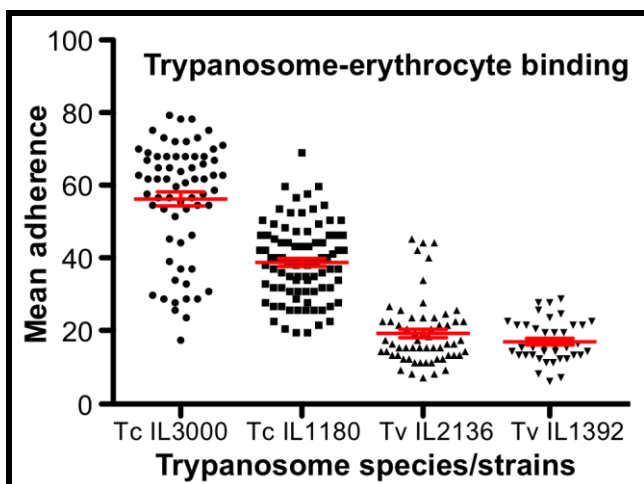
Figure 4.13: Comparison of trypanosome-erythrocyte adherence capacity in *T. congolense* (*Tc* IL3000, *Tc* IL1180) and *T. vivax* (*Tc* IL2136, *Tv* IL1392). Rabbit erythrocytes were used for trypanosome adherence assays and incubation of trypanosome-erythrocyte mixture was done at 37⁰C for 30 min in TDB. Each dot in the graph represents a mean value of three replicates (n= 300 trypanosomes/replicate). The was no significant difference in mean adherence between *T. vivax* strains ($p > 0.05$), whereas mean adherence in *Tc*IL3000 was significantly higher than in *Tc* IL1180 ($p < 0.05$)

Table 4.8: The adherence behaviour of trypanosome species was investigated using various host erythrocytes. The purified trypanosomes and host RBCs were mixed (trypanosome:RBC ratio of 1:1) and incubated at 37°C for 30 min in serum-free medium. Subsequently, the adherent and freely swimming parasites were scored. Three replicates were included (n=300 trypanosomes/replicate) for generation of means ± SE.

Trypanosome	Mean erythrocyte adherence (%) at 37°C				
	Mouse	Rat	Pig	Goat	Sheep*
T. vivax IL 2136	0	0	6±0.58	4±0.58	ND
T. vivax IL 1392	0	0	13.67±2.67	2±0.58	9.50±0.68
T. b. brucei IL Tat 1.4	0	0	0	ND	ND
T. brucei KETRI 2710	0	0	0	ND	ND
T. evansi KETRI 4009	0	0	ND	0	ND
T. congolense IL 1180	0	0	54.33±3.18	64.67±6.84	ND
T. congolense IL 3000	0	0	ND	60.67±1.33	ND
T. congolense KETRI 3827	0	0	ND	ND	88.33±1.45

*Adherence was directly quantified from infected sheep blood obtained from the ear vein and placed on a microscopic slide for microscopy. ND: not determined.

4.6.2 Influence of neuraminidase, trypsin, chymotrypsin and diminazene aceturate on adherence

i) Neuraminidase assays

Initially, the rabbit erythrocytes were incubated with 5 U/ml of neuraminidase for 1 hr at 37°C. This resulted into complete lysis of purified rabbit erythrocytes. Reduction of enzyme concentration to 1 U/ml still caused RBC lysis. We then investigated the effects on erythrocytes

of 10 mM phosphate buffer with or without 0.03% BSA (used in dissolving lyophilized neuraminidase). Both buffer and BSA did not lyse rabbit erythrocytes even after 2 hr of incubation at 37°C. Therefore, we concluded that neuraminidase caused lysis of the red cells and thus the subsequent experiments were done using lower concentration of the enzyme. At 0.2 Units/ml, neuraminidase caused minimal lysis of RBCs and thus allowed scoring of the adherence. After 20 min of incubation in presence of neuraminidase, the mean adherence in *T. vivax* reduced by more than 90% (**Fig. 4.14 A**). Minimal adherence was observed in *T. congolense* and *T. vivax* after neuraminidase treatment (30 min, 37°C) (**Fig. 4.14 B; Table 4.9**). Similarly, trypsin and diminazene aceturate decreased mean adherence of trypanosomes to erythrocytes (**Fig. 4.14 C, E**). However, mixture of chymotrypsin-treated erythrocytes with untreated trypanosomes led to increased adherence in *T. congolense* (**Fig. 4.14 D; Table 4.9**).

Table 4.9: Percentage change in mean trypanosome-erythrocyte adherence before and after treatment with 0.2U neuraminidase, 0.02% trypsin, 0.02% chymotrypsin and 0.02% diminazene aceturate. After treatment, mean adherence decreased in all trypanosome species studied suggesting that chemicals that are administered to the host could affect trypanosome motility behaviour. For instance, diminazene aceturate is a trypanocide that is commonly administered to treat livestock infected with trypanosomes.

Trypanosome	Neuraminidase			Trypsin	
	RBC-treated+ Untreated trypanosome	Trypanosome-treated+ Untreated RBC	Both RBC + trypanosome treated	RBC-treated + Untreated trypanosome	Trypanosome-treated + Untreated RBC
T. vivax IL 2136	-92.68	-85.37	-80.49	-86.96	-84.78
T. congolense IL 1180	-90.57	-88.68	-87.74	-69.70	-78.35
T. congolense IL 3000	-95.63	-85.25	-96.17	-77.20	-89.64

Continuation of **Table 4.9**

Trypanosome	Chymotrypsin			Diminazene aceturate		
	RBC-treated + Untreated trypanosome	Trypanosome-treated + Untreated RBC	Both RBC + trypanosome treated	RBCs treated, washed + Untreated trypanosome	RBCs-treated, unwashed+ Untreated trypanosome	Trypanosomes treated + Untreated RBC
Tv IL 2136	**-39.73**	-76.71	-68.49	-86.05	-86.05	-97.67
Tc IL 1180	**34.23**	-96.39	-93.69	-83.48	-55.65	-74.78
Tc IL 3000	**14.74**	-94.10	-93.12	-81.40	-76.16	-94.77

The minus sign (-) denotes percentage decrease in mean adherence. Chymotrypsin-treated RBCs mixed with the untreated trypanosomes promoted adherence (*see* values highlighted in bold). *Tv: T. vivax, Tc: T. congolense.*

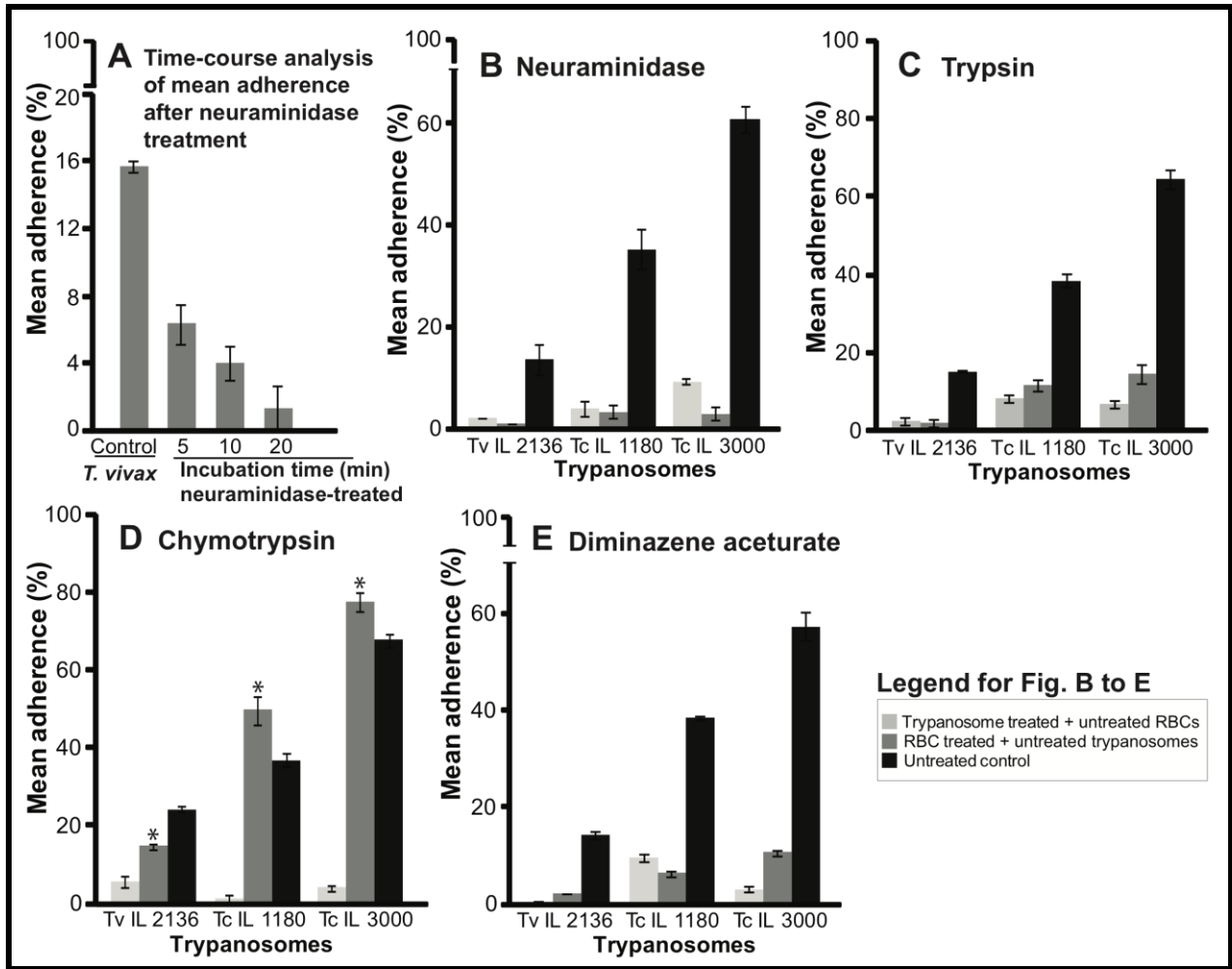**Figure 4.14: Influence of neuraminidase, trypsin, chymotrypsin and diminazene aceturate treatment on trypanosome adherence to the erythrocytes.**

(A) Optimization of incubation duration following neuraminidase treatment to study its effects on trypanosome-RBC adherence. Incubation for 20 min (at 37°C with 0.2 U neuraminidase) caused 91.5% reduction in mean adherence.

(B) Shows the effect of neuraminidase on trypanosome binding to rabbit erythrocyte. The findings indicate that neuraminidase treatment inhibits trypanosome-RBC adherence.

Similarly, treatment (30 min incubation at 37°C) with 0.02% trypsin **(C)**, 0.02% chymotrypsin **(D)** and 0.02% diminazene aceturate **(E)** reduced mean adherence %. Trypsin reduced mean adherence by over 70% (**Table 4.9**) relative to the untreated control. Chymotrypsin also reduced mean adherence %, but only when trypanosomes were treated. Asterisk* denotes increased adherence.

4.6.3 Effect of sugars on adherence behaviour

The influence of sugars on *in vitro* adherence of trypanosomes to erythrocytes was investigated and the findings showed reduction in binding capacities when trypanosomes were treated with lactose and exposed to untreated erythrocytes (**Fig. 4.15 A**). In contrast, treatment of the erythrocytes with lactose did not reduce adherence to untreated parasites (**Fig. 4.15 B**).
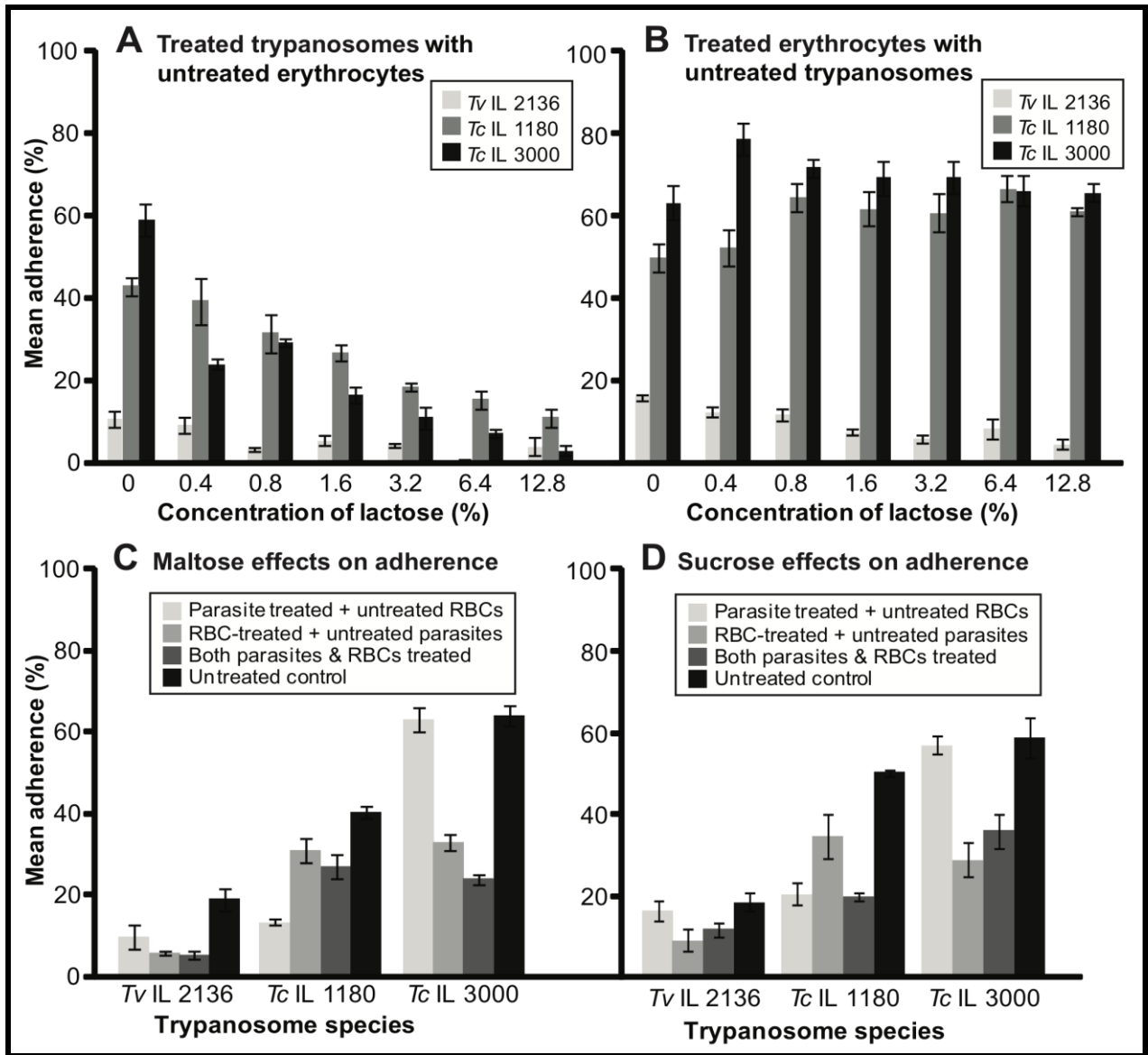
Figure 4.15: Influence of sugars on trypanosome-erythrocytes adherence.
A: Treatment of trypanosomes with increasing concentration of lactose caused reduction in mean adherence.
B: Treatment of erythrocytes with increasing lactose concentration does not affect trypanosome-RBC adherence.
C: Treatment with 2% maltose generally caused reduced mean adherence. However, there were no major changes in mean adherence of *T. congolense* when **maltose-treated parasites** were incubated with the **untreated erythrocytes**.
D: Sucrose treatment (2%) produced results that were similar to the effects of 2% maltose. Thus, sucrose treatment caused reduction in mean adherence. There were minimal changes in mean adherence in *T. vivax* and *T. congolense* when **sucrose-treated parasites** were incubated with the **untreated erythrocytes**.

Generally, there was reduction of mean adherence after treatment of both trypanosomes and RBCs with maltose (**Fig. 4.15 C**) and sucrose (**Fig. 4.15 D**).

4.6.4 Adherence studies of wild-type trypanosomes in infected rabbits

4.6.4.1 Identification of trypanosomes by polymerase chain reaction

Seven rabbits were used to support tsetse fly colony collected from Shimba Hills. All rabbits acquired infection with wild type trypanosomes transmitted by tsetse flies during feeding process. Identification of trypanosome species was done through PCR-amplification of ITS region for sequencing (**Fig. 4.16 A, B**). Sequencing of the wild-type trypanosomes in seven infected rabbits showed presence of *T. congolense* and *T. evansi* and most infections were mixed (**Fig. 4.16 B**). *T. vivax* and *T. brucei* infections were not detected in infected rabbits, though our sample size was small (n= 7 rabbits).

The prevalence of bovine trypanosomosis was recently investigated in selected locations around Shimba Hills National Reserve (Mbahin *et al.,* 2013) as a preamble to the on-going large field trials aimed at deployment of tsetse repellent technology. *T. congolense* and *T. vivax* species were reported as the main causative agents of trypanosomosis in the region. The sampled cattle graze near Shimba Hills National Reserve, hence increasing chances of coming into contact with tsetse flies. We indirectly sampled the trypanosome species circulating in tsetse fly colony obtained from the same region during the same time and our sequence data showed presence of *T. congolense* and *T. evansi* infections. We presume that *T. evansi* infections were also present in infected cattle that were sampled- but perhaps mistakenly scored as *T. vivax* since Mbahin *et al* (2013) relied on microscopy for distinguishing trypanosome species based on their morphological and motility variations.

The mean trypanosome-erythrocyte adherence in rabbit 1 and 7 with mixed infection of *T. congolense* and *T. evansi* was 28.67 ± 3.18% and 17 ± 2.65%, respectively (**Fig. 4.16 C**). The rest of the infected rabbits succumbed to the infection before investigation of trypanosome adherence. Distinguishing features such as presence or absence of free flagellum, swimming speeds/patterns and lengths of the parasites were used to distinguish *T. congolense* from *T. evansi* in mixed infections.
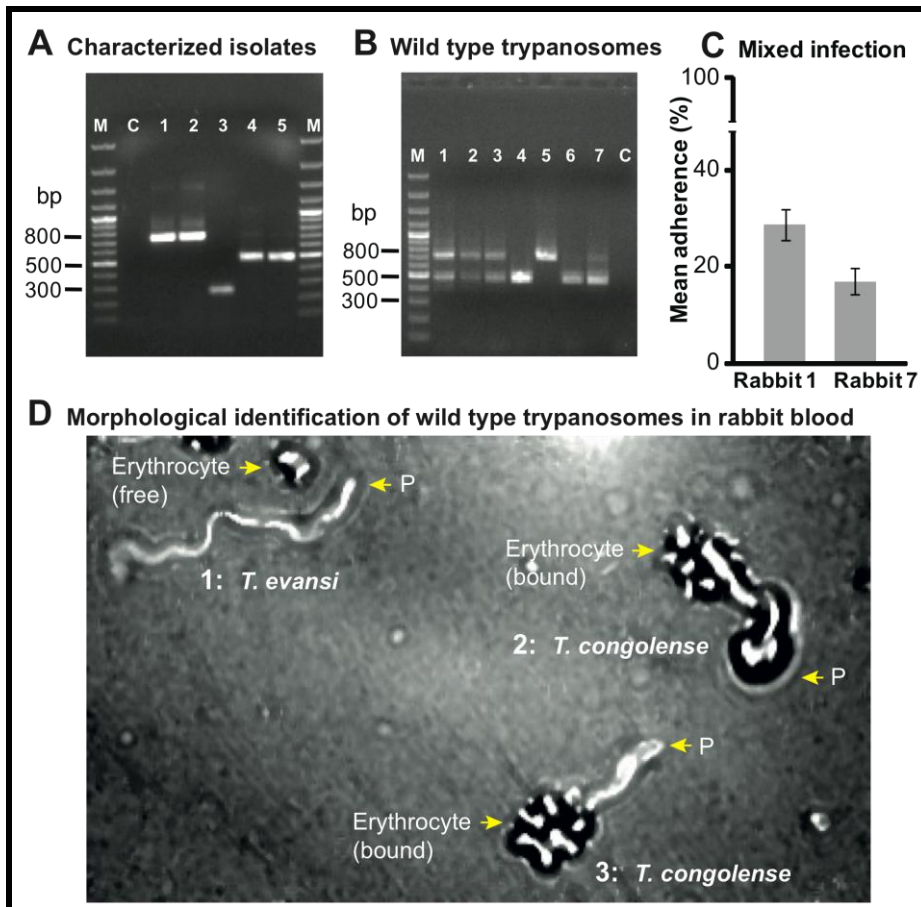
Figure 4.16: Molecular and morphological identification of trypanosome species.
(A) Trypanosome species were identified through amplification of ITS gene. PCR products were visualized on a 1% ethidium bromide-stained agarose gel under UV illumination**. M:** 100 bp DNA ladder, GeneRuler™ **C:** Negative control, **1:** *T. congolense* KETRI 3827, **2:** *T. congolense* IL 1180, **3:** *T. vivax* IL 1392, **4:** *T. b. brucei* IL Tat 1.4, **5:** *T. evansi* KETRI 4009.
(B) PCR targeting ITS gene showed mixed trypanosome infections in the infected rabbit 1, 2, 3 and 7 due to occurrence of two bands, upper and lower bands per lane. These bands were excised from the agarose gel and purified for sequencing. BLAST algorithm of the NCBI GenBank returned best hits as *T. congolense* (620-700 bp) and *T. evansi* ITS gene (480 bp)*.* **M:** 100 bp DNA ladder, GeneRuler **1-7:** Rabbit blood infected with wild type parasites, **C:** Uninfected rabbit blood.
(C) Quantitation of mean trypanosome-erythrocyte adherence in rabbit 1 and 2 with mixed infections identified to be *T. congolense* and *T. evansi*. The mean adherence percentage at parasitaemia score of 6.31×10^7 trypanosomes/ml was higher in rabbit 1 (28.67 ± 3.18) than in rabbit 7 (17 ± 2.65). Data = Mean ± SE.
(D) A snapshot acquired from high-speed movie of infected rabbit 1 blood (*see* Fig. C above) containing mixed infection with wild-type trypanosomes that were identified through sequencing to be *T. congolense* and *T. evansi*. *T. congolense* and *T. evansi* were morphologically distinguished based on the parasite morphology, cell length (*T. evansi* is much longer than *T. congolense*) and motility behaviour. Only *T. congolense* was adherent to the rabbit erythrocytes (*see* **Fig. 4.16 D** parasite number 2 and 3), but not *T. evansi* (**Fig. 4.16 D** parasite number 1). **P**= posterior end of trypanosome.

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We observed from high-speed movies of the rabbit wet blood films that the wild type *T. evansi* did not bind rabbit erythrocytes, whereas most wild type *T. congolense* attached to the red cells (**Fig. 4.16 D**). This finding was important because it confirmed that both characterized laboratory strain of *T. evansi* KETRI 2479 and the wild-type *T. evansi* exhibited the same behaviour of not binding to rabbit erythrocytes. In addition, the findings showed that the data from *in vitro* studies in TDB reflected true adherence behaviour in the mammalian bloodstream.

5.0 Discussion

5.1 Trypanosome motility

African trypanosomes are able to thrive in diverse hostile environments ranging from midgut of tsetse fly to the circulation of many vertebrates. They employ different survival tactics in the mammalian host that include: VSG switching (Cross, 1990, 1996; Borst and Ulbert, 2001; McCulloch, 2004; Matthews, 2005), immunosuppression (Sternberg, 1998) and swimming-dependent antibody clearance (Engstler *et al.,* 2007).

Motility is crucial for trypanosome survival, differentiation and completion of life cycle in the tsetse fly and vertebrate host (Vickerman, 1985; Vickerman *et al.,* 1988; Broadhead *et al.,* 2006). Definitely, the conditions in tsetse fly and vertebrate host differ in regard to temperature, viscosity, chemical composition, blood pressure, and glucose concentration, among others. Variations in environmental conditions in different hosts can influence trypanosome motility. Consequently, conditions in the mammalian host may be responsible for host-preference in trypanosome species. For instance, there are few known rodent-adapted *T. vivax* isolates*,* unlike *T. congolense* with many mouse-infective strains*.* Many laboratories are investigating factors that affect swimming parasites. For instance, chemical cues and the partial pressure of oxygen in blood have been suggested to lack any influence over trypanosome swimming velocities, and notably, these parasites do not secrete any motility-promoting factor (Heddergott *et al.,* 2012). However, higher viscosity of culture medium increases the number of persistent swimmers and results in efficient swimming (Heddergott *et al.,* 2012). The inertial forces are insignificant at micron scale; hence do not affect locomotion in trypanosomes and flagellate sperm that swim at very low Reynolds numbers (Purcell, 1977).

We investigated motility variations in trypanosome species in various culture media. All trypanosome species studied exhibited three major swimming patterns (tumbling, intermediate and persistent swimming patterns) in all culture environments. However, the relative proportions of swimming patterns varied in different trypanosome species. Notably, the proportion of tumblers in *T. congolense* in mouse blood was higher (78 - 92%) than in all other species. In contrast, *T. vivax*, *T. brucei* and *T. evansi* had the lower proportions of tumblers, with majority of the parasites being intermediate and persistent swimmers. The findings in sheep, rabbit and rat wet blood films followed a similar trend of swimming patterns as occurred in mouse blood.

However, in sheep blood, the number of persistent swimmers in *T. vivax* increased by 28%. Differences in motility patterns could be exploited for preliminary identification of trypanosome species by microscopy, particularly during field studies where molecular diagnostic techniques are not available. Thus, the predominant tumbling motion can be used for diagnosis of *T. congolense* infections.

In order to understand reasons for variations in motility, the details of swimming were investigated at cellular level. Swimming velocities and patterns were analyzed and possible factors that affected motility were investigated. We focused on the analysis trypanosome motility because efficient clearance of host-directed antibodies requires directional motility and higher swimming speeds. Parasite motility in the mammalian bloodstream was simulated by changing viscosity of the culture media and by using micropillar arrays to mimic blood cell parameters.

5.3 Swimming speeds

Trypanosomes species average swimming speeds followed the following order, from fastest to the slowest swimmers: *T. vivax*; *T. brucei*; *T. evansi*; *T. congolense.* The mean velocities of *T. vivax* in mouse and sheep blood were not significantly different (p=0.5874), contrary to *T. congolense* whose mean speed was significantly reduced in sheep (p=0.0005). *T. congolense* that adhered to sheep erythrocytes swam lower swimming velocities that free-swimming parasites.

The above results indicate that trypanosome motility is highly variable as variations in swimming speeds occurred even between strains of the same species. Also, these variations occurred between individual parasites in the same population as well as between species. Differences in swimming speeds between trypanosome species led to our initially hypothesis that *T. vivax* should clear from its surface host-derived antibodies more efficiently and faster than slower swimmers such as *T. congolense*. Surprisingly, *T. congolense* was able to endocytose surface-bound antibodies at a similar rate to *T. vivax* and *T. brucei.* This implied that short irregular intervals of higher speed observed in *T. congolense* (**Fig. 4.5 A**) might just be enough for antibody clearance. All species studied, apart from *T. congolense,* displayed efficient swimming characterized by higher velocities at certain viscosity as well as in the pillars. This suggests that antibody removal occurs more efficiently in the bloodstream of mammalian host.

In order to elucidate specific reasons for varied swimming speeds, we focused on directional swimmers in mouse blood. In general, swimming speeds from beat-to-beat varied and

characteristic beat frequencies were observed in each species i.e. highest beat frequency in *T. vivax* (28.66 Hz) and lowest in *T. congolense* (5.14 Hz). Trypanosomes, from the same population, swimming at the same beat frequency swam at significantly different speeds, thus we focused on the trypanosome morphology and swimming environment (viscosity and pillar arrays) as main factors influencing swimming speed.

5.3.1 Trypanosome morphology

Surface morphology studies in trypanosomes revealed important details on the cell body shapes and flagella orientation that could influence swimming.

Persistent swimmers in *T. vivax* population consisted of two morphotypes that swam at different velocities due to their cell body shapes (compare **Fig. 4.6 A** and **B**). The 'fastest' persistent swimmers- that comprised of 1% of the population- portrayed rigid, straighter/slender body conformation and swam at maximum speeds of above 100 μm/s in mouse blood. On the other hand, the 'slower' persistent swimmers- with average speed of 29.52 ± 19.3 µm/s - displayed flexible and wavier cell body. The force of the wave is greater when the cell is straighter, thus explaining the reason for higher swimming speeds in the 'fastest' persistent swimmers.

The attached flagellum of *T. brucei* MiTat 1.6 was described to run from the flagellar pocket, at the posterior region of an s-shaped cell body, in a left-hand 180° turn around the cell and continuing along a thinning body to the flexible anterior end (Heddergott et al., 2012). In comparison, we found that flagellum of the bloodstream forms of *T. brucei* ILTat 1.4 followed a right hand turn of 180°. Both *T. vivax* and *T. evansi* had a right-handed flagellum turn. In *T. congolense,* a straight flagellum- that attached to the cell body- originated from the flagellar pocket and projected towards cells' anterior pole. Only *T. congolense* lacked free flagellum among all trypanosome species studied. Straight flagellar conformation and absence of free flagellum could be the reason for higher proportion of tumblers in *T. congolense* than in any other species. The net contribution, to trypanosome motion, by the free part of flagellum to overall motility was suggested to be larger than that of the attached one (Heddergott *et al.,* 2012). Therefore, trypanosome with free flagellum will generate higher force for directional movement of the cell, than those lacking. Perhaps, straight flagellum that lacks free part in *T. congolense* does not generate enough force to propel the cell directionally to higher velocities and thus could explain lower swimming speeds in all three major motion patterns. Together, the above findings

suggest that evolution of trypanosomes has shaped flagellar morphologies into three distinct conformations; straight, left and right depending on the parasite species/strains. Further studies to investigate influence on motility of these flagella morphologies will solve an obvious question- why evolution has shaped these variations in flagellar forms when they can all perform a similar function of facilitating cell propulsion.

Plane-rotational swimming occurs in *T. brucei* as a result of interaction of the following two forces that act in opposite direction; the force of flagellar beat together with the hydrodynamic drag force, and rotational movement occurred due to the asymmetrical nature of the cell body (Heddergott *et al.,* 2012). We extended the same approach to study other species and found that rotational swimming also occurred in *T. vivax, T. congolense* and *T. evansi.* Therefore, the physics of swimming remains the same in all trypanosomes, but the individual parasites change the direction of flagellar beat to produce forward or backward swimming. The default swimming direction in trypanosomes is forwards, in order to allow antibody-VSG complexes to be sorted towards flagella pocket at the posterior region.

5.3.2 The influence of viscosity and pillar arrays on motility

Swimming speeds in *T. brucei* and *T. evansi* increased with rise in viscosity. In *T. vivax,* viscosity at 0.2% methylcellulose increased its speeds slightly, but further increase in viscosity caused concomitant reduction in speeds. Changes in viscosity did not cause significant changes in swimming speeds of *T. congolense.* Final concentration of 0.4% methylcellulose results in a viscosity of 5.2 mPas, which is comparable to fast flowing blood (4-5 mPas), whereas 0.6% methylcellulose (viscosity = 25 mPas) is in the upper range of reported blood viscosity. Further increase of *T. evansi* swimming speeds at 0.8% methylcellulose, higher viscosity than that of blood, implies that this species could be adapted to swimming efficiently in dense surroundings such as cell tissues- unlike in *T. vivax*. Swimming speeds of *T. brucei* reached a plateau phase from concentrations around 0.4- 0.6% methylcellulose.

All trypanosome species, except *T. congolense*, swam efficiently at maximum speeds exceeding 100 μm/s in the pillars with correct size and spacing (**Table 4.3; Fig 4.9)**. The proportion of fast directional swimmers in *T. vivax, T. brucei* and *T. evansi* increased in the pillars. Contrary to our expectations, *T. congolense* lacked directional swimmers (all parasites tumbled) in the pillar arrays. Thus, motility behaviour of *T. congolense* was similar in all tested culture environments

(TDB, blood, varying culture media viscosity, and in pillar arrays). Our findings suggest that *T. congolense* is adapted to swim at low speeds in the bloodstream of vertebrate host. It is unlikely that all sixteen tested combinations of pillar arrays were unfavourable for directional motility in *T. congolense*. The smaller size of *T. congolense* with stiff cell body and lack of free flagellum contributes to making these cells the slowest swimmers.

Highest speeds achieved by *T. vivax* and *T. evansi* in the pillars suggest that these parasites are adapted to swim more efficiently in the crowded environment of the mammalian bloodstream as reported previously in *T. brucei* (Heddergott *et al.,* 2012). We recorded highest average speeds in heterogeneous pillars (**Table 4.5**) than in the homogeneous arrays- even though in few cases maximum speeds were attained in the latter. This showed that trypanosomes swim efficiently in the bloodstream characterized by blood cells with different sizes (i.e. heterogeneous blood). The speed of the parasites varied during swimming depending on the; interaction of dynamic cell curvature with pillars, the diameter and spacing of the pillars, morphology of the parasites, adherence to the erythrocytes, etc. In addition, higher speeds were recorded in mouse blood than in TDB suggesting that trypanosome motility is affected by the conditions of culture environment.

In *T. evansi* more that 50% of the parasites swam persistently backwards in mouse wet blood films, but this motility behaviour was not observed in the other studied species under the same conditions. Addition of methylcellulose to the mouse blood led to substantial reduction in proportion of continuous backward swimmers as only one persistent backward swimmer was recorded. Furthermore, continuous backward swimming was uncommon in *T. evansi* swimmers in the pillar arrays. We, therefore, concluded that reversed swimming was due to the effects of environmental conditions on the microscope slide such as viscosity and also arrangement of blood cells.

The above findings show that trypanosome motility is influenced by the conditions of the swimming environment. For example, in sheep blood, *T. congolense* and *T. vivax* showed remarkable motility behaviour of binding to sheep erythrocytes, but not to murine (mouse and rat) blood cells.

5.4 Trypanosome-erythrocyte adherence

In order to evade host immune destruction, trypanosomes coat their surfaces with SIA derived from host's cell membranes (biological mimicry) and this enables the parasites to survive in tsetse fly midgut as well as in the bloodstream of mammalian host (Previato *et al.,* 1985). Trypanosomes adhere to red cells through SIA (Banks, 1978, 1979) and our findings are in agreement because treatment of rabbit erythrocytes with 0.2 U/ml of neuraminidase prevented adherence to *T. congolense.* It is not clear what this adherence implies either to the host or to the survival of the parasite. Thus, we hypothesize that when surface VSGs of the parasite press against sialic acid residues of erythrocytes, negative charges are created that in turn create a charge barrier between the two membranes because the surface charge of BSF-*T. congolense* is negatively charged (Lanham, 1968). This charge barrier may protect the parasite from immune recognition and could be the reason for observed gliding motion of bound RBC(s) over trypanosome surface during swimming. Increased trypanosome-erythrocyte adherence in Ndama cattle was suggested to cause slower release of erythrocyte SIA thus partly contributing to its resistance to trypanosomiasis (Esievo *et al.,* 1986). Thus, the parasite may not be primarily involved in cleaving SIAs from the hosts' RBCs, but require the negatively charged SIA on RBCs to induce binding. Perhaps, this could be the main reason for re-sialylation of host cell glycomolecules (by TS) that lead to generation of receptors for trypanosome adherence (Schenkman *et al.,* 1991).

The findings of this study provided more insight into the erythrocyte-binding behaviour in trypanosome species. Using high-speed video microscopy, we showed that *T. congolense* and *T. vivax* do not bind mouse and rat erythrocytes but adhere to rabbit, sheep, goat, pig and cattle erythrocytes. However, *T. brucei* and *T. evansi* were non-adherent to all host erythrocytes tested.

As previously reported, most erythrocytes adhered at the anterior region of the parasite rather than posterior (Banks, 1979). On average, more than 60% of bound RBCs were attached to the anterior region; with over 50% of the binding occurring at the very anterior tip. The very posterior tip of the parasite rarely bound blood cells. Perhaps, the hydrodynamic drag forces acting backwards on the surface of forward swimming parasite prevented binding to the very posterior tip. The anterior tip is used to maneuver between blood cells hence interacted and bound more blood cells. Blood cells bound to the flagellar tip remained attached at the same region or glided over the cell body. Gliding of the bound erythrocyte on the parasite surface was

more common in *T. vivax* than in *T. congolense* and consequently, fast swimming *T. vivax* was able to loose bound RBCs more easily than *T. congolense* which is adapted to swim at slower speeds, perhaps, to avoid loss of bound erythrocytes. This could also explain the lower mean adherence rates observed in *T. vivax* in comparison to *T. congolense*. For instance, 88.3% of *T. congolense* KETRI 3827 adhered to erythrocytes in sheep blood, whereas only 9.5% of the parasites were adherent in *T. vivax* IL 1392. Trypanosome-erythrocyte binding does not influence the tumbling motion of parasites because the number of tumblers in *T. congolense* KETRI 3827 was similar in both mouse (no adherence) and sheep (adherence occurs).

In vitro binding of trypanosomes to the host erythrocytes occurred best at temperature range of 24 - 37°C, and in presence of host sera (sera from cow, horse, goat and pig promoted erythrocyte binding in *T. congolense*, but not in *T. vivax*). Additionally, higher mean adherence was achieved when whole blood (rather than isolating erythrocytes) was used in adherence assays. Therefore, whole blood promoted trypanosome-RBCs adherence suggesting that the extracellular components in the whole blood (e.g. serum and components such as proteins, free serum sialic acids), that were lost during wash steps, enhanced adherence.

In addition, the effects of various sugars and specific enzymes on trypanosome-RBC adherence were investigated. Treatment of blood cells with neuraminidase, trypsin and diminazene aceturate led to significant decrease in mean adherence ($p>0.05$). In contrast, treatment of blood cells with chymotrypsin led to higher mean adherence when mixed with untreated parasites. However, when trypanosomes were given the same treatment (with chymotrypsin) followed by exposure to untreated erythrocytes, the mean adherence reduced significantly, suggesting that chymotrypsin treatment destroyed the binding factors on trypanosome surface. Similarly, lactose treatment on trypanosome led to reduced binding, but not when blood cells were treated. Treatment of either parasites, or blood cells or both with maltose and sucrose caused minimal reduction in mean adherence in some cases.

In summary, erythrocyte adherence in African trypanosomes depends on the trypanosome species and strains as well as the source of host erythrocyte, among other factors such as the temperature and host sera. Factors responsible for adherence of some trypanosomes species to erythrocytes and blood vessels and in some specific hosts have not been fully understood. The type and distribution of surface sialic acids depends on the animal species, cell type and function and currently, cow is known to have the highest number of different sialic acids (Schauer, 2004).

Differences in binding to host erythrocytes could be due to variations in the type and content of sialic acid. Lack of adherence to murine erythrocytes in all species studied here suggests that trypanosomes don't need sialic acids in these abnormal hosts of African trypanosomes. It is also possible that binding site on the trypanosome surface has specific requirements to bind to particular sialic acid types that may be absent in cases lacking adherence. The surface protein S of transmissible gastroenteritis coronavirus binds to sialic acids of erythrocytes causing agglutination. However, single point mutations in the S protein results into loss of erythrocyte agglutination ability (Krempl *et al.,* 2000). Similarly, mutations in the surface proteins responsible for sialic acid recognition could be the reason for lack of adherence to murine erythrocytes. Detailed profiling of sialic acid types and amounts on the erythrocytes from different hosts will provide an insight into their roles in parasite binding.

Following trypanosome infection in sheep, the mean PCV values dropped progressively with time, suggesting that the host cannot replenish erythrocytes at a faster rate to counter anaemia progression. The occurrence of anaemia during trypanosome infection is believed to result from cleavage of SIA on the surface of host erythrocytes by the trypanosomal sialidases followed by phagocytosis of de-sialylated erythrocytes. Consequently, removal of SIA destabilizes the membrane charges and may result into formation of erythrocyte aggregates thus causing various pathological conditions (Samoilov *et al.,* 2002; Sangeetha *et al.,* 2005).

Previous reports also suggested that flagellum beating against erythrocytes causes mechanical injury leading to anaemia (Vickerman and Tetley, 1978). We analyzed all recorded high-speed videos of swimming parasites that interacted with blood cells mostly by using their anterior regions and in doing so; they do not rapture erythrocyte membranes. However, the shapes of blood cells were temporarily deformed when the parasite pressed its flagellum against them.

Based on their distinct motility behaviour, *T. congolense* and *T. vivax* infections can be easily distinguished, for instance in trypanosome-infected sheep, by quantifying mean erythrocyte adherence and by investigating their characteristic motility patterns and speeds.

Variations in motility among trypanosome species could be responsible for trypanosome species-specific differences in virulence patterns and disease pathogenesis. Variations in disease pathogenesis include: differences in prepatency periods, pp, in *T. congolense* (pp= 10 days) and *T. vivax* (pp= 1 day) infections in sheep, despite infection with equal number of parasites (starting inoculum = 10^6 trypanosomes); different rates of parasite proliferation that influences PCV

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levels. Trends in mean PCV decline in sheep, infected with trypanosomes, followed the prepatent period of the parasite. For instance, PCV levels dropped much earlier in *T. vivax* infection than *T. congolense*. Infection with hemorrhagic strains of *T. vivax* have been shown to result in death in cattle within 2 weeks, whereas relatively virulent isolates of *T. congolense* can cause death in 6-10 weeks after challenge (Murray *et al.,* 1983). Investigation into factors responsible for differences in the pathogenesis of African trypanosomiasis is important in development of control measures. For instance, variations in trypanosome motility may affect tissue-to-tissue migration, which influences disease pathogenesis. In particular, *T. brucei* ssp. and *T. evansi* can leave the blood vessels and invade other tissues (Losos and Ikede, 1972). In contrast, *T. congolense* is strictly an intravascular parasite that binds to the capillary walls and to erythrocytes in infected cattle (Banks, 1978). On the other hand, *T. vivax* can invade internal organs, cross the blood brain barrier and invade central nervous system, CNS (Batista *et al.,* 2007). Traversal of the blood-brain barrier by CNS-tropic trypanosomes, such as *T. b. gambiense* and *T. b. rhodesiense*, requires calcium signaling that is induced by parasite cysteine protease (Nikolskaia *et al.,* 2006).

Many studies have been performed to explain pathogenesis of African trypanosomiasis in man and his livestock. These studies were mostly done in model hosts such as mice and rats for obvious reasons; that it is cheaper to maintain the colony and they can easily be expanded in shorter time, in addition to being excellent hosts to grow trypanosomes in high numbers. However, our investigations showed marked differences in trypanosome motility behavior in ruminants than in murine species. This suggests that experiments performed in murine models may not necessarily reflect true behaviour of trypanosomes in the ruminant hosts.

Conclusion

Trypanosomes adopt diverse motility behaviour for survival in the hostile environments of mammalian hosts. Adaptations in motility include multiple swimming patterns and ability to swim in forward or reverse direction. The same parasite can adopt different motility patterns during swimming. Their ability to sense and respond appropriately to the environmental signals such as mechanical resistance (that induces reversal of swimming), viscosity, erythrocyte adherence, among others factors enable these parasites to proliferate in wide host range. Most *T. congolense* bind erythrocytes and could be the reason for their strict intravascular life. Judging from its motility behaviour under different environmental conditions, *T. congolense* is optimized

to swim at slow speeds so as to prevent lose of erythrocytes, unlike in fast swimming *T. vivax*. Combination of motility, endocytosis of host-derived antibodies and erythrocyte-adherence helps these parasites to survive and proliferate in the harsh conditions of the host bloodstream. Together, these variations in trypanosome motility might be the reason for species-specific differences in virulence and pathology patterns observed in mammalian hosts. Finally, comparison of trypanosome species has revealed some fundamental differences that can be used to differentiate them. For instance, *T. congolense* can be diagnosed based on swimming pattern (tumbling), adherence to blood cells and flagellum orientation.

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Appendix

Abbreviations

Technical Abbreviations

AAT	Animal African Trypanosomiasis
AT	African Trypanosomiasis
BSA	Bovine Serum Albumin
BSF	Bloodstream form
CNS	Central Nervous System
DAPI	4', 6-Diamidino-2-Phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene-diamine tetraacetic acid
FCS	Fetal Calf Serum
Fps	Frames per second
g	Gravity (centrifugation)
GPI	Glycosylphosphatidylinositol
HAT	Human African Trypanosomiasis
HCT	Haematocrit Centrifugation Technique
Ig	Immunoglobulin
LED	Light Emitting Diode
M	Molar
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PCV	Packed Cell Volume
PSG	Phosphate Saline Glucose
RCF	Relative Centrifugal Force
RNA*i*	RNA-Interference
RPM	Revolutions per minute
SD	Standard deviation
SE	Standard error

Sp or *Spec.*	Species
Sulfo-NHS-AMCA	Sulfosuccinimidyl-7-amino-4-methylcoumarin-3-acetate
TB	Terabyte
TDB	Trypanosome Dilution Buffer
TIFF	Tagged image file format
VSG	Variant Surface Glycoproteins
Z-Stack	Multiple images taken at different focus distances

Organizations

DFG	Deutsche Forschungsgemeinschaft
icipe	International Centre of Insect Physiology and Ecology
ILRI	International Livestock Research institute
ILCA	International Livestock Centre for Africa
ILRAD	International Laboratory for Research on Animal Diseases
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KALRO-BRI	Kenya Agricultural and Livestock Research Organization- Biotechnology Research Institute
PATTEC	Pan African Tsetse and Trypanosomiasis Eradication Campaign
WHO	World Health Organization

Eidesstattliche Erklärung

Die vorliegende Arbeit wurde Betreut durch Prof. Dr. Markus Engstler (Universität Würzburg) Dr. Daniel Masiga und Dr. Francis McOdimba (*icipe,* Nairobi) von 01/2011 bis 05/2015 angefertigt.

Gemäß § 4, Abs. 3, Nr. 3, 5 und 8 der Promotionsordnung der Fakultät für Biologie der Bayerischen Julius-Maximilians-Universität Würzburg erkläre ich hiermit ehrenwörtlich, dass ich die vorliegende Dissertation selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Ich erkläre weiterhin, dass die vorliegende Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Weiterhin erkläre ich, dass ich außer den mit dem Zulassungsantrag urkundlich vorgelegten Graden keine weiteren akademischen Grade erworben habe oder zu erwerben versucht habe.

2015

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Curriculum Vitae

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Education

Since January 2011:	Doctoral Research Fellow registered at the Julius-Maximilians-Universität Würzburg (Germany), Department of Cell and Developmental Biology.
2007-2011:	Master of Science in Biochemistry, Jomo Kenyatta University of Agriculture and Technology (JKUAT, Kenya).
2003-2007:	Bachelor of Science in Biochemistry and Molecular Biology, JKUAT (*First Class Honours*).
1998-2001:	Kenya Certificate of Secondary Education, St. Paul Secondary School (Marsabit, Kenya).

Publications

Peer-reviewed articles

1. **Joel L. Bargul**, Jamin Jung, Francis A. McOdimba, Collins O. Omogo, Vincent O. Adung'a, Timothy Krüger, Daniel K. Masiga, Markus Engstler. Species-specific Adaptations of Trypanosome Morphology and Motility to the Mammalian Host (Submitted to *PLoS pathogens*).

2. J. B. Benoit, I. A. Hansen, G. M. Attardo, V. Michalkova, P. O. Mireji, **J. L. Bargul**, L. L. Drake, D. K. Masiga, S. Aksoy, Aquaporins are critical for provision of water during lactation and intrauterine progeny hydration to maintain tsetse fly reproductive success. *PLoS Negl. Trop. Dis.* 8, e2517 (2014).

3. Saliou Niassy, Sevgan Subramanian, Sunday Ekesi, **Joel L. Bargul**, Jandouwe Villinger, and Nguya K. Maniania (2013). Use of *Metarhizium anisopliae* Chitinase Genes for Genotyping and Virulence Characterization. *BioMed Research International*, article vol. 2013 ID 465213, 9 pages, doi: 10.1155/2013/465213

Manuscripts in preparation

1. **Joel L. Bargul** *et al*. *In vitro* adherence to erythrocytes by African trypanosomes.
2. **Joel L. Bargul**, Erick O. Aroko, Daniel K. Masiga. Comparative and functional analysis of *Drip-a* aquaporin homologs in tsetse flies.

Published Abstracts

1. **Joel L. Bargul**, Francis A. McOdimba, Jamin Jung, Vincent O. Adunga, Timothy Krueger, Daniel K. Masiga, Markus Engstler (2014). Variations in Swimming Patterns and Behaviour of African Trypanosomes in Mammalian Hosts Depicts Adaptation to Survive in Diverse Environments. Pg. 86-87. In: **Proceedings of the British Society for Parasitology, 52ⁿᵈ Annual Spring meeting and Trypanosomiasis and Leishmaniasis symposium, 6-9ᵗʰ April 2014, University of Cambridge, UK.**
2. **Bargul JL,** Wamunyokoli FW, Masiga DK (2011). Localization, life-stage expression and RNA*i*-mediated gene silencing of an aquaporin-like gene from tsetse fly (*Glossina pallidipes)* in SEMIO-11 workshop on *Insect chemical ecology and multilevel pest management towards food security and sustainable development.* 13 - 15 November 2011.
3. **Bargul JL**, Wamunyokoli FW, Masiga DK (2010). Gene cloning and Expression of aquaporin-like gene from tsetse fly, *Glossina pallidipes.* Pg 34. In: **Proceedings of the JKUAT Scientific, Technological and Industrialization Conference, Nairobi, Kenya.**

Work Experience

- **22nd September 2011- to Date:** Tutorial Fellow, Department of Biochemistry, JKUAT (Nairobi, Kenya). *Teaching experience in the fields of:* Molecular Biology, Biochemistry, Entomology, Lab safety and procedures, Environmental science and Bioengineering.
- **September 2007 - 21st September 2011**: Teaching Assistant, Department of Biochemistry (JKUAT).
- **July-September 2007:** Research assistant, *icipe* (Duduville Headquarters, Nairobi).

Trainings, Conferences and Workshops

- **14th – 24th June 2015:** Attended the "Sao Paulo School of Advanced Science on Neglected Diseases Drug Discovery- focus on Kinetoplastids (SPSAS-ND3)" held by the Brazillian Biosciences National Laboratory (LNBio) at the Brazillian Center for Research in Energy and Materials (CNPEM). Poster title: *Clearance of antibodies in African trypanosomes.*
- **6th - 9th April 2014:** Participated at the British Society for Parasitology 52nd Annual Spring meeting at the University of Cambridge, UK. Title of presentation: *Motility Behaviour of African trypanosomes.*
- **20th - 30th March 2013:** Attended EMBO (European Molecular Biology Organization) practical course on Imaging and Microscopy at CSIR-Pretoria, South Africa. Theme: Innovative microscopy to understand fundamental biology. Oral presentation: *High-speed microscopy videos of African trypanosomes reveal unique motility variations.*
- **26th June - 2ndJuly 2012:** Attended conference in Bad Honnef (Germany) funded by Deutsche Forschungsgemeinschaft (DFG). Theme: German-African collaboration Projects in Infectology. Presented both oral talk and poster. **Oral presentation:** *Survival strategies in African Trypanosomes; Relationship between motility and antibody removal during infections.*
- **22nd - 29th March 2011:** Attended **DFG Conference** in Accra, Ghana. Title of the presentation: *Antibody clearance as a possible virulence factor in African trypanosomes.*
- **December 2010:** Attended Global Exchange Lecture Course on Bioinformatics and Comparative Genome Analysis, Institut Pasteur Tunis, Tunisia. Organized by EMBO. Topic: Aquaporins of tsetse fly, *Glossina pallidipes*.

Fellowships and Awards

- **January 2011-December 2013: Deutsche Forschungsgemeinschaft (DFG) grant** for PhD Fellowship to study a defense strategy of African trypanosomes in vertebrate hosts. Tenable at *icipe* (Kenya) and The University of Wuerzburg, Germany
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- **2008-2010:** World Federation of Scientist (WFS) and *icipe's* DRIP fellowship tenable at *icipe* for MSc research project
- **2007-2011:** JKUAT's scholarship (fee waiver for both 1st years' course work and 2nd years' research study) to undertake MSc. in Biochemistry.
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- **August 2011:** Awarded Best Fellow Prize (included a certificate and cash award of **USD 3186/40**) by the World Federation of Scientists (WFS)- Kenya National Scholarship Programme - in recognition for research excellence (MSc Research) in Insect Science
- **2005/2006; 2004/2005; 2003/2004:** Won (for three consecutive years) *Babaroa Trust Awards-JKUAT* from the Vice-Chancellor, JKUAT, for outstanding academic performance at The Department of Biological Science, JKUAT

Acknowledgements