Crosstalk between Trypanosoma brucei and the tsetse fly

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Michael Oberle

aus Full-Reuental (AG)

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Herr Prof. Dr. Eberhard Parlow
Dekan

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Summary

Trypanosoma brucei cause the fatal disease sleeping sickness in humans and the morbid disease nagana in animals. About 36 sub-Saharan African countries are affected by these diseases. The parasites are transmitted by tsetse flies (*Glossina spp.*) exclusively where they colonise the alimentary tract and the salivary glands. The trypanosomes establish first in the midgut as procyclic forms from where they colonise then the proventriculus (that connects the mid- with the foregut) and migrate later as epimastigote forms into the salivary glands via the foregut and proboscis. In the salivary glands epimastigote forms attach to the epithelium and give rise to the mammalian infective forms, the metacyclics. This complex journey through the fly involves a complex interaction between vector and parasite where both counterparts specifically regulate genes.
In this thesis we wanted to shed light into this complex crosstalk with three projects:

During transmission through the fly, trypanosomes are frequently severely reduced when they invade a new compartment. Trypanosomes either recover and develop an infection or fail to establish an infection and are eliminated by the tsetse fly's defence. Such severe reductions represent barriers that can influence the diversity of a trypanosome population. In two similar approaches we established a model to analyse how diversity of trypanosomes is influenced during their life cycle. Short variable DNA sequences were integrated into the trypanosome's genome to establish an artificial diversity. These transfected trypanosomes were cyclically transmitted through flies and mice. Tag DNA was isolated from infected flies and/ or mice and identified by sequencing. This allowed us to monitor diversity of the trypanosomes throughout their life cycle. We found that diversity was moderately reduced in the tsetse fly's midgut but that migration into the salivary glands decreases the diversity. This decrease is mainly due to a shift in relative frequency which leads to a very uneven distribution of the tags. The diversity constantly decreased during mouse infection due to the constant gain of trypanosomes bearing the dominant tag. Surprisingly, the number of different tags was not reduced during the whole life cycle of the trypanosomes.
The two anti-microbial peptides (AMPs), attacin and defensin, of tsetse flies were reported to play an important role in eliminating trypanosomes in the midgut. The mRNA of these AMPs was shown to be up-regulated upon trypanosome infection and it was hypothesised that

procyclins might specifically induce its activation. We wanted to test this with different trypanosome strains as well with trypanosomes with incomplete or deleted procyclin coats. Tsetse flies were infected and mRNA isolated after various times of trypanosome exposition. None of the flies showed an up-regulated level of attacin and defensin mRNA. This result is in strong contradiction to some publications dealing with AMP regulation in infected tsetse flies. The tsetse flies, from the colony in Bratislava (Slovakia), show a high level of attacin and defensin mRNA in teneral flies (what not all *G. m. morsitans* do), show a midgut infection rate of about 50% (which is high compared to the infection rate in other laboratories), and are infected sometimes with the salivary gland hypertrophy virus (SGHV). It is very possible that attacin and defensin are not always up-regulated and that its activation is dependent on tsetse colony and origin.
During the establishment in the midgut trypanosomes express procyclins, a stage specific surface protein coat that was suggested to protect against proteolytic enzymes or to be important to direct the parasite in the host. To test this hypothesis all procyclin genes were deleted and tsetse fly infection experiments were carried out. Interestingly, the null-mutant (Δprocyclin) was able to infect the midgut comparable to wild type trypanosomes, disclosing that procyclins are not needed for the establishment in the midgut and that probably free glycosylphosphatidylinositol (GPI) anchors, which are loaded with procyclins in wild type trypanosomes, overtook their function. Surprisingly, Δprocyclin was able to infect the salivary glands even though at very low rates, which reflects difficulties of trypanosomes to re-load the free GPIs with epimastigote specific surface proteins (e.g. BARP) for efficient migration. In competition, Δprocyclin was completely overgrown by wild type trypanosomes in the tsetse midgut, reflecting the selective advantage of a procyclin coat.

The three projects revealed that cyclical transmission through the tsetse fly bottlenecks the ingested trypanosome population mainly during migration into the salivary glands. The AMPs attacin and defensin might not account for such a bottleneck. AMP up-regulation in trypanosome challenged flies might be dependent on their origin. Furthermore, an incomplete or deleted procyclin coat did not differentially activate the immune response. But a complete procyclin coat ensures the trypanosomes a selective advantage to establish in the midgut and to efficiently colonise the salivary glands.

Zusammenfassung

Trypanosoma brucei spp. ist der Erreger der afrikanischen Schlafkrankheit beim Menschen. Die Trypanosomen werden von den blutsaugenden Tsetse Fliegen (*Glossina spp.*), übertragen. Laut WHO ist die Bevölkerung in mehr als 36 Ländern südlich der Sahara betroffen. Zur Behandlung der tödlich verlaufenden Kankheit stehen wenige, zum Teil alte und auch toxische Medikamente zur Verfügung. Zudem erschwert das Aufkommen von resistenten Trypanosomen den Genesungserfolg.

Im gegensatz zu Wildtieren, die für den Parasiten als Reservoir agieren, können Haustiere an einer Trypanosomeninfektion tödlich erkranken. Nagana, wie die Krankheit bei Tieren genannt wird, beeinträchtigt die betroffene Bevölkerung vorallem in wirtschaflicher Hinsicht (Verluste bis zu US$ 4.5 Mia. jährlich) und verunmöglicht die Entwicklung der Bevölkerung in Tsetse-Gebieten.

Wenn Tsetsefliegen auf einem infizierten Tier Blut saugen, nehmen sie die Trypanosomen auf, welche in den Mitteldarm gelangen, wo sie zu prozyklischen Formen differenzieren. Die prozyklischen Formen exprimieren Prozycline auf the Oberflächen (bestehend aus EP und GPEET). die Trypanosomen etablieren sich im Mitteldarm von wo sie den Proventriculus befallen (Verbindung zwischen Mittel- und Vorderdarm). Von dort migrieren sie weiter in die Speicheldrüse via Vorderdarm und Proboscis (= Stechrüssel). In der Speicheldrüse wandeln sich die Trypanosomen zu den säugetierinfektiösen Formen um (die sogenannten metazyclischen Formen).

Während der Passage durch die Fliege wird die Trypanosomenpopulation zum Teil sehr stark reduziert. Dies passiert vorallem zu Beginn einer Mitteldarminfektion und bei der Migration in die Speicheldrüse. Die Trypanosomen werden auf wenige Individuen reduziert, können sich aber danach wieder vermehren. Wir untersuchten, ob diese Reduktionen die Diversität der Trypanosomen beeinträchtigt. Trypanosomen wurden mit kurzen, in ihrer Sequenz unterschiedlichen DNA (= 'Tags'), transfektiert um eine künstliche Diversität zu generieren. Tsetsefliegen wurden mit diesen transfektierten Trypanosomen infiziert. Fliegen mit infizierten Speicheldrüsen wurden zur Übertragung der Trypanosomen auf Mäusen gefüttert. Die Mitteldärme und Speicheldrüsen der positiven Fliegen wurden seziert und Kapillarblut der infizierten Mäuse entnommen um die 'Tag'-Sequenzen und ihre Verteilung zu analysieren. Im Fliegenmitteldarm wurde die Frequenz der 'Tags' nur geringfügig verändert,

jedoch nicht während der Migration in die Speicheldrüse. In der Speicheldrüse fanden wir wenige sehr dominante aber viele rezessive 'Tags'. Die Anzahl unterschiedlicher 'Tags' war nicht reduziert. Die Mausinfektionen zeigten, dass viele unterschiedliche rezessive 'Tags' neben dominanten existierten. Dies deutet darauf hin dass in der Maus mehrere ‚Subpopulationen' co-existieren können. Schliesslich haben wir gesehen, dass die Anzahl der 'Tags' während des ganzen Zyklus der Trypanosomen mehr oder weniger stabil bleibt.
Die Differenzierung zu prozyclischen Formen im Mitteldarm löst gemäss verschiedenen Publikationen eine Immunantwort aus. Vorallem die beiden antimikrobiellen Peptide (AMP) Attacin und Defensin werden dabei aufreguliert. Es wurde berichtet, dass die Zusammensetzung des Prozyclinmantels der Trypanosomen die Aktivierung dierser AMP beeinflussen kann. Wir wollten testen, ob diese AMP mit verschiedenen Trypanosomenstämmen ebenfalls aktiviert werden, und ob Trypanosomen mit unvollständigem Prozyclin-mantel oder sogar ganz fehlendem Prozyclin eine Immunantwort auslösen können. Infektionsexperimente zeigten, dass Tsetsefliegen keine AMP aufregulierten, weder nach Infektion mit Wildtyp noch mit mutierten Trypanosomen. Diese Resultate stehen im Widerspruch mit einigen publizierten Analysen, zeigen aber auch, dass Fliegen unterschiedlicher Herkunft auf Trypanosomeninfektion anscheinend unterschiedlich reagieren. Schlussfolgernd können wir sagen, dass Attacin und Defensin nicht immer, wie bisher angenommen, in trypanosomeninfi-zierten Fliegen aufreguliert werden und dass diese AMPs auch nicht zwingend verantwotlich sind für die Bekämpfung der Infektion.
Wir konnten zeigen, dass Prozycline für eine zyklische Übertragung in der Fliege nicht essentiell sind. Diese ist erstaunlich, da allgemein angenommen wurde, dass der Prozyclinmantel die Trypanosomen im Mitteldarm vor Verdauungsenzymen und Abwehrmechanismen schützt. Im Labor wurden Trypanosomen generiert, die kein Prozyclin mehr exprimieren (=Δprocyclins). Diese konnten den Mitteldarm von Tsetsefliegen infizieren und erreichten eine ähnliche Infektionsrate wie die Trypanosomen mit einem Prozyklinmantel, jedoch nicht annährend eine so hohe Speicheldrüseninfektionsrate. Offensichtlich können andere Oberflächenproteine der Trypanosomen Schutzfunktionen im Mitteldarm übernehmen (zum Beispiel freie Glycosylphosphatidylinositol-Anker). Es scheint aber, dass das Beladen der 'nackten' Trypanosomen mit Proteinen, die essentiell für die Migration in die Speicheldrüse sind, problematisch ist. Dies würde die sehr tiefe Infektionsrate in der Speicheldrüse erklären.

1. Introduction: Crosstalk between *Trypanosoma brucei* and the tsetse fly

General introduction

The protozoan parasite *Trypanosoma brucei ssp.* causes sleeping sickness in humans and Nagana in cattle. For transmission, the trypanosomes are strictly bound to its vector, the tsetse fly (*Glossina spp.*).

Sleeping sickness (human African trypanosomiasis, HAT) is fatal if untreated and Nagana (animal African trypanosomiasis, AAT) causes high mortality in cattle leading to severe economic losses. Trypanosomiasis occurs in more than 30 countries in sub-Saharan Africa (Fig. 1.1A) and is bound to the distribution of the tsetse flies (Fig. 1.3). Mainly rural regions in Africa are affected hindering economical and medical improvement.

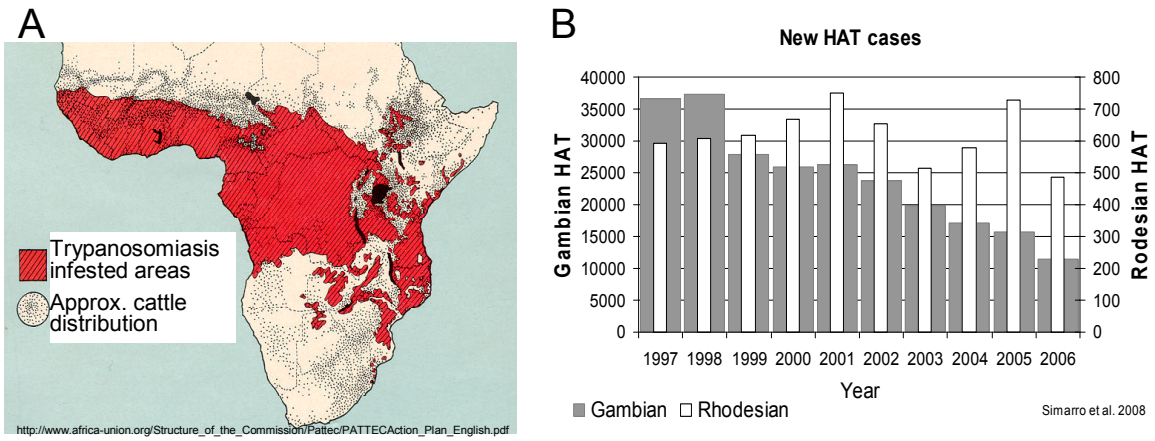

Fig. 1.1**:** A) Map of Africa showing trypanosomiasis infected areas (red) and the approximate distribution of cattle (black dots) (PATTEC: Plan of action, June 2001, http://www.africa-union.org). B) Gambian (grey) and the Rhodesian (white) HAT cases per year from 1997 to 2006 (Simarro et al. 2008).

HAT is caused by *T. b. rhodesiense* and *T. b. gambiense* which show restricted distribution in Africa and different clinical symptoms. *T. b. rhodesiense* occurs in East Africa and is mainly transmitted by the savannah type tsetse flies (morsitans group flies; see Phylogeny/ characterisation). The main hosts are wild animals which can be infected without symptoms (reservoir for the human disease). This has the consequence that infected humans are the 'wrong' hosts leading to an acute disease. Control of the disease by active surveillance of the

affected population and treatment of infected patients does not reduce the prevalence (Simarro et al. 2008) because transmission from the reservoir to man is not reduced with this strategy (Njiru et al. 2004). Less than 1000 patients are treated annually against *T. b. rhodesiense* (Fig. 1.1B) which gives the Rhodesian HAT a minor medical importance compared to the Gambian HAT in terms of patient numbers.
T. b. gambiense is spread in central and West Africa and is mainly transmitted by the palpalis group flies (see Phylogeny/ characterisation). Gambian HAT causes the chronic form of the disease. Humans represent the main host for *T. b. gambiense* while only a few reservoir animal species could be identified, even though extensive researches (Simo et al. 2006). In the 1990 half a million people was suggested to be infected with *T. b. gambiense* but only about 25,000 patients were treated per year. Since 1997 the situation had improved due to successful active surveillances in central/ west African countries (Fig. 1.1B). Important factors for the success were an increased awareness of the disease, the minor role of animal reservoir in *T. b. gambiense* epidemiology and political stabilisation in certain areas (Simarro et al. 2008).

Four different drugs are currently in use: suramin (against Rhodesian HAT) and pentamidine (against Gambian HAT) to treat the first stage of the disease (when the trypanosomes are in the blood and lymph). Eflornithine (only active against Gambian HAT) and melarsoprol are used to treat the second stage, when trypanosomes have crossed the blood-brain-barrier and colonise the central nervous system. The few drugs are toxic, expensive, difficult to administer and because of the rising resistance not always effective (Legros et al. 2002, Brun and Balmer 2006).

Beside the medical concerns, the socio-economic burden of African trypanosomiasis is very severe. The African governments initiated the Pan-African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC) which employs an approach to eradicate tsetse flies with the use of sterile insect technique (SIT) and additional approaches to control tsetse flies (see Tsetse control programs) (www.african-union.org n.d., www.fao.org n.d., Kabayo 2002).
The increase of resistance of animal infective trypanosomes (*T. b. brucei*, *T. congolense*, and *T. vivax*) is even more severe than in human infective trypanosomes. In certain regions up to 100% of *T. congolense* isolates show resistance to the most commonly used drugs (isometamedium chloride and diminazene aceturate) (Mamoudou et al. 2008).
In regions where the risk of trypanosomiasis is high, farming is unfeasible and most people live in poverty. Where the disease is tolerable, approximately 50 million cattle are held at risk

with up to 50% calf-mortality and abortion (Fig. 1.1A). The financial loss is estimated to be US$ 4.5 billion annually due to loss in milk and meat production, expenditures for trypanocidal drugs, flat agricultural productions because of sick domestic animals, and other costs that are needed to cope with the disease (Kabayo 2002).

Glossina – the vector

Phylogeny/ characterisation

A total of 33 tsetse taxa were described with about 22 different species (Krafsur 2008) and in principle all are able to transmit trypanosomes. However, some species are more efficient vectors than others and have greater medical and economical impact due to higher transmission capacity of trypanosomiasis (WHO 1998). The first species described was *G. morsitans* by Westwood (1851) and *G. frezili* was the last description of a tsetse fly species in 1987 (Gouteux 1987).

Glossina is the only genus in the family of the *Glossinidae*, which together with the two bat fly families (*Streblidae* and *Nycteribiidae*), and the louse fly family (*Hippoboscidae*) is subordinated in the super-family of the *Hippoboscoidea* and placed in the group *Calyptratea* in the order *Diptera* (Leak 1999, Krafsur 2008, Dyer et al. 2008) (Fig. 1.2). According to Petersen et al. (2007) the tsetse flies are clearly distinct from the louse fly and two bat fly families which are classified as *Pupiparia* and are described as well adapted ectoparasites. All *Hippoboscoidea* feeds on blood and are viviparous i.e. the larva develops in the reproductive tract and is fed with the 'milk' produced by the female fly (Krafsur 2008, Petersen et al. 2007).

Glossina can be sub-divided into three groups: the *fusca*, the *palpalis* and the *morsitans* group. The groups are separated according to different structural complexes of the genitalia (including supporting criteria of body hairs and behavioural specialities) (Leak 1999) as well as differences in the ecological niches (see Distribution). Modern analysis using isoenzyme electrophoresis and sequencing of a ribosomal internal transcribed spacer (ITS) region confirmed this grouping and lead to a wide acceptance that the groups are mostly monophyletic (Peterson et al. 2007, Dyers et al. 2008). The integration of *G. austeni* into the morsitans group is still discussed (Gooding et al. 1991, Chen et al. 1999) as well as the elevation of some sub-species to the species level (Dyer et al. 2008). The *fusca* group is

phylogenetically the oldest group of which the *morsitans* and the *palpalis* group flies have evolved (Leak 1999) (Fig. 1.2).

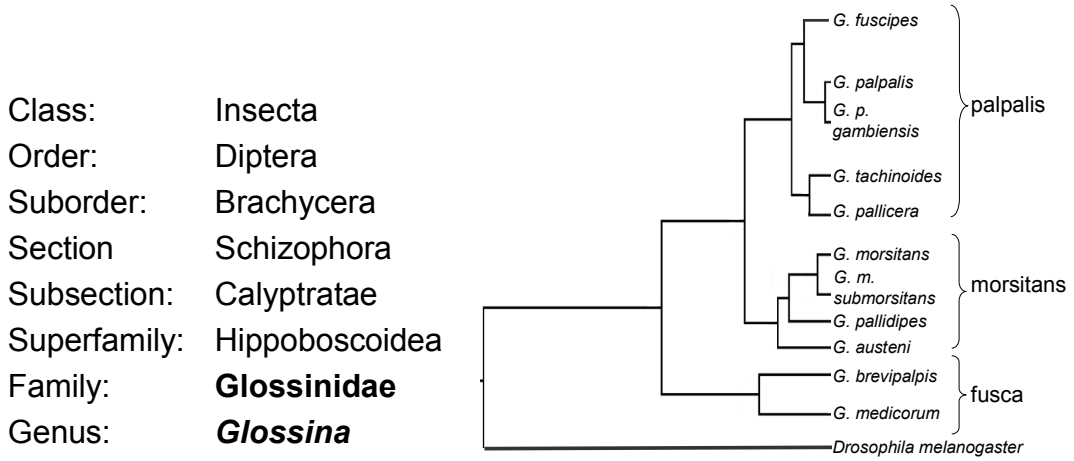

Fig. 1.2**:** Phylogeny of *Glossina* with its separation of the subgroups (*palpalis*, *morsitans*, and *fusca*) modified from Dyer et al. 2008

Tsetse flies are non-described in colour. The most prominent appearance is the glossa (=palp) in front of the head, a sustained shield that protects the proboscis in idle position (Fig. 1.4). The proboscis, the piercing mouth parts, is composed by the labrum, the labium and the hypopharynx (Leak 1999). Further distinctive feature of the genus *Glossina* is the presence of the hatched cell in the wing venation and the secondary branching of hairs on the third segment of the antenna (Molyneux and Ashford 1983).

Distribution

The distribution of African trypanosomiasis is restricted to the distribution of tsetse flies, which can be located roughly in the geographical area between south of the Sahara and north of Kalahari (Fig. 1.3). Tsetse flies are temperature sensitive and the distribution is hence bound to this climate factor as well as vegetation, humidity, and presence of host animals (Leak 1999). The temperature sensitivity affects the adult as well as pupal stage and suggests a temperature dependent distribution: High temperature (> 35°C) leads to an increased mortality in adult flies whereas low temperature (14° to 16°C) impedes pupae from hatching

(Terblanche et al. 2008). Adult flies use microclimatic differences in the habitat to mitigate temperature related stress (Terblanche et al. 2008).

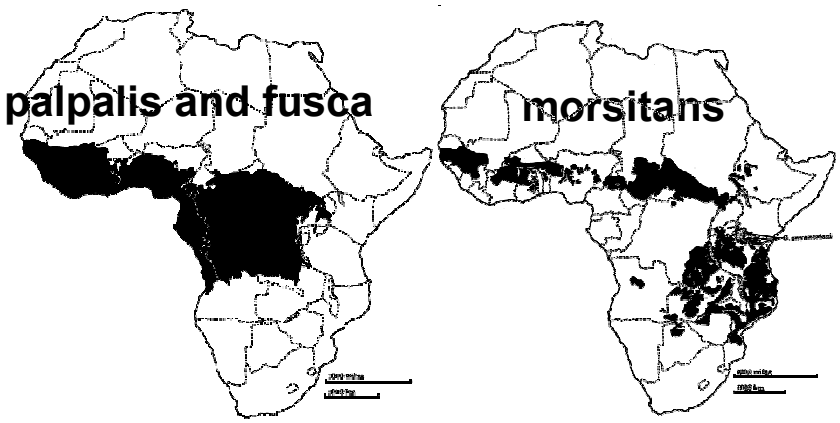

Fig. 1.3**:** Distribution of *morsitans*, *palpalis*, and *fusca* group species. The distribution of *palpalis* and *fusca* group species largely overlaps (from http://www.fao.org/).

The three phylogenetic distinct tsetse fly groups inhabit ecological diverse niches. The *fusca* group is found in the forest, the *palpalis* group is mainly distributed along rivers. Both groups are found in the equatorial zone in the rainforest (Fig. 1.3). The *morsitans* group flies inhabits the savannah zones in west, east and southern Africa where the flies rely on light vegetation with bushes and small trees for resting and sun protection (Fig. 1.3). The three tsetse fly groups are also known as forest, river, and savannah group flies according to their ecological niches.

Biology

A female Glossina needs to mate only once in her lifetime where she takes up a spermatophore from the first mating male a few days after emergence. All the eggs are fertilised from this spermatophore. The female produces up to 8 larvae during its lifetime of 3-5 month. The slow reproductive rate (one generation time under optimal conditions is 43 days) is compensated by the high survival rate of adult female flies and the pupae (Krafsur 2008).

The three larval instars develop in the uterus of the female where they are fed from the milk gland. During the feeding, the endosymbionts *Sodalis* and *Wigglesworthia* (see 1.2.7.

Symbionts of tsetse flies), are transmitted to the offspring (Attardo et al. 2008). An inseminated female tsetse fly deposits a third instar larva about every 10 day. Deposited larvae immediately bury themselves into the moist soil or sand through peristaltic movement, and pupate. The black puparium remains in the ground for a period of about 30 days until the adult fly emerges as a teneral fly (Leak 1999). Most pupae hatch during the day around midday, depending on the daily pattern of temperature change (Zdarek and Denlinger 1995).

Glossina species are all heamatophagous, and both sexes are dependent on blood meals. Hosts are found by olfactory and visual recognition. *Glossina* species require food approximately every two days and starvation longer than 4 to 6 days leads to death (Leak 1999). The probing of a fly is heat stimulated. The piercing mouth parts disrupt several capillaries under the skin and the saliva containing a powerful anticoagulant is continuously injected into the wound (Leak 1999). The size of a blood meal is around 20 to 60 mg and takes about 60 seconds. The choice of host species is considerably dependent on the host species of the previous blood meal and the starvation status of the tsetse fly. Bouyer et al. (2007) showed that flies tend to select similar or related host species for their blood meals.

Tsetse control programs

One reason why sleeping sickness declined in the late 1960's was the consequent and continuous spraying of insecticides (for example dichlorodiphenyltrichloroethane = DDT). The high cost was the main problem leading to a prematurely cessation of the spraying programme (Molyneux and Ashford 1983, Simarro et al. 2008).
In 1994 the International Atomic Energy Agency (IAEA) initiated the program for the eradication of the tsetse fly in Zansibar by displacing wild (fertile) males by gamma-sterilised ones (sterile insect technique = SIT) (Vreysen et al. 2000). The release of totally 8.5 Mio sterile males within 3 years led to an embryonic arrest in the female population and finally to the collapse of the tsetse population on that island. PATTEC together with IAEA plans to employ SIT on the mainland of Africa even though its feasibility on the Continent with several tsetse species and dynamic population is doubtful (Rogers and Randolph 2002; Simarro et al. 2008) and the release of male tsetse flies in inhabited regions might lead to an increased trypanosomiasis during sterile male release which were shown to be capable to transmit trypanosomes (Moloo et al. 1982).
Tsetse control by selective shooting of potential reservoir game animals or bush clearing (important tsetse resting sites) did not meet the expected success and is not practised anymore

(Molyneux and Ashford 1983, Leak 1999, WHO 1998). The use of insecticides directly poured on cattle (pour-on) reduced infection rate in cattle in some areas but not the tsetse density (Rowlands et al. 2001). Successful pour-on method is dependent on vegetation, invasion pressure of surrounding tsetse populations, cattle as well as wild animal reservoir density, and the use of insecticide formulation (Rowlands et al. 2001).

Traps with blue (long distance visual attractant) and black (short distance visual attractant and high landing response) clothes show a high selectivity to tsetse flies. These traps are widely used in Africa and are sometimes additionally equipped with olfactory attractants (e.g. acetone, octenol a.o.). Tsetse flies are killed either by an insecticide (on treated traps) or they are caught in the trap and subsequently die (Leak 1999). The traps are very effective, economic, and easy to maintain (WHO 1998). The combination of tsetse traps placed around inhabited areas and insecticides (pour-on and aerial spraying) is widely used in Africa and provides a certain protection against tsetse flies and trypanosomiasis.

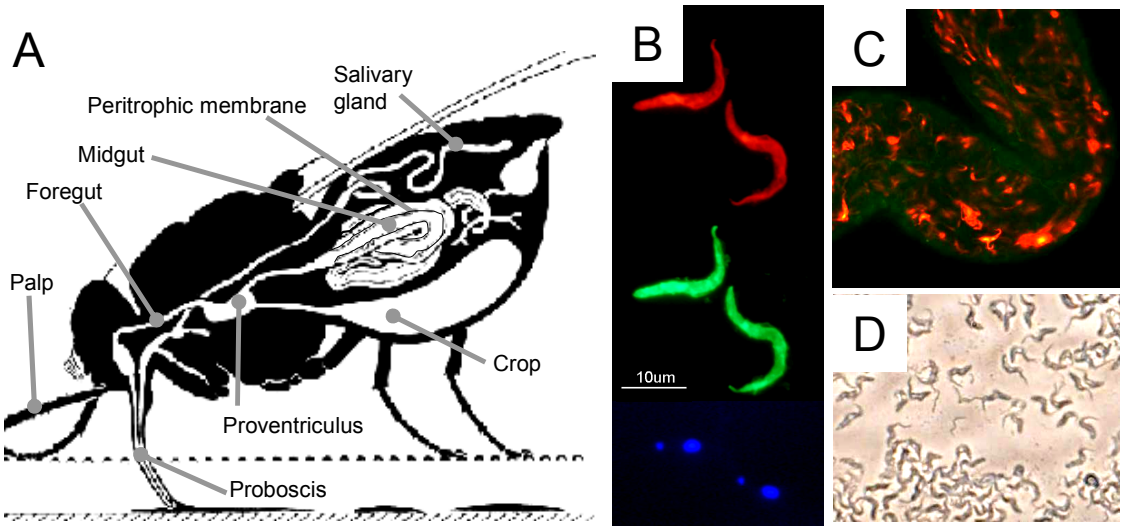

Fig. 1.4: A) Cross-section though a tsetse fly (modified from Geigy and Herbig 1955) with described organs affected during the cyclical transmission of trypanosomes. The microscopic pictures show trypanosomes isolated from infected tsetse flies; B**)** anti-EP (red) and anti-GPEET (green) immune fluorescence (IF) and DAPI (kinetoplast and nucleus in blue) stained trypanosomes derived from the midgut; C**)** infected salivary glands with RFP (red fluorescent protein) transfected trypanosomes; D**)** extruded metacyclic forms on a glass slide.

Tsetse defence against trypanosomes

Tsetse flies are the only vectors for *Trypanosoma bruei* and *Trypanosoma congolense*. *Trypanosoma vivax* can be transmitted mechanically by various blood sucking insects (Cherenet et al. 2006). The specific interaction between trypanosomes and tsetse flies is of great interest to find effective methods to minimise or block transmission.

A passage though a fly contains many barriers against trypanosomes in the midgut as well as during migration and colonisation of the salivary glands (see Life cycle). A first crucial step is the establishment in the midgut. Non adapted trypanosomes are killed by proteases within a few hours (Sbicego et al. 1999) which might also happen in the midgut lumen. Within a few days trypanosomes evade into the ectoperitrophic space, which is separated from the gut lumen by the peritrophic membrane (PM) (Fig. 1.4A), secreted in the proventriculus (cardia) (Lehane 1997). In teneral flies (young flies that did not have a blood meal) the PM is incomplete and trypanosomes can reach the ectoperitrophic space by circumnavigating its posterior end in the midgut (Lehane and Msangi 1991). This may be one reason for their higher susceptibility to trypanosomes compared to older flies. The PM was sought to be an important barrier to trypanosomes. Hence, it was shown that the ectoperitrophic space can also be reached by penetration of the PM (Ellis and Evans 1977) what weakens its importance as a defence against trypanosome infection.

A further defence was suggested to be lectins (Maudlin and Welburn 1987, 1994), since infection rate was significantly increased when feeding with lectin-binding D-glucosamine (Maudlin and Welburn 1988, 1994, Welburn et al. 1994). The direct effect of lectins on trypanosomes was never shown but enhanced trypanosome growth *in vitro* as well as an increased mortality of the flies when treated with D-glucosamine lead to the assumption that other mechanism than direct inactivation of lectins could take place (Peacock et al. 2006, Roditi and Lehane 2008).

Reactive oxygen species (ROS) produced by oxidase in the gut epithelium provide an important defence against a range of microbial infections in *Drosophila* (Ha et al. 2005) as well as in tsetse flies (Hao et al. 2003, 2007, Lehane et al. 2003). Trypanosome and bacterial infection result in a different regulation of ROS; infection with trypanosomes lead to repression of nitric oxide (NO) and its synthase (NOS) activation and a significant elevation of H_2O_2 whereas bacterial challenge provoked the exact opposite (Hao et al. 2003). The different regulations of immune related genes in the tsetse gut tissue upon trypanosome or bacteria challenge suggested pathogen dependent recognition pathways (Lehane et al. 2003).

Antimicrobial immune response

The model organism *Drosophila melanogaster* is also used for basic research on dipteran innate immune system, which showed that flies possess an efficient and well developed defence against a range of microbes. For the recognition of microbes two different pathways, Toll and Imd (= immune deficiency) pathways, are activated by the specific binding of so called pattern recognition receptors (PRR's) (Lemaitre and Hoffmann 2007). These bindings are specific and lead to the activation of only one or both pathways, or can even suppress an immune response (Aggarwal and Silverman 2008). Many PRR's were identified to specifically recognize and bind bacteria and/ or fungi, but up to now none of them was described to bind protozoans (Boulanger et al. 2006, Schmid-Hempel 2005). The activation of the Imd and Toll pathway leads to the production of antimicrobial peptides (AMP's). Diptericine, attacin, drosocin, cecropin, defensin, drosomycin, and metchnikowin are well characterised and described in *Drosophila* (Lemaitre and Hoffmann 2007).

AMP's were also described in tsetse flies (Hao et al. 2001, 2003, Hao and Aksoy 2002, Lehane et al. 2008). Efficient AMP transcription was shown in the fat body (Hao et al. 2001) and in the proventriculus (Hao et al. 2003) by northern blot analysis after microbial challenge. Bacterial infection resulted in a fast (within hours) up-regulation of attacin and defensin while trypanosome infections showed an unambiguously distinct AMP-activation pattern (Hao et al. 2001, Wang et al. 2008). Bloodstream trypanosomes were not but procyclics were recognized by the tsetse fly's immune response. Six days after infection, attacin and defensin transcript level was high in the fat body of infected flies. It was hypothesised that differentiation into procyclic forms trigger the tsetse fly immune response (Hao et al. 2001).

Thee different attacins (attA, attB and attD) are organised on three different clusters in the tsetse genome. AttA and attB are closely related and show high sequence similarity (denoted as attA/B), while attD is different (Wang et al. 2008). All three attacins show an increased transcript level in the fat bodies but a low level in the midgut/proventriculus tissue ten days after trypanosome infection (Wang et al. 2008) which suggest that trypanosomes might repress the activation of attacin in their proximity. This would make sense since recombinant attacin peptides were demonstrated to have trypanocidal activity *in vitro* as well as in the tsetse fly when added to the blood meal (Hu and Aksoy 2005).

Transcript level of attacin and defensin was high in the fat body 10 days after infection whether or not trypanosomes in the midgut were cleared and 20 days after infection in parasite positive flies only (Hao et al. 2001). But the consistent up-regulation of these AMPs upon trypanosome infection could not always be confirmed. Attacin and cecropin peptides in

the hemolymph were detected 6 days after infection but not later (Boulanger et al. 2002) even though it is known from *Drosophila* that AMP's can be very consistent and its detection in the hemolymph is possible for up to two weeks after repression of their mRNA (Uttenweiler-Joseph et al. 1998).

Symbionts of tsetse flies

Insects whose diet consists of a single source of food, such as blood, establish symbiotic relationships with micro-organisms to provide nutritional supplementation. PCR-based phylogenetic studies have revealed the presence of two *Enterobacteriaceae* species (*Wigglesworthia glossinida* and *Sodalis glossinidus*) and one *Rickettsia* species (*Wolbachia*) in various tissues of the tsetse fly (O'Neill et al. 1993, Cheng and Aksoy 1999, Aksoy 2000, Kramer et al. 2003).

Both *Enterobacteriaceae* symbionts are transmitted via secretion of the milk gland (Aksoy et al. 1997), while *Wolbachia* is transmitted transovarially to the offspring tsetse fly larva. The elimination of the symbionts as a consequence of antibiotic therapy results in a severe decrease in egg production (Nogge 1976). The absence of the symbiont after an antibiotic treatment can be restored by feeding the tsetse flies with thiamidine, pantothenic acid, pyridoxine, folic acid, and biotin. Therefore, gut symbionts provide tsetse flies with important vitamin B metabolites (Nogge 1978).

The obligate primary symbiont *Wigglesworthia* lives in the mycetom that consist of specialized epithelial cell in the anterior midgut (Aksoy 1995b, Aksoy et al. 1995). *Wigglesworthia* co-evolved with the ancestor tsetse fly about 50 to 80 millions years ago (Chen et al. 1999) and constitute a distinct lineage of *Proteobacteria* related to the P-endosymbionts of aphids, *Buchnera aphidicola* (Aksoy 1995b). Based on endosymbiont phylogeny, the *fusca* flies constitute the most ancient subgenus, which confirm the phylogeny of the tsetse flies (Leak 1999). Wigglesworthia expresse a 60 kD chaperonin, which seems to be a major protein in the midgut (Aksoy 1995a).

The secondary symbiont Sodalis *glossinidus* lives inter- and intra-cellularly in the tsetse midgut, muscle, fat body, haemolymph, milk gland, and salivary gland tissue (Cheng and Aksoy 1999). *Sodalis* isolated from different tsetse species are almost identical, indicating either a horizontal transfer between species or an independent acquisition of the bacterium by each species (Chen et al. 1999, Weiss et al. 2006). The resistance to attacin (Hu and Aksoy 2005) and its influence of susceptibility on trypanosome infection demonstrated that *Sodalis* closely interact with the tsetse fly and its microbial patogens (Welburn et al. 1993). Unlike

many obligate symbionts, it has been possible to culture *Sodalis in vitro* (Welburn et al. 1987). Antibiotic-resistant *Sodalis* were produced which successfully replaced the wild type symbionts in antibiotic treated female flies and infected the progeny larva (Weiss et al. 2006). This ability opens the way for paratransgenesis experiments where symbionts express foreign genes to block trypanosome infection in the fly (Aksoy et al. 2008).

Trypanosomes

General description

The order *Kinetoplastida* harbours the families *Bodonina* (largely free living protozoans) and *Trypanosomatina* (exclusively parasitic protozoans). The genus Trypanosoma is divided in the section *Stercoraria* (South American trypanosomes including *T. cruzi* causing Chagas disease) and *Salivaria* containing the African trypanosomes (Fig. 1.5A).

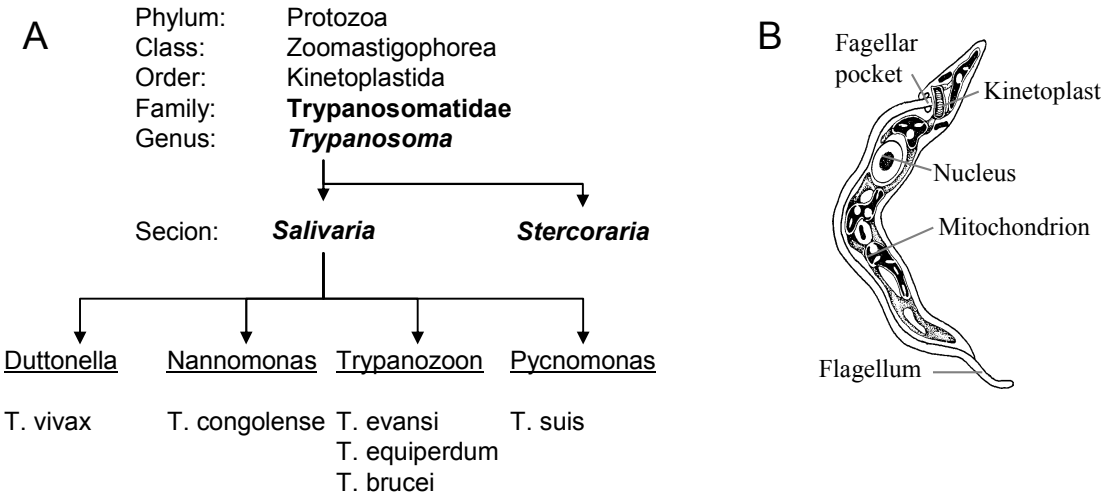

Fig. 1.5: A) Taxonomy of trypanosomes (according to Molyneux and Ashford 1983); B) Ultra-structure of a procyclic trypanosome (organelles are indicated).

Trypanosomes are flagellated unicellular organisms. The flagellum originates from the basal bodies and arises from the flagellar pocket along the body to the anterior end of the cell (Fig.

1.5B). The flagellum is attached along the cell body building an undulating membrane. Trypanosomes have a single mitochondrion containing the kinetoplast (mitochondrial DNA). The basal body is always located close to the kinetoplast. A dense microtubule network underlies the surface membrane, giving the trypanosome its typical shape. Classical description of trypanosome life cycles stages refer to distinct morphological shapes, the position of the kinetoplast in relation to the nucleus, the extension of the flagellum and the expression of stage specific surface proteins (e.g. VSG's and procyclins; see below) (Vickerman 1985). During its developmental cycle, the trypanosome alternates between proliferative phases and non-proliferative phases (Vickerman 1985) (Fig. 1.6).

Life cycle

The trypanosomes shuttle between the mammalian host and the tsetse fly vector which are strikingly dissimilar. To cope with the two different environments a close interaction between parasite and host lead to massive gene regulation. Adaptation mainly affects the mitochondrion (due to the change of metabolism) and the composition of the surface proteins (Vickerman 1985).

The trypanosomes enter the tsetse fly's midgut with the blood meal originating from an infected mammalian host. During feeding, the engorged blood is stored in the crop, from where it is transported via the proventriculus into the midgut (Fig. 1.4A). The bloodstream forms differentiate into procyclic forms within a few hours (see Differentiation). Early procyclic trypanosomes express GPEET and EP (named according to the amino acid single letter code of the tandem repeat at the N-terminus of the surface proteins) (Fig. 1.4B) whereas late procyclics express only EP on the surface (see Midgut infection).

The trypanosomes reach after two (Glättli 1988) to four days (Vickerman 1985) the ectoperitrophic space where the proliferating procyclics establish themselves in the anterior part of the midgut (Fig. 1.6). After six days, trypanosomes arrive in the proventriculus by penetration of the peritrophic membrane at the site of its excretion and they transform into mesocyclic forms (Van den Abbeele et al. 1999). During migration into the salivary glands trypanosomes further differentiate into epimastigote forms (Sharma et al. 2008). The asymmetrically dividing epimastigote forms are highly motile and play therefore a crucial role in completing the journey into the paired ducts of the salivary glands. After the asymmetrical division, the short sister cells are hypothesised to attach to the epithelium and differentiate

into the attached epimastigotes that represent proliferative stage in the salivary glands (Van den Abbeele et al. 1999, Sharma et al. 2008) (see Migration).

Via two intermediate stages epimastigote forms transform into the mammalian infective metacyclic trypanosomes, which are not attached and can be found in the saliva (Fig. 1.4DFig. 1.6). The two intermediate stages are either described as derivative of epimastigote forms (Van den Abbeele et al. 1999) or as pre-metacyclic stages (Vickerman 1985). The dividing attached intermediate stages in the salivary glands give rise to the metacyclic forms (see Maturation).

Metacyclic forms express a variable surface glycoprotein (VSG) coat consisting of about 10^7 proteins (Cross 1990). More than 1000 different genes in the trypanosomes genome code for VSG (Cross 1978). The genetic repertoire of the VSG's allows trypanosomes to cover their surface with new variants what protects them against the mammalian specific immune response (see VSG) as well as the complement-mediated destruction of the pathogen (Donelson et al. 1998).

Injected into a mammalian host during a blood meal of an infected tsetse fly, trypanosomes provoke a local inflammatory reaction in the dermal tissue from where they enter in the lymphatic system and the bloodstream. In the blood two morphologies are present: long slender and short stumpy (Fig. 1.6). The long slender forms divide by binary fission whereas short stumpy trypanosomes are in a cell cycle arrest.

Cell density is the main trigger that differentiates long slender into short stumpy forms (Reuner et al. 1997, Nolan et al. 2000, Matthews et al. 2004, Vassella and Boshart 1996) what regulates the parasite population in the mammalian host. The non-dividing short stumpy trypanosomes are pre-adapted for the further growth in the tsetse fly's midgut (see Differentiation).

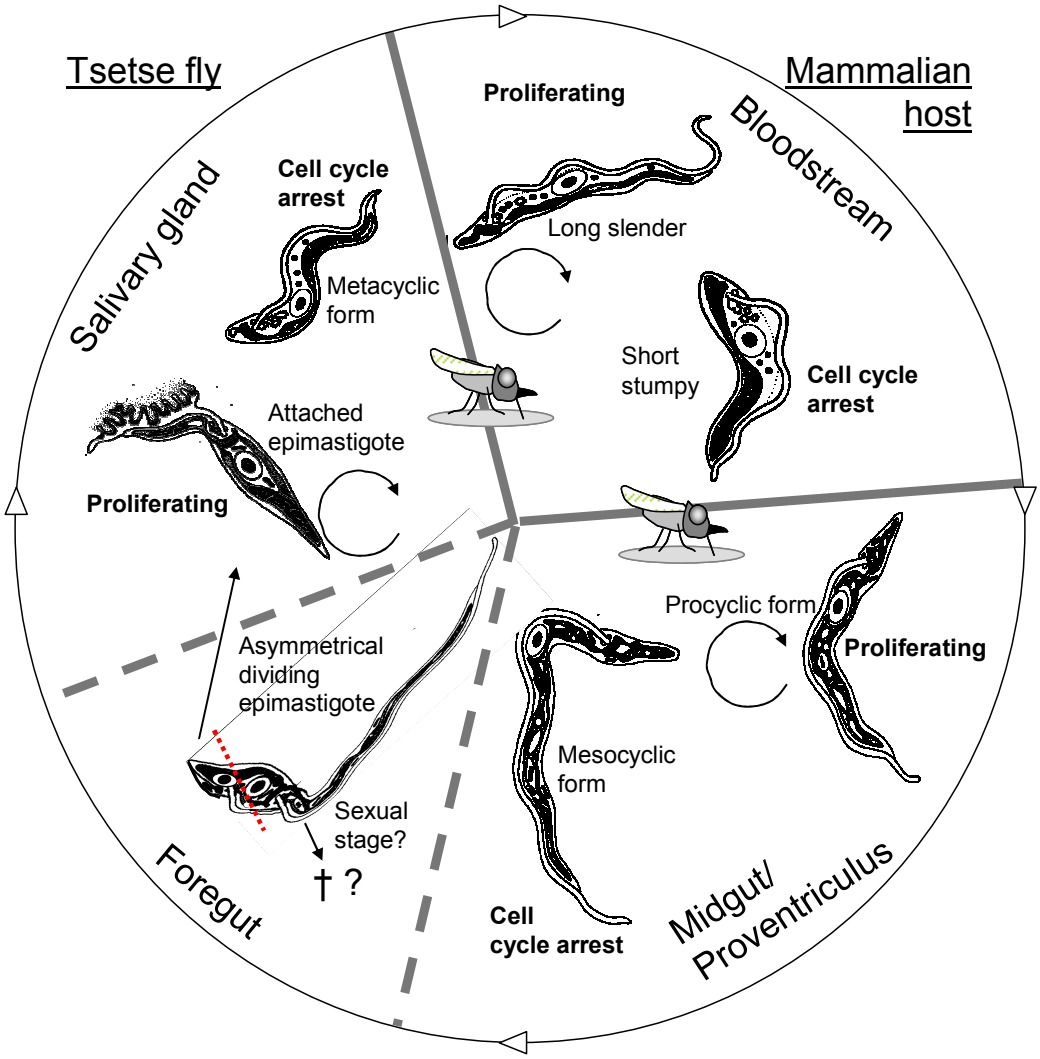

Fig. 1.6**:** The life cycle of *Trypanosoma brucei* can be divided into three segments (the mammalian bloodstream, the tsetse fly midgut/ proventriculus, and the salivary glands) each containing proliferating and cell cycle arrested stages and one or several intermediate stages. The long slender, the procyclic, and the attached epimastigote forms proliferate and ensure massive parasite production. Induced by various triggers (e.g. density in the bloodstream) fractions of proliferating forms differentiate into the cell cycle arrested stages. In the salivary glands and the bloodstream intermediate form are described (see text). In the midgut, no forms are described as intermediate. Possibly, the elongated procyclic forms in the proventriculus (described in Sharma et al. 2008) might present such forms. The asymmetrically dividing epimastigote forms, which migrate into the salivary glands, do not fit in any of the described cell cycle status. Their division lead to long and short epimastigote forms (dotted line). The short forms become attached epimastigote forms and the long epimastigote sister cells performed the transport and most probably die afterwards (indicated with †; Sharma et al. 2008). It is hypothesised that these asymmetrical dividing epimastigotes represent the mating stage of the trypanosomes (Gibson et al. 2008).

Differentiation

In the bloodstream two morphologically different forms are present, the proliferative long slender (ls) and the non-proliferative short stumpy (ss) forms. The ss represent the pre-adapted forms for the successful colonisation of the insect midgut. Differentiation from ls to ss is induced by a cell density mediated parasite signal (Reuner et al. 1997) present in the medium of cultured trypanosomes (stumpy induction factor = SIF) (Vassella et al. 1997). The induction of ss can be achived *in vitro* by cultivation on an agarose matrix (Vassella and Boshart 1996). In peak parasitaemia the fraction of ss forms increases but decreases in parasitaemia minimum. The sole energy source of ls is glucose (and its transformation into ATP by glycolysis) whereas ss have the ability to metabolise substrates like proline and alpha-ketoglutarate and survive in the absence of glucose (similar to procyclics that process proline in the Krebs cycle for ATP production) (Matthews et al. 2004; Vickerman 1985). This metabolic change, the higher resistance to proteolytic enzyms (Sbicego et al. 1999) and alterations in pH (Nolan et al. 2000) assigns the ss as the pre-adapted stage for the insect midgut environment. The active form of the *T. brucei* protein tyrosine phosphatase (TbPTP1) keeps the trypanosomes in the cell cycle arrested ss stage whereas TbPTP1 inactivation in the fly's midgut releases the trypanosomes to develop further to the procyclic stage (Szöőr et al. 2006). Ingested pre-adapted ss forms transform into the procyclic forms whereas the ls, not being able to transform, die.

The differentiation to procyclic forms can be induced *in vitro* by citrate and cis-aconitate and a drop in temperature (Blundell et al. 1998, Vassella et al. 2000, Sbicego et al. 1999, Brun and Schönenberger 1981). During the differentiation in the midgut VSG's are more and more replaced by procyclins. The change from VSG to procyclin coat is a continuous process. First procyclins are detectable 4 to 8 hours and VSG's are lost during 12 to 16 hours after initiation (Roditi et al. 1989, 1987, Blundell et al. 1998). The co-existence of VSG's (which protect against undigested complement or antibodies present in the blood) and procyclins (that shield against proteolytic enzymes in the midgut) on the surface during a short period might be of a selective advantage for the trypanosomes (Roditi et al. 1989).

Midgut infection

Trypanosomes in the insect midgut are subjected to a range of defence mechanisms (see Tsetse defence against trypanosomes). This leads to a loss of midgut infection in a large proportion of flies even under optimal laboratory conditions (more than 50%) within the first

3 to 5 days (Gibson and Bailey 2003). Trypanosomes have to cope with these conditions in the midgut by escaping the gut lumen and activating a procyclin coat.

First analysis by antibodies showed antigenically indistinguishable surface of tsetse midgut forms in contrast with the highly diverse VSG's of the bloodstream forms (Honigberg et al. 1976). Molecular Studies on the structure of *T. brucei* midgut forms revealed a Glu-Pro repetitive (EP-repeat) surface protein and was identified as procyclin protein (Mowatt and Clayton 1987, 1988, Roditi et al. 1989, 1987, Richardson et al. 1986). On the surface of *T. congolense* midgut forms a glutamate and alanine-rich protein (GARP) (Bayne et al. 1993, Beecroft et al. 1993) was found to be a functional equivalent of EP in *T. brucei* (Ruepp et al. 1999). In early midgut infection a second procyclin with a characteristic 5 to 6 penta-peptide repeat (GPEET) is, beside EP, dominantly expressed on the surface of *T. brucei* (Butikofer et al. 1997). Both, EP and GPEET, are attached to the membrane by a glycosylphosphatidyl-inositol (GPI) anchor (Treumann et al. 1997).

Three variants of EP (EP 1 - 3) and one of GPEET are located on four different loci (Roditi et al. 1998). The organisation of the procyclin genes of AnTat 1.1 (Vassella et al. 2001) is shown in Fig. 1.7.

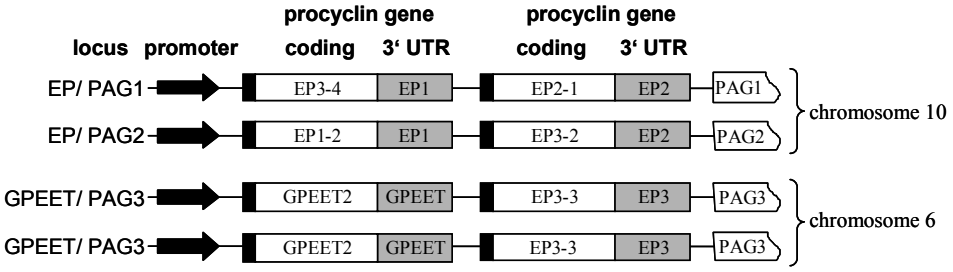

Fig. 1.7: Procyclin expression sites of AnTat 1.1. The sequences of *T. brucei* 427 stock gives the nomenclature for the 3' un-translated region (UTR) (grey). The 5' UTR (black) are highly conserved. On the chromosome 10 EP3 gene is followed by EP2 gene on the locus EP/PAG1 and on the locus EP/PAG2 the first gene is EP1 followed by an unusual form of EP3 gene. On chromosome 6 both loci (GPEET/PAG3) carry a GPEET2 gene followed by an EP3 gene.

Procyclin expression is regulated post transcriptionally and its regulatory element is in the 3'untranslated region (UTR) (Hehl and Roditi 1994, Furger et al. 1997, Vassella et al. 2000). The GPEET mRNA has its regulatory element in the second (out of three) stem loop between

nucleotides 140 and 200 (Vassella et al. 2000). Ongoing projects show that ALBA proteins are responsible for the specific stability of the GPEET mRNA (A. Güttinger et al., unpublished).

EP and GPEET are functionally distinct. Both are genetically induced and expressed on the surface. GPEET is expressed in early procyclic forms during a period of about 7 days (Vassella et al. 2000). In late (= established) midgut infections trypanosomes express solely EP on the surface.

To study the functions of EP and GPEET several knock-outs were generated. The EP-null mutant of the 427 strain grew *in vitro* comparable to its wild type but established considerably lower midgut infection (Ruepp et al. 1997).This suggested that EP is important for the trypanosome survival in the tsetse midgut (Roditi et al. 1998). It was not possible to delete both GPEET genes in the strain 427 which was considered to play a role in parasite-parasite interaction or as a receptor for a soluble ligand (Ruepp et al. 1997). Due to its conserved expression during the establishment in the midgut (the first seven days) GPEET was also discussed to play a role in host-parasite interaction (Vassella et al. 2000).

Using the fly transmissible strain AnTat 1.1 it was possible to knock-out all four procyclins to generate the Δprocylin (Vassella et al. 2009, page 91). Very surprisingly the Δprocylin was able to infect the salivary glands, querying the function of procyclin as either an important shield against proteolytic factors in the midgut or as a key factor for path finding during the insect cycle (Roditi et al. 1998). The transmission rate of Δprocyclin was significantly reduced whereas midgut infection rate was comparable to wild type infection rate. This implies that the absence of the procyclins in the midgut might not be problematic, since the free GPIs can take over its function, but the charging of the GPIs could bear some difficulties which plays an important role for the establishing a mature infection.

Migration

A few publications reported that trypanosomes might cross the epithelium of the midgut and infect the salivary glands via the hemolymph (Mshelbwala 1972, Otieno 1973). Reports on trypanolytic factors in the hemolymph detected by *in vitro* assays (Croft et al. 1982) and the absence of further observation in recent times, led to the wide acceptance that this path is not meaningful for the cycle in the tsetse fly. The most accepted pathway to the salivary glands is via the proventriculus and foregut. The trypanosomes cross the soft part of the peritrophic membrane (where it is secreted) to reach the proventriculus. The mesocyclic forms in the

proventriculus are longer than the procyclic forms (Van den Abbeele et al. 1999, Vickerman 1985) but still express EP (Sharma et al. 2008). With increasing length (= advanced differentiation towards the long epimastigote forms) the abundance of EP weakens (Sharma et al. 2008). The gain of body size in mesocyclics leads to the rearrangement of the nucleus (which moves to the posterior end of the cell and is situated close to the kinetoplast) and the generation of epimastigote forms (Sharma et al. 2008). The asymmetric division of epimastigotes give rise to the short epimastigote forms that colonise the salivary glands where they express the stage specific brucei alanine-rich protein (BARP) (Urwyler et al. 2007). The long sister epimastigote which served as a vehicle, has no further function and dies.
The migrating epimastigote forms were either detected during a defined period from about 8 to 24 days after infection (Van den Abbeele et al. 1999) or continuously (Sharma et al. 2008). The migration is a very important phase for the parasites ensuring the completion of the life cycle. In a high fraction of flies trypanosomes fail to migrate into the salivary glands (about 50% to 80% of midgut infected flies prevent salivary gland infections).

Maturation

The attached epimastigote forms divide and give rise to the metacyclic trypanosomes. The epimastigotes are the only forms in the salivary glands being able to divide; metacyclics are in a cell cycle arrest (Vickerman 1985; Sharma et al. 2008). First metacyclic forms appear after about 12 days (Van den Abbeele et al. 1999). Under laboratory conditions about 10 to 20% of flies infected with *T. brucei* develop a mature infection; in the field this number is much lower (< 1%). Metacyclics are the vertebrate infective forms and express a restricted set of variable surface glycoproteins (VSGs) (Cross 1990).

VSG

Up to 10^7 VSG's build a dense coat and cover the complete trypanosome surface (Cross 1990). The continuous change to new, for the host's specific immune response unknown, VSG coats, ensures the trypanosomes to survive in the bloodstream of the mammalian host. Trypanosomes have about 1000 or 2000 different VSG genes covering more than 2% of the total genome. A large fraction of the silent *vsg* (-reservoir) is present on mini-chromosomes (Pays and Nolan 1998). At least 20 expression sites (ES), located in telomeric regions, can be found in the genome (Stockdale et al. 2008). Only one of them is active at a time, the others are silenced by the integration of base J (β-D-glucosylhydroxy-methyluracil) (Pays and Nolan

1998). Each of the ES has up to 10 associated genes (esag's). The function is not always clear for some of the esag's, but many of them code for membrane proteins (Pays and Nolan 1998). In active ES, RNA polymerases transcribe all esag's including the VSG gene at their end (after 45 to 60 kb). In contrast to the ls the ss forms do not undergo antigenic variation anymore (Vickerman 1985, Stockdale et al. 2008) by reducing the transcription activity of the ES. In inactive ES, the RNA processing and elongation is inefficient and leads to the abortion of transcription after a few hundred bases with the result that VSGs are not transcribed anymore (Amiguet-Vercher et al. 2004, Vanhamme et al. 2001).

VSG switching in trypanosomes represents the stochastic change of the VSG coat to new variants to escape the host's specific immune response. These variants grow and establish a next parasitemia peak which triggers the development of specific immune response against the dominant VSG variants and its elimination leading to the selection of new variants. Two main switching mechanisms are described: the activation of a new ES (*in situ* activation) and the recombination of VSG DNA into an active ES (gene conversion and recombination) (Pays and Nolan 1998, Stockdale et al. 2008). About 10^{-7} (in laboratory adapted strains) to 10^{-2} (in wild type strains) VSG switches per cell and generation occur spontaneously (Cross 1990). Trypanosomes showed to express a restricted number of different VSG variants at a time but what mechanisms regulate the ordered expression of the VSG's is not known. Intensive cross-talk between ES (intra-genome) and between trypanosomes cells (inter-genome) must take place to fulfil the observed pattern of the VSG switching (Vickerman 1989). To deal with this complex mechanism diverse models were suggested (here three examples are given):

A) It was observed that trypanosomes expressing different VSG's showed small differences in growth rate (Aslam and Turner 1992) which was suggested to be a possible mechanism for the ordered appearance of different VSG variants (Frank and Barbour 2006). Morrison et al. (2005) showed that different growth phenotypes of trypanosomes expressing different VSG's do not explain the high degree of ordered VSG expression.

B) The existence of trypanosomes with two active ES (show two different VSG's on the surface during the course of switching) led to the hypothesis that these 'doubly expressing' (DE) trypanosomes interact with the host's immune response and generate the ordered appearance of VSG's (Agur et al. 1989). In this model a high number of different VSG's are suggested to be permanently expressed in a 'cloud' at a very low rate (>0.005) and hence under the detection level for the immune system. This model predicts an ordered appearance of VSG's even though all switches have similar probabilities. The exaggerated importance of the DE's and the complexity led to a low acceptance of this model.

C) Different switching probabilities are hypothesised to lead to an ordered (hierarchical) VSG expression pattern. The hierarchy depends on distinct probability for the activation of a specific VSG variant. The probability of activation is dependent on the locus in the genome and the sequences of the flanking regions of the VSG gene (Morrison et al. 2005). The observed preference of transcriptional switching mechanism between ES compared to the recombinant switching mechanism supporting this hypothesis (Aitcheson et al. 2005).
Beside the hierarchical expression of VSG's Turner (1999) hypothesised a high switching rate in the beginning of infection and a decrease of switching rate during infection. The hierarchic VSG expression (e.g. after variant A follows B, then C, and so on) was suggested to be reversible: Syringe-passage of trypanosomes expressing one VSG variant (e.g. variant D) into a new host stops the expression of the hierarchically following VSG variant (e.g. variant E) and resets the order by expressing the variant from the start of the hierarchy (variant A) (Turner 1999).

Genetic diversity and multiple strain infection

The genotypic diversity of trypanosome isolates and stocks were analysed with multi-locus enzyme electrophoresis (MLEE) in the past and with micro- and mini-satellite technique in recent times. The finding that some *T. brucei* isolates showed a high diversity but others not, denoted important implications for the evolutionary and epidemiological significance of the parasite.
T. b. gambiense type I isolated from patients in Democratic Republic of Congo and in Cameroon showed only polymorphism between isolates of the two countries but not among isolates from one country (Morrison et al. 2008). This suggested a very limited genetic exchange and allowed a clear and tight clustering of *T. b. gambiense* type I (Koffi et al. 2007). Much higher diversity was detected in *T. b. brucei* and *T. b. rhodesiense* isolates a finding that implies a high rate of multiple infections in the field (see Table 1.1). An average of about 10% of multiple infections was considered to appear in Africa without geographical restriction (Balmer and Caccone 2008). This implies a high rate of gene exchange and can therefore influence the ability to acquire and distribute traits of medical and economic importance (MacLeod et al. 1999).
Two major disadvantages come along with the satellite technique: (i) dominant genotypes in a sample might mask a single infection and (ii) minor genotypes might be lost because field

isolates have to be cultivated (*in vitro* or *in vivo*) to gain appropriate cell number prior to analysis.

Host	Multiple strain infection		Countries	Reference
	%	No.		
All host	3.1	Ca. 900	Africa	(Godfrey et al. 1990)
Tsetse fly	42.9	28	Kenya, Uganda	(A MacLeod et al. 1999)
Cattle	18	50	Uganda	(A. MacLeod et al. 2000)
Human	8.7	23	Ivory Coast	(Truc et al. 2002)
All host	8.8	137	Africa	(Balmer and Caccone 2008)

Table 1.1: Overview of published reports on multiple strains identified in *T. brucei* isolates (modified from Balmer and Caccone 2008)

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2. Rational and aim of the project

Close interaction between trypanosomes and the host leads to specific gene regulation in both counterparts. The mammalian host as well as the tsetse fly vector recruit a whole set of genes to fight the parasite. Besides specific gene regulation, mainly the vector deploys also mechanical barriers against the parasite. Trypanosomes on the other hand gained strategies to cope with these defence mechanisms and successfully infect hosts and vectors.

'Bottleneck'

Trypanosomes undergo a very complex life cycle in the vector and mammalian host where they have to conquer diverse barriers and hurdles. Such barriers reduce trypanosome population size and might lead to parasite clearance. In successful cases, the population recover and gain in size.

Trypanosomes are very regenerative: a single trypanosome is sufficient to infect a tsetse fly midgut (Maudlin and Welburn 1989) and hypothetically a similar low number might be enough to colonise the salivary gland of a fly (Peacock et al. 2007). Only a single metacyclic trypanosome might be enough to infect a mouse (Gingrich et al. 1981).

A reduction of the population size followed by a boost represents a bottleneck. Trypanosomes experience several bottlenecks during the cycle in the tsetse fly (Gibson and Bailey 2003, Van den Abbeele et al. 1999) but also during transmission from vector to the host (and vice-versa). The diminution takes also place in the mammalian host when the specific immune response eliminates trypanosomes that do not switch to a new VSG coat in a useful time. Bottlenecks can also come in action during treatment or after treatment when trypanosomes re-colonise the bloodstream (relapse), or when some trypanosomes gain resistance.

Such reduction might negatively influence the heterogeneity of trypanosome populations. Loss of heterogeneity has important implications for the acquisition and maintenance of traits - such as human serum-resistance, drug resistance, and/ or virulence. This has never been quantified in vector transmitted diseases.

In this project we want to estimate if the loss of trypanosome number during a bottleneck has an impact on the heterogeneity. The aims are:

- to generate a diversity with different tags (DNA-markers) integrated into the trypanosome genome.
- to analyse the heterogeneity of the tags during the cycle in the tsetse fly.

- to analyse the heterogeneity during the whole cycle (in the tsetse fly and mouse) including the natural transfection.

In this project, we established a model that allows to monitor the diversity of a trypanosome population during their life cycle.

Fly immunity

Tsetse flies demonstrate a high resistance to trypanosome infection which is reflected in the low infection rate (1 – 2%) of wild caught flies. The immune response of the tsetse flies is considered to play an important role for the resistance since some anti-microbial peptides (AMP's) are up-regulated upon trypanosome infection (Aksoy et al. 2003). The expression of procyclins in the midgut is considered to elicit an AMP response (Hao et al. 2001) whereas its composition (presence or absence of GPEET) is suggested to be crucial (Hu et al. 2008). This led to reassess the function of procyclins: is there a function of the procyclin coat that specifically activates the immune response?

The aims are:

- to systematically test with defined mutant trypanosomes (Δprocyclin and ΔGPEET generated in the laboratory of I. Roditi) if EP, GPEET, or both of them elicit a tsetse immune response.
- to analyse the regulation of attacin and defensin after wild type infection using different trypanosome strains.

One dominantly expressed putative lectin in the midgut tissue shows an extended run of 59 EP-repeats at the C-terminus, and is named tsetseEP (Chandra et al. 2004). This finding was interesting since trypanosomes also express an EP-repeat when they colonize the midgut. Its function is not clearly known but was shown to be up-regulated in the midgut epithelium upon bacterial challenge (Haines et al. 2005). Only two publications deal with the tsetseEP and more knowledge is highly needed.

The aim is to analyse the tsetseEP regulation upon bacterial and trypanosome infection.

Delta procyclin

The specific expression of the EP and GPEET might indicate a very smooth crosstalk between tsetse fly and trypanosomes and represents the outcome of long lasting evolution between these two counterparts. To find out the specific function of EP and GPEET several knock-out were generated and tsetse experiments were conducted (Roditi and Lehane 2008). Surprisingly, all of them were able to heavily infect the midgut leading to the general question about the function of procyclins and its need during the cycle in the tsetse fly. All these experiments were done in trypanosomes that were not able to infect the salivary glands. In a new approach we wanted to analyse the function of procyclin in a fly transmissible strain. The aims are:

- to generate a procyclin knock-out in the AnTat 1.1 strain.
- to test if a procyclin null-mutant can infect the salivary glands.
- to characterise the infectivity of the procyclin knock-out.
- to analyse the fitness of the procyclin null-mutant in competition with wild type trypanosome *in vitro* and *in vivo*.

I participated since several years in this project and my contribution shows nicely that research is a very interacting field where people from different laboratories collaborate with each other.

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3. Bottlenecks in the cycle of *T. brucei* through tsetse flies and mice

Michael Oberle[1+2], Oliver Balmer[1], Reto Brun[1], Isabel Roditi[2]

[1]Swiss Tropical Institute, Socinstrasse 57, 4051 Basel

[2]Institute of Cell Biology, Balzerstrasse 4, 3012 Bern

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Abstract

Trypanosoma brucei infects mammals and is transmitted by tsetse flies (*Glossina spp.*). Its life cycle is complex and comprises different developmental stages in the vector and the mammalian host where trypanosome numbers are repeatedly reduced. A decrease in population size can result in a reduction of the pathogen's fitness due to loss of genetic diversity. In this study, we analysed how trypanosome population diversity is influenced during their life cycle. Eight different DNA tags (40bp) were integrated into the genome of trypanosomes and used to monitor diversity during fly and the mouse passage. Tsetse flies were infected with the tagged trypanosomes. Salivary gland positive tsetse flies infected mice by feeding on them. Infected midgut and salivary glands of these flies were dissected and blood samples from infected mice were collected over a period of 7 – 10 weeks. DNA was extracted and tags were amplified by PCR and identified by 454 pyro-sequencing. We found that the number of tags was maintained in the midgut, the salivary glands and also during mouse infection. This was surprising since trypanosomes face severe bottlenecks during fly passage. The relative frequencies of some tags shifted during migration, which resulted that only a few tags were dominant in the salivary glands. During chronic mouse infection, minor tags co-existed with dominant ones, and were transmitted to new insect hosts. This model allows to monitor the population dynamics of a vector transmitted protozoan parasite.

Introduction

Bottlenecks are events that drastically reduce the effective size of a population (Nei et al. 1975). The individuals that overcome such a bottleneck recover and establish a new population. Often, a population that has gone though a bottleneck shows reduced genetic diversity due to the loss of locus variability. The reduction of diversity is profoundly dependent on the size of the bottleneck (Nei et al. 1975). Many pathogens experience frequent bottlenecks during transmission as studies with RNA viruses and malaria parasites has shown (Iglesia and Elena 2007, Bergstrom et al. 1999, Mackinnon et al. 2005). Such transmission bottlenecks were described to reduce fitness of pathogens due to loss of genetic diversity (Iglesia and Elena 2007, Bergstrom et al. 1999, Mackinnon et al. 2005). In malaria, transmission through a mosquito (a bottleneck) resulted in a decrease of virulence to the

mammalian host which balanced determinants for virulence and protected the parasite from a 'dead-end' virulence evolution (Mackinnon et al. 2005).

The parasite *Trypanosoma brucei* infects mammals and is transmitted by a blood-feeding insect vector, the tsetse fly (*Glossina spp.*). Trypanosomes occur in sub-Saharan Africa and cause the fatal disease sleeping sickness in humans (Simarro et al. 2008) and nagana in cattle. Owing to nagana an annual economic loss of up to US$4 billion is estimated (Kabayo 2002). Trypanosomes can infect their vector and host very effectively: a single parasite is sufficient to infect a tsetse fly (Maudlin and Welburn 1989) and one bite of an infected fly is sufficient to infect a mammalian host (Thuita et al. 2008) with a minimal infective dose of one metacyclic trypanosome (Gingrich et al. 1981). The high infectivity implies that trypanosomes could easily cope with very narrow bottlenecks. If transmission bottlenecks are so small and so frequent, trypanosomes might not only risk loss of fitness but also the accumulation of deleterious mutations (Ebert 1998). Trypanosomes have the ability to adapt quickly to changing environments. Resistance to drugs can be acquired *de novo* in sub-curative treated infected mammals, but it is not known if, and how efficiently such traits are maintained during cyclical transmission through the tsetse flies in the field. Under laboratory conditions drug-resistant trypanosomes could be passaged through tsetse flies (Van den Bossche et al. 2006, Diack et al. 1997). The term bottleneck has been often used to describe the observed reduction in parasite numbers during the trypanosome's life cycle (Peacock et al. 2007, Lehane et al. 2003, Bingle et al. 2001, Hu et al. 2008, Harmsen 1973). Transmission bottlenecks could influence the diversity of trypanosome populations and hence the distribution of drug resistance but this is dependent on their size. A systematic analysis of bottlenecks is still pending.

The trypanosome life cycle is complex and comprises several stages in the vector and the mammalian host where their numbers are frequently reduced. A tsetse fly takes up trypanosomes while feeding on an infected mammalian host. The trypanosomes reach the midgut with the blood meal where they differentiate into procyclic forms. In the midgut more than 99% of the ingested trypanosomes are killed during the first three to five days (Gibson and Bailey 2003, Van den Abbeele et al. 1999) (Fig. 3.1A). There might be several reasons for this reduction in population: enzymes in the midgut, oxidative radicals (Hao et al. 2003), gene activation in the gut epithelium with putative immune-related functions (Lehane et al. 2003), and the peritrophic membrane (a mechanical barrier), which trypanosomes have to circumvent to reach the ectoperitrophic space (Lehane and Msangi 1991). Trypanosomes reach the proventriculus and may migrate to the salivary glands via the foregut, and the

proboscis (Gibson and Bailey 2003, Van den Abbeele et al. 1999, Sharma et al. 2008). It has been proposed that only a few trypanosomes undertake this journey and that epimastigote forms are the only forms capable of founding a population in the salivary glands (Gibson et al. 2008, Peacock et al. 2007). In a large fraction of tsetse flies with infected midguts (> 50%) trypanosomes fail to infect the salivary glands (Fig. 3.1B).

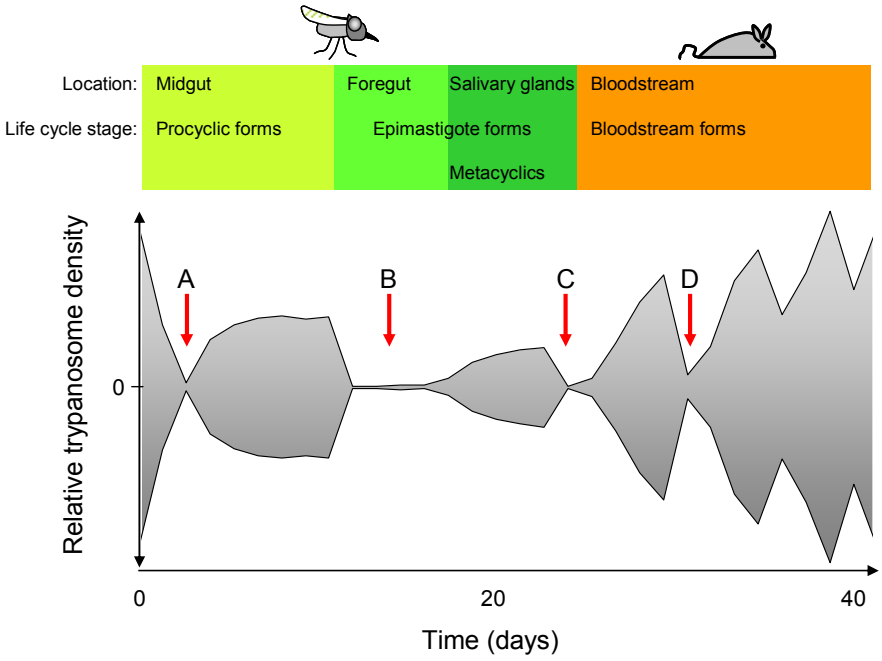

Fig. 3.1: Illustration of reductions in population size during the life cycle of *Trypanosoma brucei*. Relative population density is given on the y-axis. Trypanosomes are taken up by a tsetse fly, in the midgut the population collapses during the adaptation to the environment (up to 1000-fold reduction) (A) and recovers thereafter. Only a few trypanosomes at a time are presumed to migrate to the salivary glands, but migration takes place during an extended period or even continuously when trypanosomes are present in the midgut (B). Long epimastigote forms can reach the salivary glands where they establish an infection and give rise to mammalian infective metacyclic forms. During a blood meal, metacyclic forms are injected into a mammalian host. The injection and the relocation of trypanosomes from the site of injection into the bloodstream may reduce its number (C). In the mammalian host population is periodically reduced in size owing to the adaptive immune response and also owing to the differentiation of long slender into non-dividing short stumpy forms (D).

The factors that hinder migration to the salivary glands are not known and it is under debate whether migration is restricted to a defined period (Van den Abbeele et al. 1999) or whether trypanosomes migrate continuously (Sharma et al. 2008). Epimastigote forms in the salivary glands attach to the epithelium and proliferate. They give rise to the mammalian-infective metacyclic forms (Vickerman 1985). During a blood meal several hundred metacyclic forms are injected into the mammalian host (Gingrich et al. 1981). The injection of trypanosomes and their relocation into the bloodstream via the lymphatic system might again reduce their numbers (Fig. 3.1C). The metacyclic forms differentiate into long slender bloodstream forms. They proliferate in the bloodstream and evade the host's specific immune response by stochastic switching of the variant surface glycoprotein (VSG) coat (Cross 1990, Pays 2005). The activation of the mammalian immune response against dominant variants of VSGs selectively kills trypanosomes. Additionally, the differentiation of the long slender forms into the non-dividing stumpy forms caused by a factor released by the parasite themselves (Vassella et al. 1997) leads to a substantial reduction in population size in the mammalian host (Fig. 3.1D).
Under experimental conditions, a salivary gland infection rate of 5 – 20% is reached, but in the field this rate is even lower (0.1 – 1%). Fly passage seems to be a severe barrier to trypanosomes, but what happens to the diversity of such a population? And what happens to the diversity in a mammalian host? We established a model that allowed us to monitor parasite diversity during fly and the mouse passage, including transmission from the vector to the mammalian host and vice versa. We tagged trypanosomes with short DNA tags, integrated into a non-transcribed region of the genome, and dissected infected midguts and salivary glands of tsetse flies and sampled blood of infected mice. The tags were subsequently identified by deep sequencing. The analysis of the tags revealed new insights into how the frequency and distribution of trypanosomes is influenced during the life cycle.

Results

Experimental design. Eight different 40mer tags were inserted into the plasmids pTag 1 – 8 upstream of its promoter. Procyclic forms of AnTat 1.1 were cloned *in vitro* and one clone was passaged through a tsetse fly and a mouse to ensure its transmissibility (Fig. 3.2). The bloodstream forms were triggered to differentiate to procyclic forms *in vitro* and transfected separately with the plasmids. The individual transfectants grew at similar rates in SDM-79

(Fig. 3.2). Cultures of the eight clones were mixed and used to infect tsetse flies, of which 50% developed a midgut infection and 8.75 % produced metacyclic forms. Three flies A, B, and C with infected salivary glands were selected and allowed to feed on one mouse each (45, 31, and 27 days after fly infection, respectively). Parasites were first detected 6, 7, and 4 days after the infective bite in mice A, B, and C, respectively. Subsequently, the salivary glands and midguts of the flies were dissected and DNA isolated for tag analysis. Tail blood samples were collected from each mouse to monitor the parasitaemia and DNA was prepared from five samples for tag analysis in the weeks 1, 2, 3, and 4 and at the termination of the experiment after 7 to 10 weeks. Two batches of ten teneral flies were fed on mouse C 18 and 30 days post infection. Midguts were dissected after 10 and 12 days and one positive midgut from each batch was taken for tag analysis (fly D and fly E in Fig. 3.3). Tags were amplified by PCR, sequenced by 454 massively parallel pyro-sequencing and analysed for their frequency and distribution. In total, we identified 30,592 sequences, an average of 1330 tags per sample.

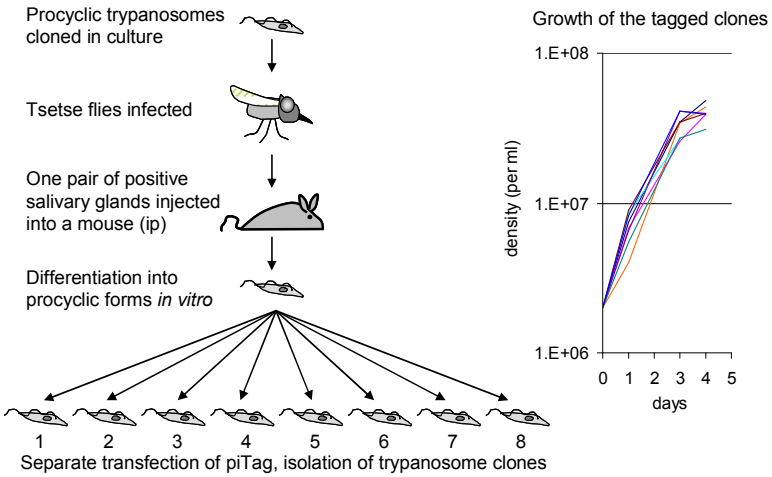

Fig. 3.2: Cloning procedure: Cloned procyclic trypanosomes were passaged though a tsetse fly and a mouse and bloodstream forms were triggered to differentiate to procyclic forms *in vitro* (left panel). The plasmids piTag 1 - 8 were then transfected separately. The eight tagged clones grew at similar rates in SDM-79 (each colour correspons to a trypanosome clone with a different tag) (right panel).

As a common pattern in all three experiments (Fig. 3.3) a large number of tags was detected in the midgut, many of them at high frequency (> 5%, coloured in red). All eight tags were

detected in the three flies and each midgut contained at least six different tags. This demonstrates that the cultured procyclic trypanosomes maintained their diversity in the gut lumen of the flies. A second common feature was the smaller number of tags detected in the mice with only one dominant tag at later stages of infection. And the third common pattern in all three mouse infections was the sporadic appearance of very minor tags.

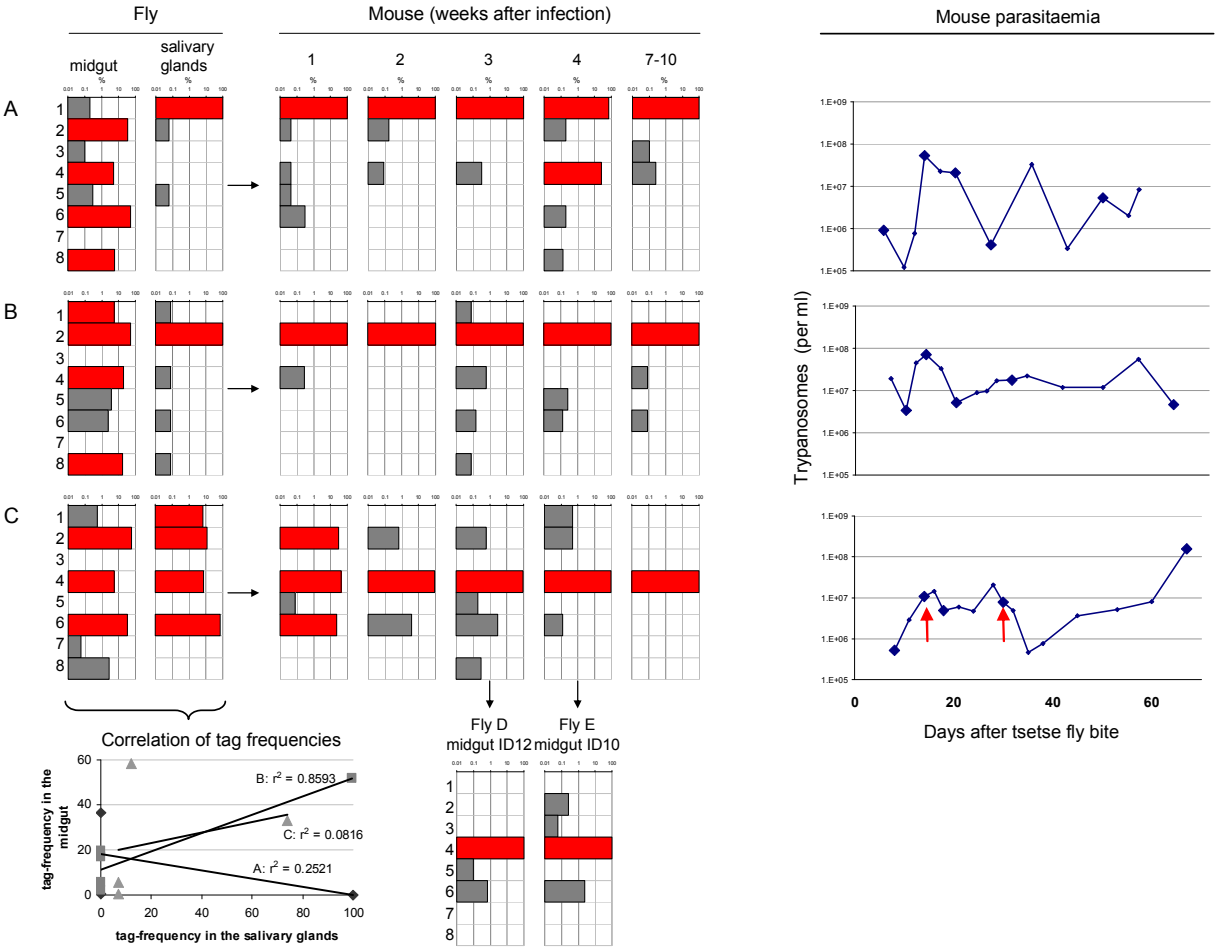

Fig. 3.3: Tag diversity during the life cycle of trypanosomes in three different experiments. The bars in each chart indicate the presence and frequency of the tags on a log scale ranging from 0.01 - 100 per cent where dominant tags are highlighted in red (>5%). Tsetse flies were infected with procyclic trypanosomes with an even distribution of the tags. Three flies (A, B, and C) had infected midgut (mg) and salivary glands (sg). These flies each infected one mouse whose parasitaemia was monitored (right panel). Five blood samples were taken from each mouse for analysis of the tags (indicated by large diamonds in the right panel) after 1, 2, 3, and 4 weeks and at the termination of the experiment after 7 - 10 weeks. Flies D and E were infected on mouse C 18 and 30 days after infection (indicated with the red arrows in the

right panel). Midguts were dissected after 12 and 10 days. Relative frequencies of the tags in the midgut and in the salivary glands were plotted and correlation coefficient (r^2) was calculated (correlation of tag frequencies).

The frequency of individual tags in the salivary glands changed compared to the midgut (Fig. 3.3). This was most striking in fly A, in which tag 1 was minor (0.2%) and tag 6 dominant (52%) in the midgut, whereas in the salivary glands tag 1 was dominant (99.9%) and tag 6 undetectable. In fly B, tag 2 (99.7%) was dominant in the salivary glands. This resulted in a very uneven distribution of the tag frequencies in the two flies. In fly C, the frequencies of the tags 1, 2, 4, and 6 were detectable in the salivary glands with relative frequencies ranging from 6.8 - 74%. These findings showed that tags that are dominant in the midgut are not necessarily so in the salivary glands and that their relative frequencies were sometimes altered. The dissimilar distribution of the tag frequencies in the midgut and salivary glands is reflected by the diverse correlation coefficients: $r^2 = 0.08$, 0.86, and 0.25, for the flies A, B, and C respectively (Fig. 3.3). This confirmed the analysis of a pilot study where the distribution of the tag frequency was also dissimilar between midgut and the salivary gland sample (data not shown).

The distribution and frequency of tags in the mouse mirrors the corresponding salivary glands infection. The dominant tag in the salivary glands retained its dominance in mice A and B from the first sample onwards. In mouse C, tags 2 (32%), 4 (43%), and 6 (25%) were present in the first week of infection. Tag 4 became progressively more dominant and was the only dominant tag from the second week onwards and finally the only one detectable after ten weeks. Tag 1, with the frequency of 6.9% in a salivary glands of fly C, became very minor in the following mouse infection and was detected only once (0.6%) after four weeks. The presence of one dominant tag and a few minor tags during mouse infections led to a very uneven distribution of the frequencies of individual tags, which showed up to 1000-fold variation between the tags.

A different tag was dominant in each of the mouse infections (tags 1, 2, and 4 in mice A, B, and C) indicating that all trypanosomes bearing a different tag are equally fit. In all three experiments minor tags were detectable throughout the mouse infection. Some of the minor tags were found in only one sample, whereas others occurred in several samples. Tags 3 and 8 in mouse A, and tags 1 and 8 in mice B and C each came up in only one sample, however, with frequencies of 0.08 – 0.5%. Tag 7 was not detected in any of the mouse samples. Taken

together, a minimum of six tags were detected in each mouse with a range of tag frequency of 0.08 – 100%.

The parasitaemias showed some common patterns even though the inoculum was different in the three mice (Fig. 3.3, right panel). The number of trypanosomes injected during a blood meal can vary from a few hundred to thousand (Gingrich et al. 1981, Thuita et al. 2008) and the number of trypanosomes that reach the bloodstream is unknown. Despite this variable inoculum, the first peak of parasitaemia in all mice appeared on day 14 or 15 post-infection. After this first peak, the parasitaemia showed different patterns in the mice, a phenomenon well described elsewhere (Morrison et al. 2005).

The parasitaemia fluctuated three orders of magnitude (10^5 – 10^8 trypanosomes per ml) but this had no influence on the number of different tags detected. We found no correlation between mouse parasitaemia and number of tags detected (data not shown).

The analysis of the tags from flies D and E revealed two interesting outcomes. First, the tag that was dominant in the mouse remained so in the fly's midgut (Fig. 3.3). Second, minor tags in the mouse blood were retained by the flies. For example minor tags with frequencies of 0.5 – 3% in the mouse were detectable in the flies' midgut (tags 2, 5, and 6). Interestingly, tag 3 was detectable in fly E even though it was under the detection limit in all the mouse samples of experiment C. The two flies together took up five of the six tags detected in donor mouse. The parasitaemia at days 18 and 30 was $4.9x10^6$ and $7.7x10^6$ ml^{-1}, respectively. Assuming the flies took up 20µl of blood, about 1 - $1.5x10^5$ trypanosomes might have reached the midgut of the two flies. If approximately 1% survived the decrease of the population in the midgut (Van den Abbeele et al. 1999) even minor tags might be present in 2 - 30 individual trypanosomes, which could establish an infection in the fly's midgut (data not shown). The maintenance of such a large number of different tags after natural transmission by a tsetse fly is surprising. Any trypanosome present in the midgut is capable of migrating to the salivary glands and establishing a dominant population, regardless of the integrated tag and its abundance.

Discussion

We found a large number of different tags throughout the life cycle of trypanosomes. This finding is highly interesting since trypanosomes face severe reductions in population size each time they adapt to a new environment (in the midgut, salivary glands, and bloodstream)

but this seems to have little influence on the diversity of the tags. We showed furthermore that some reductions in trypanosome numbers were additionally accompanied by changes in the relative frequency of the tags. This change led to a very uneven distribution of the tag frequency and hence to a drop in detectable number of different tags. Changes in tag frequency were detected mainly in the salivary glands, but not, or to a lesser extent in the transition to the mammalian host or from the bloodstream to the tsetse midgut.

The diversity of tags were maintained in the flies' midguts regardless of whether the flies were infected with procyclic or bloodstream forms. Massive reduction of the population size during the adaptation to the midgut environment has been reported after experimental infection with procyclic forms (Moloo et al. 1994, C. Kunz Renggli unpublished observation) but this had at most a modest influence on the diversity of the tags. Tsetse flies ingested approximately 2 - $5x10^4$ procyclic culture forms. Assuming a survival rate of only 1% each tag should be present in 30 to 60 trypanosomes, of which not all recovered equally, leading to changes in relative frequency for some of the tags.
Two tsetse flies were infected on the donor mouse C with bloodstream forms. Five different tags were found in the established midgut infections of the two flies. Calculations revealed that for some tags possibly only single trypanosomes recovered, and that a minimum of 500 - 1000 trypanosomes survived in the midgut. A similar number was recorded by (Van den Abbeele et al. 1999) and even a smaller one (20 – 100 trypanosomes) was determined by others (Gibson and Bailey 2003). Such a severe reduction of trypanosome numbers affects both the non-adapted slender and the pre-adapted short stumpy forms and leads to the subsequent loss of infection in a high percentage of flies (Van den Abbeele et al. 1999, Gibson and Bailey 2003, Welburn and Maudlin 1999). The reduction in the midgut represents a bottleneck where obviously a very small number of trypanosomes with different tags can recover leading to the co-existence of several tags. Co-existence of different *T. brucei* genotypes was also found in the midgut of wild caught tsetse flies (Stevens et al. 1994, MacLeod et al. 1999).

Migration to the salivary glands leads to a shift in relative frequency for some of the tags. Up to five tags per experiment changed their relative frequencies in the salivary glands compared to the midgut (from dominant to minor or vice versa). This change might reflect how the salivary glands were infected. In infected glands trypanosomes are tightly packed and have to compete for space. Trypanosomes that arrive first would have the advantage that they

can easily disperse in the salivary glands. Therefore, early migration might be more beneficial for successful colonisation of the salivary glands than dominance in the midgut. This 'race for space' might influence the distribution of tags and would explain why relative frequencies of tags can shift completely during migration.

Monitoring mouse infections revealed that all the tags detectable in the midgut have reached the salivary glands but some were apparently under the detection limit. In a recent study, tsetse flies were infected with two strains of trypanosomes expressing different fluorescent proteins (Peacock et al. 2007). Some salivary glands were infected with trypanosomes of only one colour and it was hypothesised that only a few trypanosomes may have migrated. The use of eight different tags and the highly sensitive detection method revealed that virtually all tags present in the midgut reach the salivary glands and that the number of tags was clearly not affected. High variability of genotypes in the salivary glands was also found in field isolates and interpreted as a proof for frequent genetic exchange in nature (MacLeod et al. 1999). Our study allows the alternative interpretation that a wide variety of different genotypes might have reached the salivary glands.

In the salivary glands, the distribution and the relative frequency of the tags seems to be reasonably stable. It was not changed in the first mouse sample (week 1) even though flies were dissected four to seven days after mouse infection. The dominant tags found in the salivary glands and in the first mouse samples, were also found in extruded saliva probes in the time between mouse infection and salivary gland dissection (data not shown). The 'race for space' would also explain why trypanosomes that arrive later can not disperse and its tags remain minor whereas dominant tags persist in the glands.

In the field, Tsetse flies may take up different genotypes that can establish infections in the midgut and their relative frequencies can change completely in the salivary glands in each fly, causing a high variety of different dominances among all infected tsetse flies. The migration could balance different frequencies of genotypes (minorities in the midgut can dominate the salivary glands) and stabilise their diversity in the population.

A large number of tags was maintained during the mouse infections but relative frequency was highly variable among the tags, which affected the detection level. Some tags were detected only once in ~6800 sequences obtained from five mouse samples (e.g. tags 1 or 8 in mouse B, Fig. 3.23). Considering this low probability for of detecting the minor tags, we conclude that all tags that were transmitted by the tsetse fly were present continuously during mouse infection.

Interestingly, the switching mechanism of VSG, which obviously occurred in the mouse, did not lead to a loss of tags during the long infection period. Variant antigen type become dominant due to stochastic switching of the active VSG gene but trigger a specific immune response which leads to their elimination and to the selection of new variants. An ordered (hierarchical) VSG expression pattern was hypothesised and represents one widely accepted model for VSG switching (Aitcheson et al. 2005, Lythgoe et al. 2007, Morrison et al. 2005). It is also widely accepted that several different VSG genes are expressed at a time (Lythgoe et al. 2007). The persistence of all tags implies two possibilities: either all trypanosomes, regardless of their inserted tag, expressed the same VSG variants (one or several different VSGs) in the same hierarchal order. Since these trypanosomes derive from a clone, all of them might have inherited the same hierarchy for VSG switching. Or that the differently tagged trypanosomes did not share a common hierarchy, which would imply that each sub-population was large enough to generate new VSG variants to prevent its elimination by the immune response. Unfortunately, we could not analyse which VSG variants were expressed by which tagged trypanosomes, since the VSG expression cannot be linked to the different tags.

Our model was established to monitor the diversity of tagged trypanosomes during the life cycle. Deep sequencing revealed the diversity of minor tags underneath dominant ones and yielded highly quantitative data about parasite population dynamics after a bottleneck. This approach is an advance over the use of fluorescently labelled trypanosomes where the number of tags is restricted and dominant tags can easily shroud minor tags when analysis is done by microscopy. We generated a diversity of eight tags that was integrated into one clone. In our model the number of tags can easily be enlarged. The use of a clone has the advantage that selective processes, whenever they emerge, are similar for all tagged trypanosomes. The similar generation time *in vitro* and the dominance of distinct tags in each of the three mouse infections (A, B, and C) demonstrated that the differently tagged trypanosomes were equally fit. This is important to study bottlenecks and allows its characterisation without interference of competition between different genotypes.

Bottlenecks were reported at several sites during the life cycle of trypanosomes (Fig. 3.1). The establishment of the infection in the midgut and the transmission to the mouse during feeding reflect bottlenecks that reduce the population size numerically. We could show that these reductions did not influence the number of different tags nor its relative frequencies.

The parasitaemias in the three mice were never below10^5 ml^{-1} and minor tags co-existed throughout mouse infections. The migration into the salivary glands did influence the tag frequency sometimes very much as discussed and shows therefore different characteristics. This finding might lead to reappraise dynamics of trypanosome populations in the field. We propose that bottlenecks in the life cycle of trypanosomes have only marginal influence on the number of different genotypes but fly transmission can alter their relative frequency.

Materials and methods

***Trypanosoma brucei brucei* AnTat 1.1** (Le Ray et al. 1977) was cloned by the micro-drop method (Brun and Schonenberger 1979). Briefly, the peripheral wells of a 96 well plate (Corning, NY, USA) were filled with sterile water. A drop of 25µl of conditioned medium (50% SDM-79 (Brun and Schonenberger 1979), 25% inactivated foetal bovine serum (FBS, Connectorate, Dietikon, Switzerland), and 25% sterile filtered medium from a one- to two-day old procyclic culture) was carefully placed at the edge of the inner wells. A micro-drop was obtained by touching the bottom of the inner wells with a gold-plated paper clip that was previously dipped into a trypanosome culture (in logarithmic growth phase; with a density of 10^4 trypanosomes ml^{-1}). The micro-drop was examined under an inverted microscope and 25 µl of conditioned medium was added to wells with single trypanosomes. Fresh SDM-79 medium was added to wells where trypanosomes grew successfully. After about two weeks, clones were transferred into culture flasks with larger volumes of medium.
Tsetse flies (see below) were infected with one clone (Fig. 3.2). The infected salivary glands of one fly were dissected and inoculated intraperitoneally into a female NMRI mouse (RCC, Ittingen, Switzerland), which developed a parasitaemia of about 10^8 trypanosomes ml^{-1}. Trypanosomes in the blood were obtained by heart puncture and aliquots with 10% glycerol were cryo-preserved. Bloodstream forms were triggered to differentiate into procyclic forms in SDM-79 medium supplemented with 10% FBS, 3 mM CCA (sodium citrate and cis-aconitate) (Brun and Schönenberger 1981), and 20mM glycerol at 27°C for 3 days (Vassella et al. 2000). Procyclic forms were cultured thereafter in the same medium as mentioned above, but without CCA.

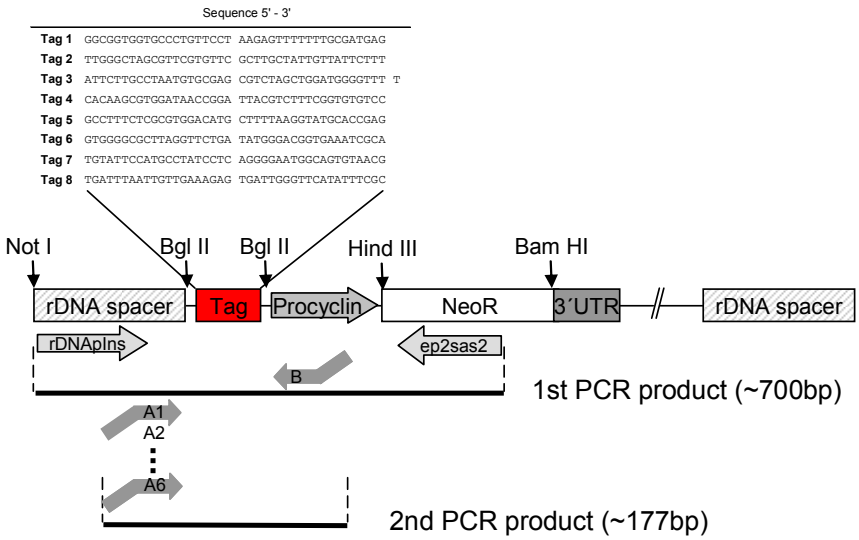

Fig. 3.4: Construction of the plasmid iTag. Eight different 40mers were integrated into the plasmid upstream of the procyclin promoter. Transcription of the neomycin resistance gene (NeoR) is controlled by the procyclin promoter and the last 19 bases of the 3´untranslated region (3´UTR) of EP2. The plasmid integrates into an rDNA spacer of the trypanosome's genome. Nested PCR was performed with the oligonucleotides rDNApIns and ep2sas2 and A1 - A6 and B (see Table 3.1).

The construct. The procyclin promoter of the EP1 gene, the neomycin resistance gene, and 19bp of the procyclin 3'untranslated region (UTR) on the plasmid pKON (Ruepp et al. 1997) was amplified by PCR using the primers Bgl II-promotor (ATAGATCTCGAAAACTCTTC-GGGA) and KO 2 (TATCTAGAGGGCACTGCAGT) (with Bgl II and Xba I sites, underlined). The conditions were: 3 min at 96°C, 30 cycles of 1 min 94°C, 1 min 55°C, and 1:30 min 72°C, followed by 10 min extension at 72°C. The PCR product was digested with Bgl II and Xba I. The plasmid pLew111 (http://tryps.rockefeller.edu) was digested with Bgl II and Nhe I to provide the backbone with the rDNA spacer and ligated with the digested PCR product (Xba I has compatible ends with Nhe I). The newly derived construct pIns has a unique Bgl II restriction site between the rDNA spacer and the procyclin promoter that was used for the insertion of the tag fragment.

An oligonucleotide, containing a variable 40mer flanked by two constant regions with Bgl II sites (ATCACGGCCGGGAGATCT$(N)_{40}$AGATCTGTGAGACCCATTAAGCTTCC) was purchased from Microsynth AG, Balgach, Switzerland. Double stranded DNA was produced by amplification with the constant flanking sequences: iTag-oligo (ATCACGGCCGGGAGATCT) and BIL-4A (GGAAGCTTAATGGGTCTCAC). The

conditions were 3 min at 96°C, 30 cycles of 1 min 94°C, 30 sec 55°C and 30 sec 72°C followed by 10 min extension at 72°C. The PCR product was inserted into pCR®2.1 TOPO® (Invitrogen, Carlsbad Ca, USA) according to the manufacturer's protocol and used to transform *E. coli* XL-1 blue. Purified plasmids were sequenced by the Sanger sequencing method. Eight tags were selected, the 46bp fragments released with Bgl II and ligated into pIns to generate pTag1 to 8 (Fig. 3.4).

Stable transfection was performed with 10µg of each plasmid (pTag1 to 8) linearised with NotI (Fig. 3.4). Each plasmid was electroporated separately into $2.5x10^7$ procyclic trypanosomes. The trypanosomes were resuspended in 450 µl Cytomix (120mM KCl, 0.15mM $CaCl_2$, 10mM K_2HPO_4, 25mM HEPES, 2mM EDTA, 5mM $MgCl_2$). Transfection and cloning by limiting dilution were carried out as described elsewhere (Vassella et al. 2000). G418 (25µg ml^{-1}) was used to select stable transfectants.

Tsetse fly (*G. m. morsitans*) pupae were obtained from the Institute of Zoology, Bratislava, Slovakia. The flies were maintained at 25°C and 70% relative humidity with 12 hours of light per day. Teneral tsetse flies (under the age of 72 hours) were infected with transfected procyclic trypanosomes. Defibrinated horse blood (TCS Bioscience Ldt, Buckingham, UK) was centrifuged, serum removed and washed 3x with PBS (phosphate buffered saline). The washed horse erythrocytes were mixed with heat-inactivated FBS and SDM-79 (in volume ratios of 5: 2: 3). The procyclic forms were mixed with the blood at a final concentration of 10^6 ml^{-1}. Every other day, the flies were fed with complete horse blood through a silicon membrane. Starting twenty days after infection, tsetse flies were examined for the presence of metacyclic forms in their saliva. Tsetse flies with a mature infection were allowed to feed on NMRI mice 2 to 4 days after the appearance of first metacyclic forms. The paired ducts of the salivary glands were extracted from the neck of the tsetse flies. This prevented contamination with midgut forms. The midgut (including the proventriculus) was then dissected out of the abdomen. The tissues were dissected on separate slides in a drop of PBS. The drops as well as the tissues were then transferred into an Eppendorf tube containing lysis buffer (see below).

Mice were kept at 22°C, 70% relative humidity and with 12 hours of light per day. To determine the parasitaemia, 10µl tail blood were mixed with 40µl sodium citrate (3.2%) and 4µl was uniformly distributed under a $20mm^2$ cover slip. The volume in one field under 200 magnification was calculated to be 4nl. For each sample, 10 - 15 fields were counted. The

parasitaemia is given as the number of trypanosomes per ml mouse blood. For the analysis of the tags 50µl of tail blood was processed.

Samples were transferred into an Eppendorf tube containing 200µl lysis buffer (100mM NaCl, 50mM Tris-HCl (pH 8), 5mM EDTA, and 1% SDS) and resuspended with a pipette tip and stored at -20°C. Prior to DNA extraction samples were incubated at 37°C with RNase A (400ng ml^{-1}) and Pronase (40µg ml^{-1}) for one and two hours, respectively.
Genomic DNA was isolated by phenol/chloroform extraction followed by ethanol precipitation and dissolved in H_2O. Extracted DNA derived from the blood was additionally purified with 0.5 starting volumes (sv) ammonium acetate (7.5M) followed by precipitation with 5 sv ethanol.

Nested PCR was carried out with the oligonucleotides rDNAsense/ ep2sas2 (Table 3.1) for the first amplification using the following conditions: 3 min at 96°C, 30 cycles of 1 min 94°C, 1 min 45°C, and 45 sec 72°C, followed by 10 min extension at 72°C. The second PCR performed with the fusion primers A and B, which consist of two regions: a template specific region for PCR amplification and a fusion region for 454 sequencing (Microsynth, Balgach, Switzerland). The primers A1 - A6 can be distinguished by a variable 6mer bar-code that connects the two regions (Table 3.1). This bar-code was used to allocate samples mixed in the same region on the pyro-sequencing plate. The primers A and B yielded products of 177bp. The PCR conditions with the primers A and B were: 3 min at 96°C, 30 cycles of 1 min 94°C, 50 sec 52°C and 30 sec 72°C followed by 10 min extension at 72°C.

The 454 picotiter plate pyro-sequencing was performed by Microsynth, Balgach, Switzerland, with the Roche Genome Sequencer FLX System as described elsewhere (Thomas et al. 2006, Margulies et al. 2005).
Five regions of a picotiter plate were used for this study: one (I) for the control DNA and four regions (II - V) for the samples (Fig. 3.5A). For the control DNA, the eight transfected cultures with the tags 1 - 8 were mixed, DNA extracted, and split into 6 aliquots. DNA from each aliquot was amplified with a different bar-coded fusion primer A1 - A6 together with the primer B (Fig. 3.5B). The samples collected from the flies and mice were amplified with the primers A1/B - A6/B as indicated in Fig. 3.5A. The bar-codes were identified with sfffile (included in the 454 software package), allowing one mismatch.

A

Oligonucleotide used	region I	region II	region III	region IV	region V
A1	control sample A1 1447seq.	FlyA sg 1611 seq.	FlyA midgut 980 seq.	mouseA ID 6 2340 seq.	mouseA ID 14 1167 seq.
A2	control sample A2 1605 seq.	mouseA ID 20 1257 seq.	mouseA ID 27 1514 seq.	mouseA ID 49 1908 seq.	FlyB sg 1266 seq.
A3	control sample A3 1052 seq.	FlyB midgut 993 seq.	mouseB ID10 1035 seq.	mouseB ID 14 1828 seq.	mouseB ID 20 1314 seq.
A4	control sample A4 1214 seq.	mouseB ID 31 1535 seq.	mouseB ID 63 1186 seq.	FlyC sg 540 seq.	FlyC midgut 792 seq.
A5	control sample A5 1125 seq.	mouseC ID 8 1248 seq.	mouseC ID 14 1180 seq.	mouseC ID 18 1033 seq.	mouseC ID 30 1794 seq.
A6	control sample A6 1476 seq.	mouseC ID 67 1366 seq.	FlyD midgut ID12 1070 seq.	FlyE midgut ID10 1635 seq.	
seq. per region	7919	8010	6965	9284	6333
total Sequences					38511

B

B

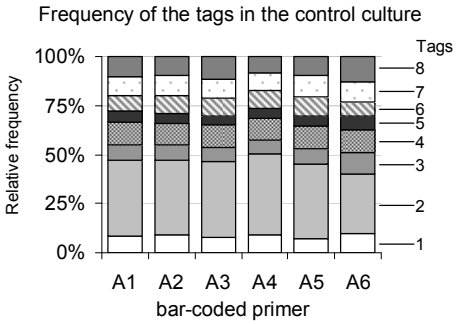

Fig. 3.5: A) Organisation of the samples on the pyro-sequencing plate. Each row represents one region (regions I - V) where six samples were mixed, each amplified with a different primer A1 - A6 and B. Number of sequences that identify the tags is given for each sample (seq.). Six aliquots of the control culture was similarly amplified (region I). B) Each bar shows the relative frequency of the tags of the control culture, amplified with the primers A1/B – A6/B. The tags are displayed on the right and bar-coded primers used for the amplification are given on the x-axis. The distribution of the tags was very similar regardless which bar-coded primer A was used. This control showed that the different bar-codes did not bias the analysis.

Tag identification was initially performed with CLC Workbench where the sequence repertoire of the complete 40mers was used to allocate the tags allowing 20% mismatch. An average of 348 tags per sample could be identified using this program. Reanalysis with MS Excel revealed 38,511 sequences identified with an average of 1330 sequences per sample (ranging from 540 to 2340 sequences). Briefly, the sequences were imported into one row of an Excel file. Each sequence was checked for appropriate length and other criteria (for example, a 3mer and a 4mer in the right position in the conserved sequence region upstream of the tag sequence with the correct distance to each other). Sequences were excluded from the analysis whenever one criterion was not fulfilled. The first five bases were used to allocate the tags. The detection sensitivity was similar for all the samples as we could not find a correlation between the number of different tags detected and the number of sequences

analysed per sample (data not shown). Fig. 3.5A summarises the number of sequences obtained from each sample.

Fig. 3.5B shows the frequencies of tags amplified from the control DNA using the primers A1 – A6. The distribution of the tags was very similar among all control samples (two-sided paired T-test; $p > 0.3$ for all combinations). This demonstrated that the bar-codes did not influence the analysis of the tags.

Oligonucleotide	Sequence, 5' - 3'
rDNApIns	GAGGACCGAATACTAATA
ep2sas2	TATAGATCTGTGAATTTTACTTTTTGGT
Fusion primers	
A 1	*GCCTCCCTCGCGCCATCAG* TACGAGAGAATGTCTTTGGCAACACAC
A 2	*GCCTCCCTCGCGCCATCAG* ATACGTAGAATGTCTTTGGCAACACAC
A 3	*GCCTCCCTCGCGCCATCAG* CGTATCAGAATGTCTTTGGCAACACAC
A 4	*GCCTCCCTCGCGCCATCAG* TCGACAAGAATGTCTTTGGCAACACAC
A 5	*GCCTCCCTCGCGCCATCAG* ACAGTGAGAATGTCTTTGGCAACACAC
A 6	*GCCTCCCTCGCGCCATCAG* CATGACAGAATGTCTTTGGCAACACAC
B	*GCCTTGCCAGCCCGCTCAG* TTCCGTGGGCCCCGACT

Table 3.1: Oligonucleotides used for the amplification of the tags by nested PCR. The primers A1 - A6 and B consist of a fusion region (in italics on the left) and a template specific region (on the right). The variable 6mer barcode of the primers A1 - A6 is underlined. The bar-code allows the allocation of samples in the same region on the pyro-sequencing plate (see Fig. 3.5A).

Acknowledgement

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4. Diversity of trypanosomes during tsetse fly transmission

Michael Oberle[1+2], Line Arni[2], Reto Brun[1] and Isabel Roditi[2]

[1]Swiss Tropical Institute, Socinstrasse 57, 4051 Basel

[2]Institute of Cell Biology, Balzerstrasse 4, 3012 Bern

Abstract

This is a pilot study of the previous chapter 3 (Bottlenecks in the cycle of *T. brucei* through tsetse flies and mice). We were mainly interested in the number of different tags detectable in the salivary glands to test if transmission of an RNAi library in procyclic trypanosomes through tsetse flies is feasible. This would allow us to generate an RNAi library in bloodstream forms. Tagged trypanosomes were produced (40 different tags) and tsetse flies infected. In parallel these trypanosomes were maintained in culture. DNA of tagged trypanosomes from infected midguts and salivary glands or from culture medium were isolated and analysed by sequencing. The results showed that the diversity of tags was not reduced after fly passage but surprisingly after a culture period of 100 days. Even though many tags passed the tsetse fly the transmission of an RNAi library is unfeasible.

Introduction

RNA interference (RNAi) was shown to efficiently knock-down gene expression in trypanosomes (Ngo et al. 1998) and to be a powerful tool to reveal the function of genes (Beverly 2003). So far, RNAi libraries were generated in procyclic trypanosomes (Morris et al. 2002) due to the much higher electroporation efficiency, and RNAi libraries were produced from well adapted laboratory strains. Due to extended period of cultivation these procyclic forms have lost the ability to be transmitted through a tsetse fly and cannot differentiate into other stages. This has two draw-backs: first, this impedes the application of such a library in bloodstream forms. Second, such trypanosomes cannot be used to employ RNAi during differentiation steps like they happen during the fly passage.
An RNAi library was recently established in the tsetse-transmissible trypanosome strain AnTat 1.1 (G. Schumann and I. Roditi, personal communication). These trypanosomes can differentiate what allows to study genes involved e.g. in the differentiation from early to late procyclic forms and would theoretically allow to generate a bloodstream form RNAi library by tsetse fly transmission. Since such an RNAi library contains about 150,000 different inserts we have to know what diversity can be passaged though the fly.
The rate of salivary gland infection is dependent on many factors (Aksoy et al. 2003) and varies consistently between different laboratories. With AnTat 1.1 we consistently obtained

salivary gland infection rates of 15 - 20%. This rate is high compared to other data but the number of trypanosomes that reach the salivary glands is not known and is difficult to estimate. We have seen that the abundances of trypanosomes in the midgut compared to the salivary glands can change (see chapter 3).

This study was done as a pilot experiment prior to the study in chapter 3. We generated 40 different tags that were integrated into the trypanosome genome. The tagged trypanosomes (which derived from a population) were isolated from tsetse midgut and salivary glands and the tags identified by sequencing. When we started the project we were interested to see how many tags could be found in the salivary glands. This should help us to estimate what diversity of an RNAi library could be expected in infected salivary glands and to estimate if its transmission through tsetse is feasible.

Materials and methods

Trypanosomes and flies

Trypanosoma brucei brucei of the pleomorphic strain AnTat 1.1 (Le Ray et al. 1977) was cultured in SDM-79 (Brun and Schonenberger 1979) with 10% FBS and 20mM glycerol (Vassella et al. 2000).

Tsetse fly (*G. m. morsitans*) pupae were obtained from the Institute of Zoology, Bratislava, Slovakia. The flies were maintained at 25°C and 70% relative humidity with 12 hours of light per day. Washed horse erythrocytes (TCS Bioscience Ldt, Buckingham, UK) were mixed with FBS, and SDM-79 (at ratios 5:2:3) and supplemented with procyclic trypanosomes to a final concentration of 10^6 ml^{-1}. Teneral male and female tsetse flies (under the age of 72 hours) were infected. Every other day, the flies were fed with full horse blood through a silicon membrane. The tsetse flies were dissected after 30 days as described in chapter 3.

Construct of the plasmid

The oligonucleotide TrypTAG (Microsynth, Balgach, Switzerland) consisted of a conserved 20mer region that annealed with BIL-4A and a variable 10mer region (Fig. 4.1A). 100ng of TrypTAG, 50ng of BIL-4A, and 50µM of dNTP were incubated in 10µl buffer (20mM Tris-HCl, pH 7.5, 50mM $MgCl_2$, 1mM DTT) and extended by Klenow (2 units) to generate a double-stranded (ds) TrypTAG (Fig. 4.1A). The plasmid pIns (see chapter 3) was digested with Bgl II and overhangs treated with Klenow to generate blunt ends. 10ng of dsTrypTAG

were ligated into 100ng pIns; dsTrypTAG can be inserted in both orientations (with the variable region up- or downstream of the conserved region). The newly generated pTryptag library was cloned in *E. coli* K12 XL-1-blue. To select clones that harboured a tag, colonies were transferred to a nylon membrane (NytranN, Scheicher & Schell, Dassel, Germany) and hybridised with the DIG-labelled oligonucleotide DIG-Bil-4A (Microsynth, Balgach, Switzerland) (see below) (Fig. 4.1B). Positive clones were picked and cultured overnight at 37°C. Plasmids were extracted and digested with Hin dIII, which gave a ~500bp fragment when the dsTrypTAG was integrated, which served as a second confirmation for its insertion (Fig. 4.1B). Plasmids of 40 clones were isolated and purified with the mini-prep kit (Qiagen, Hilden, Germany) and sequenced using the primer rDNApIns. Sequences of the tags and their orientation in the plasmid are given in the Table 4.1.

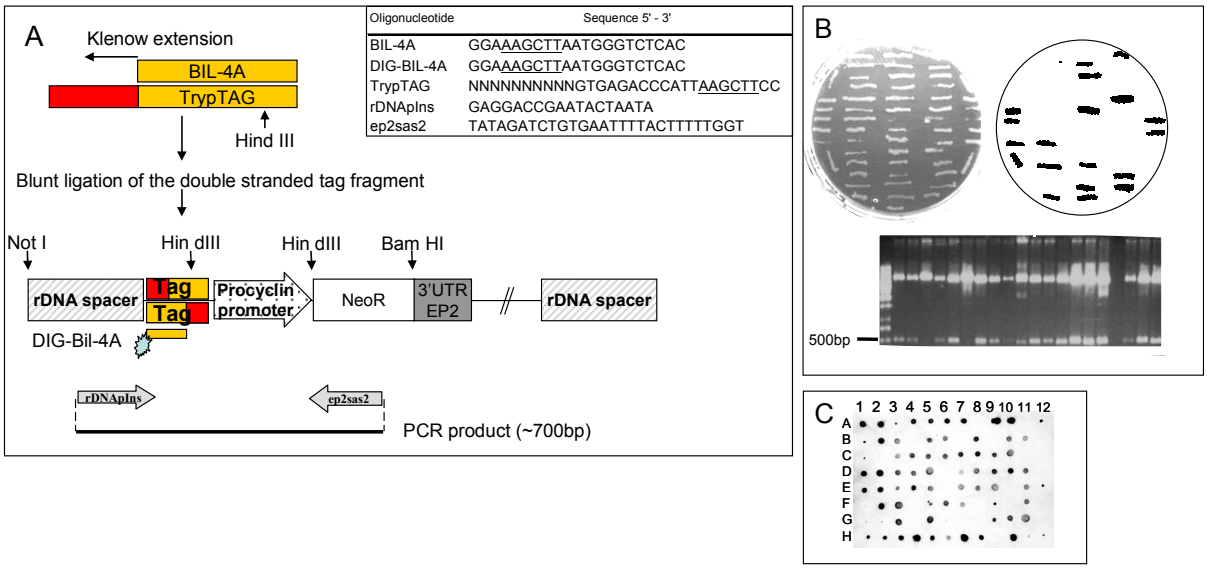


Fig. 4.1: pTryptag construct and the identification of the tag inserts. A) Establishment of dsTrypTAG by Klenow extension and the plasmid pTryptag with the restriction sites. The names of the oligonucleotides and their sequences are given in the integrated table. B) Hybridisation of filter lifts with DIG-BIL-4A. Colonies of pTryptag transfected *E. coli* and the hybridised filter lift showing colonies with the integrated plasmids (upper panel). The 500bp fragment after Hin dIII digestion of the DIG-positive plasmids is shown on the gel (lower panel). C) The 700bp PCR product (see in A) inserted into pCR 2.1 TOPO was transfected into *E. coli* and dot blots of colonies were hybridised with DIG-BIL-4A.

Tag	Bil-4A	tag	A4-liB	#
1	GGAAGCTTAATGGGTCTCAC	CAGGCGGGTT	TGAGACCCATTAAGCTTCC	49
2	GGAAGCTTAATGGGTCTCAC	CCATCAGTGG		30
3	GGAAGCTTAATGGGTCTCAC	CTTGTCTT		28
4	GGAAGCTTAATGGGTCTCAC	ATAAGGCCTG		30
5	GGAAGCTTAATGGGTCTCAC	CCGGTAGTAC		30
6		CTGTCGGTT	GTGAGACCCATTAAGCTTCC	29
7		CTATCTTGTT	GTGAGACCCATTAAGCTTCC	30
8	GGAAGCTTAATGGGTCTCAC	CCCCAGTGAG		30
9		GCCACAGGAT	GTGAGACCCATTAAGCTTCC	30
10		CGGAGCGC	GTGAGACCCATTAAGCTTCC	28
11	GGAAGCTTAATGGGTCTCAC	CTACACAAGA		30
12	GGAAGCTTAATGGGTCTCAC	CATTATCAAG		30
13		CATCATAGCT	GTGAGACCCATTAAGCTTCC	30
14	GGAAGCTTAATGGGTCTCAC	ACACAGCACC		30
15	GGAAGCTTAATGGGTCTCAC	AATTTCGCCC		30
16	GGAAGCTTAATGGGTCTCAC	ACATAAC		27
17		GGAGAGACCC	GTGAGACCCA TTAAGCTTC	30
18		ACTACGTTCG	GTGAGACCCATTAAGCTTCC	30
19	GGAAGCTTAATGGGTCTCAC	CCTTTTCGCC		30
20		A	GTGAGACCCATTAAGCTTCC	21
21		CATATATGAC	GTGAGACCCATTAAGCTTCC	30
22	GGAAGCTTAATGGGTCTCAC	TATGCATTGT		30
23	GGAAGCTTAATGGGTCTCAC	AACCGCTCAT		30
24		CGCATGGGTT	GTGAGACCCATTAAGCTTCC	30
25	GGAAGCTTAATGGGTCTCAC			20
26	GGAAGCTTAATGGGTCTCAC	TCAGAAGGTT		30
27	GGAAGCTTAATGGGTCTCAC	AACAGCATGG		30
28			GTGAGACCCATTAAGCTTCC	20
29		CTCATCTTCA	GTGAGACCCATTAAGCTTCC	30
30		GGCTCAGAAA	GTGAGACCCATTAAGCTTCC	30
31	GGAAGCTTAATGGGTCTCAC	GGACTTCTTT		30
32	GGAAGCTTAATGGGTCTCAC	GCCCCGTAAT		30
33		CCGC	GTGAGACCCATTAAGCTTCC	24
34	GGAAGCTTAATGG			13
35		CCTGGGAAGC	GTGAGACCCATTAAGCTTCC	30
36	GGAAGCTTAATGGGTCTCAC	GCGGCGTCCG		30
37	GGAAGCTTAATGGGTCTCAC	ATTAAGATGG		30
38	GGAAGCTTAATGGGTCTCAC	TCGTGG		26
39		GTTT	GTGAGACCCATTAAGCTTCC	24
40		GGCGTCC	GTGAGACCCATTAAGCTTCC	27

Table 4.1: The sequences and orientation of dsTrypTAG (see Fig. 4.1A). Tag regions contain up to 10 nucleotides.

Transfection of trypanosomes

Bacterial clones with the 40 different tags were mixed and plasmid DNA extracted with the midi-prep kit (Qiagen). 10µg of the pTryptag library was linearised with Not I. Electroporation was performed with 2 x 10^7 procyclic trypanosomes as described elsewhere (Burkhard et al. 2007) Transfected trypanosomes were diluted 1:100 and 1:1000 and wild type trypanosomes (final concentration $5x10^5$/ml) were added as feeder cells. One ml of each dilution was transferred into a 24 well plates. After about 20 hours 15µg ml^{-1} G418 was added to the wells to select transfected trypanosomes. After two weeks trypanosomes from ten wells with good growth were transferred into culture flasks where they grew to higher density. The ten cultures were mixed to obtain the culture at day 0 (Fig. 4.2) with which tsetse flies were

infected as described above. The culture was maintained in parallel to the fly infection experiment and diluted in fresh medium every other day to a cell density of $2x10^6$ ml^{-1}. Two ml of the culture was collected at day 0, and after 30 and 100 days and DNA isolated (described below).

DNA preparation and tag identification

Trypanosomes isolated from the flies (dissected organs) and culture samples were lysed in 100mM NaCl, 50mM Tris-HCl (pH 8), 5mM EDTA, and 1% SDS. Lysis was incubated with RNase and pronase at 37°C for 1 and 2 hours, respectively. DNA was then isolated by phenol/ chloroform (1:1) extraction. The tag region was amplified by PCR with the primer pair rDNApIns/ ep2sas2 as described in chapter 3. The PCR products were inserted into pCR®2.1 TOPO® (Invitrogen, Carlsbad Ca, USA) and cloned. We used 0.2 to 0.3µl of the plasmid solution, which is three to five times less than what is recommended in the manufacturer's protocol, as we realised that the yield of bacterial clones was sufficient for our purposes and that this procedure reduce costs. 50 to 100 white colonies were picked with a toothpick, then dipped into a 2ml Eppendorf tube with LB medium for overnight culture and then dotted on a positively charged nylon membrane (Roche, Mannheim, Germany). The membrane was further processed and hybridised with DIG-BIL-4A (see below). Plasmids from positive clones (Fig. 4.1C) were purified with the miniprep kit using spin columns or 96 well formats (Qiagen) and sequenced with the primer ep2sas2. The sequences were analysed and tags identified by eye.

The tags for the sample of the culture at day 100 were sequenced by pyro-sequencing described in chapter 3. Nested PCR was done with rDNApIns/ ep2sas2 (1^{st} PCR) and A6/ B (2^{nd} PCR). In total, 690 sequences were obtained.

Hybridisation

The membranes of the filter lifts and dot blots were first incubated in denaturation buffer (0.5M NaOH, 1M NaCl) and then in neutralisation buffer (1 M Tris-HCl pH7.5, 3 M NaCl) for 5 min each and then crosslinked by UV (Stratalinker). The membranes were pre-hybridised for 1 – 2 hours at 37°C with DIG easy-Hyb (Roche) and then hybridised for 2 hours at 37°C with DIG-Bil-4A (0.5µg of the 5´ Dig labelled Bil-4A was mixed with 10ml DIG easy-Hyb). Membranes were washed at room temperature 2x 5 min (2xSSC, 0.1% SDS) and at 37°C for 2x 15 min (0.5xSSC, 0.1% SDS). The further steps including the incubation with DIG antibody and CDP-Star was done according to the manufacturer's manual.

Analysis

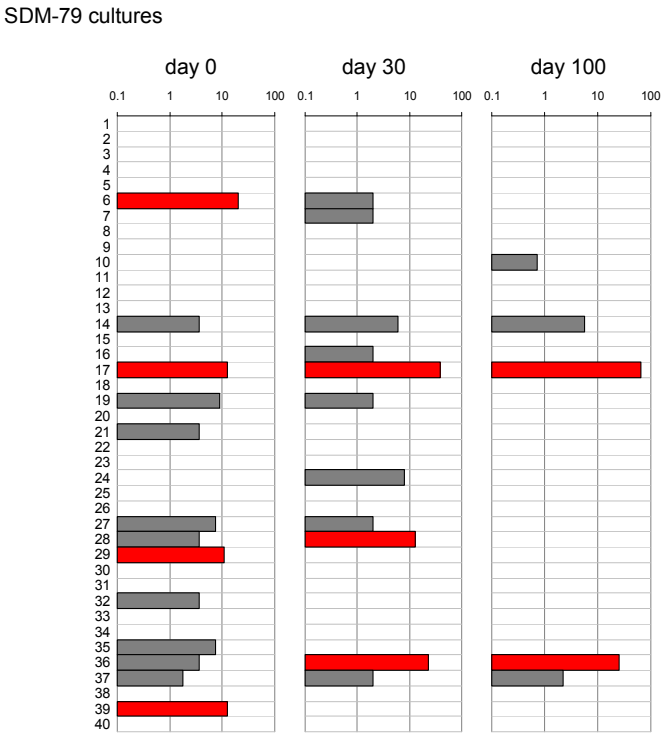
The Shannon index (H_s) is used in ecology to index diversity. H_s was calculated according to the calculation published elsewhere (Shannon 1948). The maximum Shannon index (H_{max}) is the natural logarithms of the total number of species/ tags. In this study H_{max} is 3.69 (with 40 tags).

Results

In vitro

pTryptag transfected procyclic trypanosomes (culture at day 0, Fig. 4.2) was used to infect tsetse flies (see below). The tagged trypanosomes were cultured in SDM-79 without neomycin. Trypanosomes were harvested at day 0 and after 30 and 100 days. From only 55 sequences we found 13 different tags, of which four had a frequency higher than 9% (tags 6, 17, 29, and 39) in the culture at day 0 (Fig. 4.2). The number of tags declined with duration: after 30 days only 11 tags were detected with 52 sequences, and after 100 days only 5 different tags were found, even though 690 sequences were obtained reaching a much higher sensitivity. The tags 14, 17, 36, and 37 were detectable throughout the experiment and showed an increased frequency from day 0 to 100. Some tags (21, 29, 32, and 39) were detectable in the culture at day 0 only but not afterwards whereas other tags (7, 10, 16, and 24) were detectable in the samples at day 30 or 100 only.

This result showed that the frequency of tags was influenced by the length of time in culture. In general, the frequency of a few tags increased over time, whereas the frequency of a larger fraction of tags decreased and, hence, became undetectable. The use of less than 60 sequences to analyse the number of tags and its frequency in the cultures at day 0 and 30 was by far too low, but the analysis clearly identified the most abundant tags and gave an indication of how trypanosome diversity changes in the culture. The use of 690 sequences for the trypanosomes from the culture at day 100 was certainly more appropriate to estimate the diversity and frequency of the tags. In all the cultures together we found 17 different tags with 797 sequences.



total Sequences	55	52	690
Tags	13	11	5
Shannon Index	2.36	1.81	0.91

Fig. 4.2: Tagged trypanosomes sampled from the SDM 79 culture at day 0, 30 and 100. Tags are indicated on the left. Frequencies of the tags are given as percentages. Dominant tags (> 9%) are highlighted in red.

In vivo

All the tsetse flies had a large number of different tags in the midgut as well as in the salivary glands (Fig. 4.3). Nearly 200 sequences were obtained from trypanosomes from the midgut and salivary glands of fly 10. From the two other flies, 42 to 57 tags per sample were analysed. In the fly 10 (with the largest number of sequences) 28 and 29 different tags were detected in the midgut and the glands, respectively. In the flies 22 and 42, 11 - 12 different tags were detected in their midgut and 10 tags in each pair of salivary glands. This showed that the number of different tags was constant in salivary glands and midguts in all tsetse flies and that its number was not reduced during the migration.

The number of sequences influences the number of different tags detected, the larger the number of sequences the more different tags could be found and the higher was the Shannon index. This allows to compare only samples with similar numbers of sequences. The Shannon index of each midgut sample can be compared with the one of the salivary glands (similar

numbers of sequences were done). H_s of the samples isolated from the flies 10 and 22 were similar, but not in the fly 42. In general, we cannot see a strong reduction of the Shannon index in any of the flies examined.

The correlation coefficient (r^2) was calculated for tag frequency and distribution in the midgut and salivary glands of each fly. We found a correlation of $r^2 = 0.73$ in fly 22 but not in the other two flies (Fig. 4.3). This means that dominant tags in the midgut were not necessarily dominant in the salivary glands and showed that tag frequency may change completely during transmission.

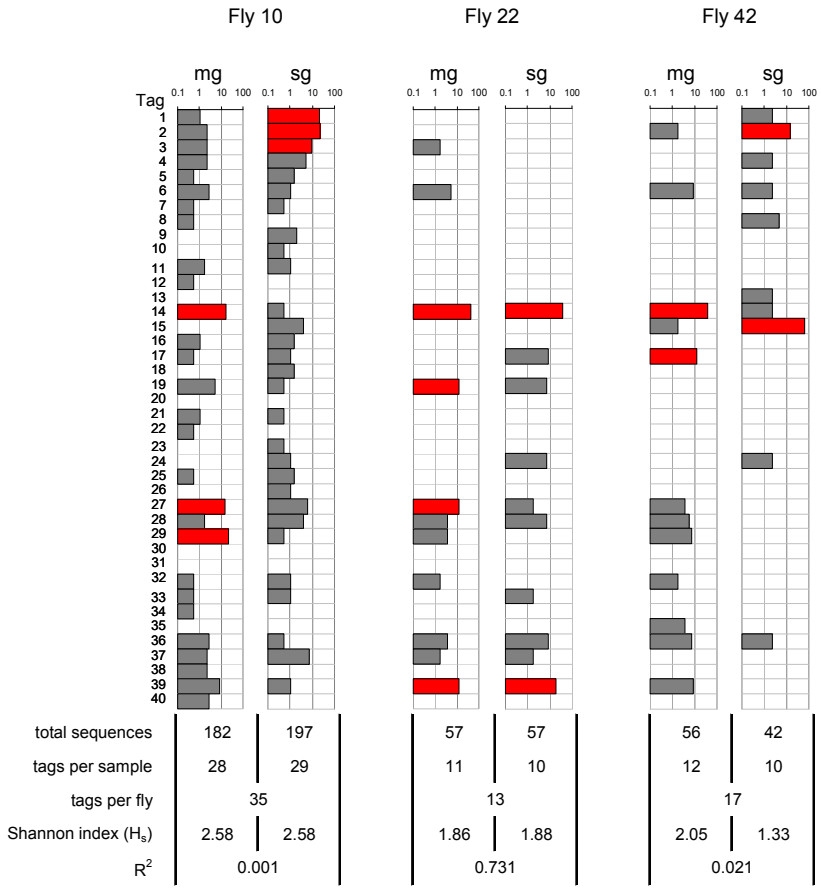


Fig. 4.3: Tagged trypanosomes sampled from the midgut (mg) and salivary glands (sg) of three different flies (fly 10, 22, and 42) 30 days after infection with procyclic trypanosomes from the SDM-79 culture at day 0 (Fig. 4.2). Frequencies of the tags are given as percentages. Dominant tags (> 9%) are highlighted in red.

Discussion

In this study we found a wide variety of different tags in the midgut as well as in the salivary glands. The large number of tags found in the salivary glands revealed that many trypanosomes were able to migrate. This confirms the results from the experiments done in chapter 3. Unfortunately, we cannot estimate the number of trypanosomes that migrate due to profound changes in the relative frequency of some tags between the midgut and salivary glands. Very pragmatically, we can say that that at least 29 trypanosomes migrated (in the salivary glands of fly 10 we found 29 different tags).

We did not discriminate between the two ducts of the salivary glands as was done previously (Gibson et al. 2006, Peacock et al. 2007). Both glands of a fly were pooled for tag analysis. Discrimination between the two salivary glands has no meaning for the purpose of this study as well as for the cyclical transmission in the field.

The number of sequences per sample was far too low. A very simplified model assuming that all 40 tags were equally distributed, calculated a minimum of 250 sequences would be necessary to find all different tags with 95% probability (Fig. 4.4).

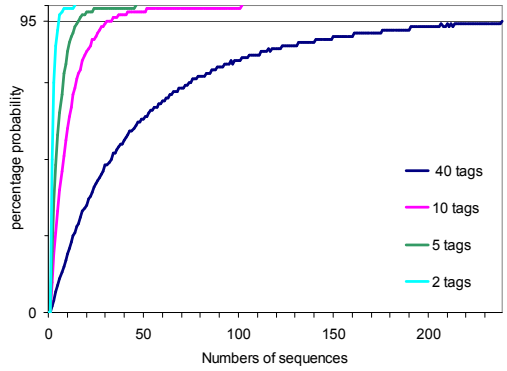


Fig. 4.4: Hypothetical model under the assumption that the tags are distributed equally. It calculates the probability to detect all tags present (y-axis) when assessing a certain number of sequences. For example: with 10 tags less than 50 sequences would be necessary to have a 95% probability to detect all the ten tags. With 40 different tags more than 250 sequences would be needed.

Whether or not we had appropriate number of sequences, we could clearly show that the number of different tags was maintained during the cyclical transmission through the tsetse

fly but declined significantly in the culture from 13 to 5 detectable tags within 100 days ($p > 0.001$, Pearsons chi square). The decline was also reflected by a > 2-fold decrease in the Shannon index (Fig. 4.5). This is an interesting observation and might explain why elongated culture of trypanosomes lead to the loss of tsetse transmissibility.

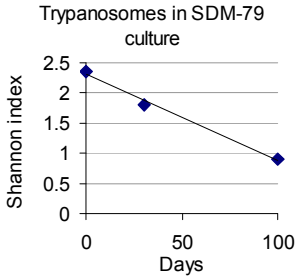


Fig. 4.5: Decline of diversity in cultured procyclic trypanosomes over time

Interestingly, trypanosomes from the culture at day 30 showed a Shannon index and number of different tags similar to what was found in the midgut of the tsetse flies 22 and 42 (all samples do have similar number of sequences). This could either indicate that the conditions in the culture medium and the tsetse fly midgut are similar and that diversity in the midgut would similarly decrease after 100 days like in the culture. The other possibility is that during the midgut and salivary glands infection some unknown mechanisms maintain a higher diversity than in the culture.

We could detect 37 out of the 40 tags in all fly samples and all the samples from the culture, in nearly 1400 sequences analysed. It could be that after electroporation three tags were lost during passage in culture and the culture at day 0 never had more than 37 different tags.

In total, 31 different tags were found in the three salivary glands. This is a recovery rate of nearly 84% of the tags (31 tags of 37), which is high considering that we analysed tags from only three infected salivary glands. The study with eight tags (in chapter 3) revealed a similar high recovery rate (87%) in three infected flies. The abundance of tags changed during migration and led to a very uneven distribution of tag frequencies in the salivary glands. This may impede the recovery of minor tags and make fly transmission to generate a bloodstream form RNAi library completely unsuitable.

Even though the recovery rate of different tags was high, establishing a bloodstream RNAi library by fly transmission of a procyclic RNAi library is unfeasible. A recent study showed a significantly increased transfection efficiency of bloodstream forms, which allows an RNAi library to be generated directly in bloodstream forms (Burkard et al. 2007), which is certainly the strategy to choose.

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5. The innate immune response of tsetse flies: do trypanosomes always elicit attacin and defensin?

Michael Oberle[1+2], Reto Brun[1] and Isabel Roditi[2]

[1]Swiss Tropical Institute, Socinstrasse 57, 4051 Basel

[2]Institute of Cell Biology, Balzerstrasse 4, 3012 Bern

Abstract

High anti-microbial peptide (AMP) transcript level was reported in the tsetse fly after trypanosome infection. In this study we analysed the regulation of the mRNA of the two well described AMPs, attacin and defensin, in flies infected with different trypanosome strains as well as with trypanosomes with deleted or incomplete procyclin coat. RNA was isolated from infected and non-infected tsetse flies and analysed on northern blots. Our results showed that none of the wild type or the mutated trypanosomes increased the transcript of these AMPs in the tsetse flies. Challenging with bacteria resulted in a fast increase of the attacin and defensin mRNA demonstrating that the fly's immune response is functioning. Possibly not all tsetse flies activate the transcripts of these AMPs upon trypanosome infection and maybe their origin plays a role. We conclude that trypanosomes do not necessarily unleash an immune response and that attacin might not play a key role in eliminating the parasites in the midgut.

Introduction

Tsetse flies (*Glossina spp*.) are the vector of the parasite *Trypanosoma brucei* that causes sleeping sickness in humans and nagana in animals. In over 36 sub-Saharan African countries more than 60 Mio people live at risk. Sleeping sickness is fatal if untreated and the drugs available are old and unsafe (Brun and Balmer 2006) leading to frequent treatment failure due to several reasons of which drug resistant trypanosomes might be one of them (Brun et al. 2001). Diagnosis of sleeping sickness is challenging in remote areas due to the examination of cerebrospinal fluid for correct staging of the disease (Simarro et al. 2008). Affected people suffer also from economic loss of up to US$ 4.5 billion annually due to the wasting disease nagana that severely increase mortality in cattle (Kabayo 2002). High rates of treatment failure of sleeping sickness patients, the lack of good diagnostic tools and the absence of new drugs in the near future make strategies to reduce or even block transmission in the vector an alternative approach against African trypanosomiasis (Aksoy et al. 2008).

When a tsetse fly takes a blood meal on an infected mammalian host, bloodstream trypanosomes are transported to the midgut. The pre-adapted short stumpy bloodstream forms are able to differentiate into procyclic forms in the tsetse midgut, which express the stage

specific procyclins. They are suggested to protect the parasite against proteolytic enzymes in the tsetse midgut (Ruepp et al. 1997) or play an important role in the further migration to the salivary glands (Vassella et al. 2009). Procyclins are composed of EP and GPEET, named according to their amino acid repeats at the C-terminus of the peptide (Mowatt and Clayton 1987, 1988, Roditi and Clayton 1999). Both are induced simultaneously (Vassella et al. 2000) and expressed during the initial seven days in the tsetse midgut (when trypanosomes are considered as early procyclic forms), after which GPEET is repressed and EP remains (representing late procyclic forms) (Vassella et al. 2000).

A large number of trypanosomes are killed during the first 3 to 5 days in the midgut (Gibson and Bailey 2003; Van den Abbeele et al. 1999) which leads to the complete elimination of the infection in a large proportion of tsetse flies. A mechanical barrier against trypanosomes is the peritrophic membrane (PM), which trypanosomes have to circumnavigate or penetrate to evade the trypanocidal environment in the midgut lumen (Lehane and Msangi 1991). An important defence against a range of microbial infections provide reactive oxygen species (ROS) produced in the gut epithelium of tsetse that are differentially induce by bacteria and trypanosomes (Hao et al. 2003, 2007, Lehane et al. 2003). Beside the activation of ROS, several genes in the tsetse gut tissue were shown to be induced differentially upon challenge, suggesting pathogen dependent recognition pathways (Lehane et al. 2003). One such gene is tsetseEP; its protein has an extended run of 59 EP repeats at the C-terminus (Chandra et al. 2004) and is up-regulated in bacteria-infected tsetse flies (Haines et al. 2005). TsetseEP can be found in the gut lumen, the haemolymph, and in the fat body (Haines et al. 2005). TsetseEP and procyclic EP co-exist in the gut lumen of infected flies but if and how they interact and if they influence each other is not known.

Some few, but persistent, publications reported the occurrence of trypanosomes in the haemolymph of the tsetse flies (Mshelbwala 1972, Otieno 1973, Kaaya et al. 1986) implying that trypanosomes penetrate the epithelium and infect the salivary glands via the haemolymph. The absence of such reports in recent times (Van den Abbeele et al. 1999, Sharma et al. 2008, Peacock et al. 2007) and the trypanocidal effect of the haemolymph (Croft et al. 1982) led to the wide acceptance that trypanosomes do not cross the gut epithelium and remain in the alimentary channel for their transmission to the salivary glands.

Tsetse flies possess an effective resistance to trypanosome infection where antimicrobial peptides (AMPs) were reported to play a significant role, as it was shown in *G. m. morsitans* (Aksoy, Gibson, and Lehane 2003). Efficient AMP transcription was mainly reported in the

tsetse fly's fat body (Hao et al. 2001) but also in the proventriculus tissue (Hao et al. 2003) after microbial challenge. Bacterial infection resulted in a fast (within hours) up-regulation of attacin and defensin while trypanosome infection showed an unambiguously distinct AMP-activation pattern in the fat body (Hao et al. 2001, Wang et al. 2008). Its up-regulation was mainly seen after infection with procyclic trypanosomes but not with bloodstream forms which led to the hypothesis that differentiation into procyclic forms trigger tsetse fly immune response (Hao et al. 2001).Upon trypanosome infection the level of attacin and defensin mRNA was increased in the fat body after six, 10, and 20 days and was still high in self-cleared flies 10 but not 20 days after infection (Hao et al. 2001). In contrary to that, a suppression subtractive hybridisation study showed no increased level of these mRNA in the fat body in self-cured tsetse flies (Lehane et al. 2008). Also the peptides attacin and cecropin were detected in the haemolymph only 6 days after infection but not later (Boulanger et al. 2002). Low abundance of attacin transcripts in the midgut tissue of trypanosome-infected flies led to the hypothesis that trypanosomes might repress the attacin activation in their proximity (Lehane et al. 2003, Wang et al. 2008). This would make sense since the trypanocidal effect of attacin was shown *in vivo* as well as *in vitro* (Hu and Aksoy 2005).
Immunogenic and non-immunogenic trypanosomes described recently were correlated with the presence or absence of GPEET. GPEET-negative trypanosomes were produced *in vitro* at high cell-density and showed no up-regulated level of attacin and defensin mRNA in infected tsetse flies while GPEET-positive trypanosomes did up-regulate these mRNAs (Hu et al. 2008). A systematic test with confirmed GPEET knock-outs and with different trypanosome stocks is pending.

In this study we analysed the regulation of the attacin and defensin mRNA in flies infected with wild type trypanosomes of different strains and trypanosome with mutated procyclin coats. Our finding revealed that *Glossina m. morsitans* from the colony in Bratislava did not up-regulate the attacin and defensin mRNA upon infection with these trypanosomes. This finding is in strong contradiction with published results (Hao et al. 2001; Aksoy 2003). Here, we raise the question, what role plays the up-regulation of AMPs in eliminating parasites from the midgut.
Furthermore we analysed the mRNA abundance of tsetseEP in non-infected and trypanosome-infected tsetse flies.

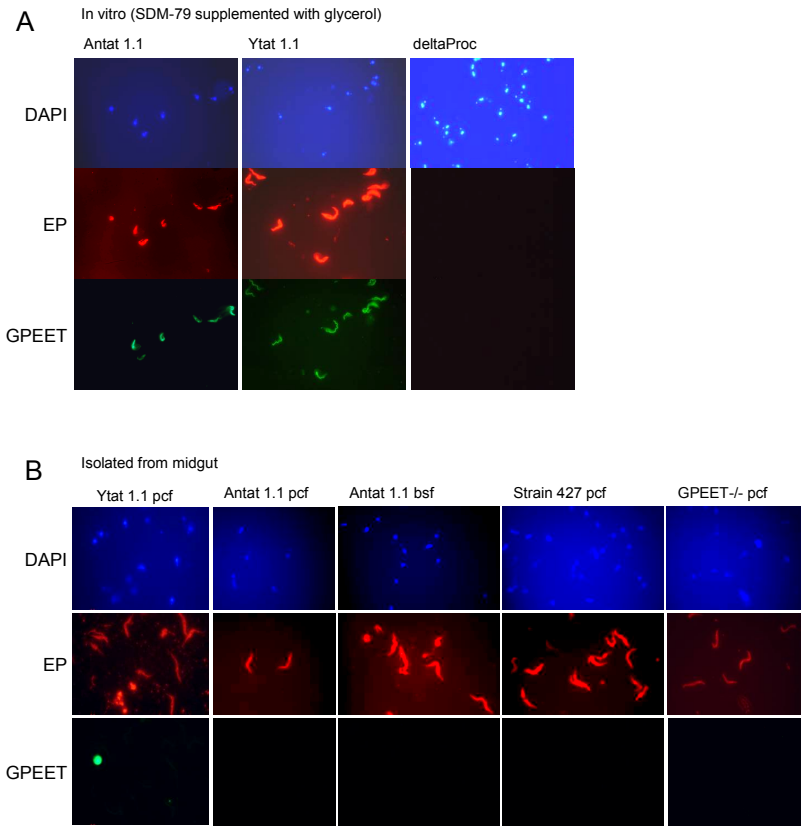

Fig. 5.1: Procyclic trypanosomes stained with anti-EP and anti-GPEET. A) Confirmation of the early state of trypanosomes prior to tsetse fly infection. Trypanosomes derive from the SDM-79 culture supplemented with glycerol. Bloodstream forms of AnTat 1.1 and Δprocyclin were differentiated *in vitro* to procyclic forms (early procyclics). Procyclic *T. b. rhodesiense* Ytat 1.1 (kindly provided by S. Aksoy) was cultured in SDM-79 without glycerol. B) Trypanosomes isolated from midgut after 8 days and later were stained with anti-EP and anti-GPEET. All trypanosomes are late procyclic forms.

Materials and methods

Tsetse flies and trypanosome strains

Glossina morsitans morsitans (from the colony of the Institute of Zoology, Bratislava, Slovakia) was used for all the fly infection studies. The flies were kept at 25°C and a relative humidity of 75-80% and received defibrinated horse blood (TCS Bioscience Ldt, Buckingham, UK) every other day.

The following trypanosome stocks were used: *Trypanosoma brucei brucei* AnTat 1.1 (Le Ray et al. 1977), STIB 247 (Geigy et al. 1975), the ‘eternal juvenile’ (I. Roditi, personal

communication) procyclic strain 427 (Cross and Manning 1973), the GPEET knock-out transgenic Antat 1.1 (= GPEET-/-), the AnTat 1.1 procyclin null-mutant (Δprocyclin, see chapter 6) , and the procyclic *T .b. rhodesiense* Ytat 1.1 strain (kindly provided by S. Aksoy). According to the experimental setup procyclic culture forms (pcf) or bloodstream forms (bsf) were used to infect teneral flies (10^6 trypanosomes ml^{-1} blood). Since it was reported that tsetse flies might not up-regulate mRNA of attacin and defensin when GPEET is not present on the surface (Hu et al., 2008), early procyclics were used for the infection. The trypanosomes were differentiated from bloodstream to early procyclic forms in SDM-79 supplemented with 3 mM CCA (sodium citrate and cis-aconitate) (Brun and Schonenberger 1979) and 20mM glycerol (Vassella et al. 2000). The expression of EP and GPEET on the surface was confirmed by fluorescent staining (Fig. 5.1A). Procyclic forms were fixed with acetone (-20°C) for 10min. Anti-GPEET antiserum (K1, rabbit) and anti-EP antiserum (monoclonal antibody anti-247 EP) were used at a dilution of 1:500. The secondary antibody, Alexa 488-conjugated anti-rabbit (Molecular Probes) was used at 1:1000 and TRITC-conjugated anti-mouse (Sigma) was used at 1:500.

For the infection with procyclic forms a mixture of washed horse erythrocytes (3x with phosphate buffered saline (PBS)), inactivated foetal bovine serum (iFBS, Connectorate, Dietikon, Switzerland), and SDM-79 (at volume ratios of 5:2:3) was used. For the infection with bloodstream forms cryopreserved stabilates of trypanosomes grown in NMRI mice (RCC, Ittingen, Switzerland) were thawed at 37°C and mixed with full horse blood. The tsetse flies were infected/ fed on an artificial membrane with 20ml blood/ 100 flies (Moloo 1971, Langley and Maly 1969).

Experiments/ Immune challenge

Immune stimulation was performed with *E. coli* K12 XL-1 blue (OD_{600} 0.6) by microinjection into the pteropleuron of the fly's thorax described elsewhere (Hao et al. 2001, Boulanger et al. 2002). Fat bodies of 5 flies were dissected into a drop of PBS after 24 hours and pooled. Total RNA of bacteria challenged flies, that showed up-regulated attacin and defensin mRNA, was used as positive controls (shown in Fig. 5.2, 5.3, and 5.6 indicated by 'E.coli').

Tsetse flies without challenge served as negative controls (control), which was carried along in every single experiment. Their first blood meal was either washed erythrocytes supplemented with SDM-79 and iFBS or full horse blood dependent of the experiment.

For dissection, flies were chilled on ice, abdomen was opened with tweezers, and a fragment of the midgut was prepared on a glass slide to determine the infectious status under the microscope. Infected flies were taken for RNA extraction.
In the experiments described in the Fig. 5.3 to 5.5, pooled fat bodies of 5 to 8 flies were analysed. *Per os* infected flies with *E. coli* (O.D. 600 = 0.07), pcf of AnTat 1.1, and with a mixture of the two were dissected 15 and 40 hours later. RNA was also extracted from pooled midguts. Groups of flies were infected with pcf of Antat 1.1, GPEET-/-, and strain 427, as well as the bloodstream form (bsf) of Antat 1.1 and STIB 247. Flies were dissected 6, 24, 54, and 100 hours or 2, 4, 7, and 12 days after infection according to the experiments demonstrated in Fig. 5.4 and Fig. 5.5. Two tailed paired T-test was employed to compare transcript abundance within groups (control, pcf AnTat 1.1 and GPEET-/-) as well as in-between groups for each dissection time point (Fig. 5.4).
For the experiment described in Fig. 5.6 pcf of AnTat 1.1, Δprocyclin and Ytat 1.1 were used for infection. Whole fly or the whole abdomen including the dissected midgut of 1 or 2 flies was used for RNA extraction.

RNA extraction and electrophoresis

Total RNA was extracted with Trizol® (Invitrogen, Carlsbad Ca, USA) as described in the manufacturer's protocol. 6µg and 15µg RNA of the pool of fat bodies or the whole fly abdomen extract, respectively, was loaded on a 1.5% TBE/ guanidine thiocyanate gel (Kyes et al. 2000). Electrophoresis was done at 90V (2-3h) and gel soaked in 10mM NaOH for 10 to 30 min. The RNA was blotted to a Hybond N+ membrane (Amersham) with 10xSSC and 10mM NaOH over night and stabilised by UV-cross linking (Stratalinker).

DIG labelled probes

The antimicrobial peptide genes attacin and defensin as well as the housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene were amplified from tsetse cDNA (kindly provided by S. Aksoy) using the following pimers:
- defensin-f ACACTATGTGCTGTTGTC, defensin-r GTGCAATAGCATACACCAC
- attacin-f CGTCGTTCTAGTGAAAGGAC, attacin-r GTTTGACCATCAAACGGACC
- GAPDH-f TAGATTGGACTGTGCGCTTG, GAPDH-r AAATGGGTGGATGGTGAGAG.
The PCR products were cloned in pCR®2.1 TOPO® (Invitrogen, Carlsbad Ca, USA) and verified by sequencing (data not shown). DIG labelled DNA probes were produced by DIG-PCR (Roche, Mannheim, Germany) using M13r/ M13f primers.

The DIG labelled probes for tsetseEP was produced by DIG-PCR of the plasmid pZL1-GmmEP-11-3p (Chandra et al. 2004) using the primers GmmEP-s1 (GGGTCAAAATGCAA-GAG) and GmmEP-as3 (AACGTTAGTAGTCAGAAAC). DIG labelled tsetseEP probe hybridised specifically to tsetseEP mRNA but not to early and late procyclic trypanosome mRNA as shown by Chandra et al. (2004) and by own data (Fig. 5.7).

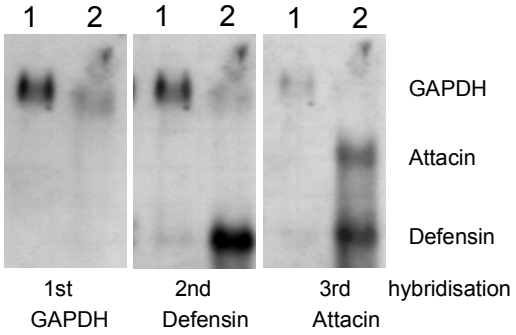

Fig. 5.2: Serial hybridisation of GAPDH (1st), defensin (2nd), and attacin (3rd) probes. RNA was extracted from fat bodies of self-cured flies 10 days after infection with pcf AnTat 1.1 (1) and flies 24h after injection with *E. coli* (2).

Hybridisation

The blots were pre-hybridised with DIG easy-Hyb solution for 2 hours and hybridised with 100 to 500 ng of each DIG labelled probe (GAPDH, attacin, and defensin) over night at 42°C. Low stringent wash 2x10min (2xSSC, 0.1%SDS) was at room temperature and high stringent wash 2x15min (0.1SSC, 0.1%SDS) at 68°C. The following washing and detection steps were done according to the manufacturer's manual (DIG hybridisation protocol).
The DIG labelled attacin, defensin, and GAPDH probes recognise bands at different sizes. The specific hybridisation was confirmed by serial hybridisation of one probe after the other on the same blot (Fig. 5.2). This allowed us to hybridise all three probes in one step. The signals on the membrane were analysed with AIDA Image Analyzer. The mRNA abundance of GAPDH was set to one in each sample and used to normalise the attacin and defensin transcripts.

Results

Mixed infection

Three groups of tsetse flies were infected *per os* with procyclic trypanosomes, *E. coli*, and a mixture of both (*E. coli*/ trypanosomes) (Fig. 5.3). A control group was fed with uninfected blood. The fat bodies of the flies were dissected 15 and 40 hours after infection. The flies infected with *E. coli* and with the mixture showed increased level of the attacin and defensin mRNA after 15, and especially after 40 hours compared to the control.

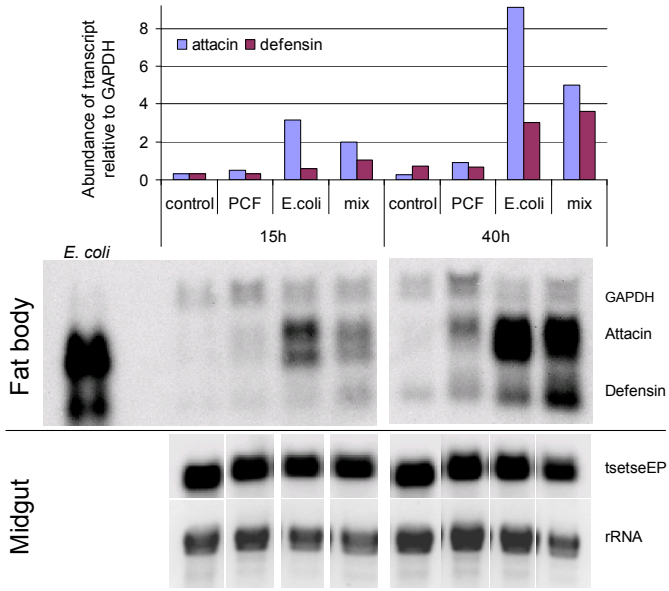

Fig. 5.3: Teneral tsetse flies were infected *per os* with AnTat 1.1 procyclic culture forms (PCF), *E. coli* or a mixture of both (mix). Control flies were fed on uninfected blood. Fat body and midgut was dissected 15 and 40 hours after feeding, RNA isolated and loaded on two different gels. RNA from fat body were hybridised with DIG labelled GAPDH, attacin and defensin probes. GAPDH was used for normalisation (GAPDH mRNA abundance is 1 for each sample). The RNA from the midguts was hybridised with tsetseEP probe. Ethidium bromide stained rRNA show the loading.

The results shows that flies can up-regulate AMP transcripts upon bacteria challenge *per os* in the fat body as shown elsewhere (Hao et al. 2001; Nayduch and Aksoy 2007; Hao et al. 2003; Hao and Aksoy 2002; Wang et al. 2008; Boulanger et al. 2002). Transcript abundance in the fat body of trypanosome infected flies was as low as in the control flies after 15 and 40 hours. The attacin mRNA level of the mixed infected flies was nearly two fold reduced compared to

the bacterial infected flies, which might indicate an immune suppressive effect of the trypanosomes.

Procyclic form infection

Procyclic trypanosomes were reported to unleash an immune response 3 to 6 days after infection but not bloodstream forms (Hao et al. 2001, Hu and Aksoy 2006) or GPEET-negative trypanosomes (Hu et al. 2008). Tsetse flies were infected with procyclic culture forms (pcf) of AnTat 1.1, GPEET-/-, and strain 427, which is known to express high amount of GPEET. An additional group was infected with AnTat 1.1 bloodstream forms (bsf). Control flies fed on non-infected blood (control) were conducted with all the infected groups.

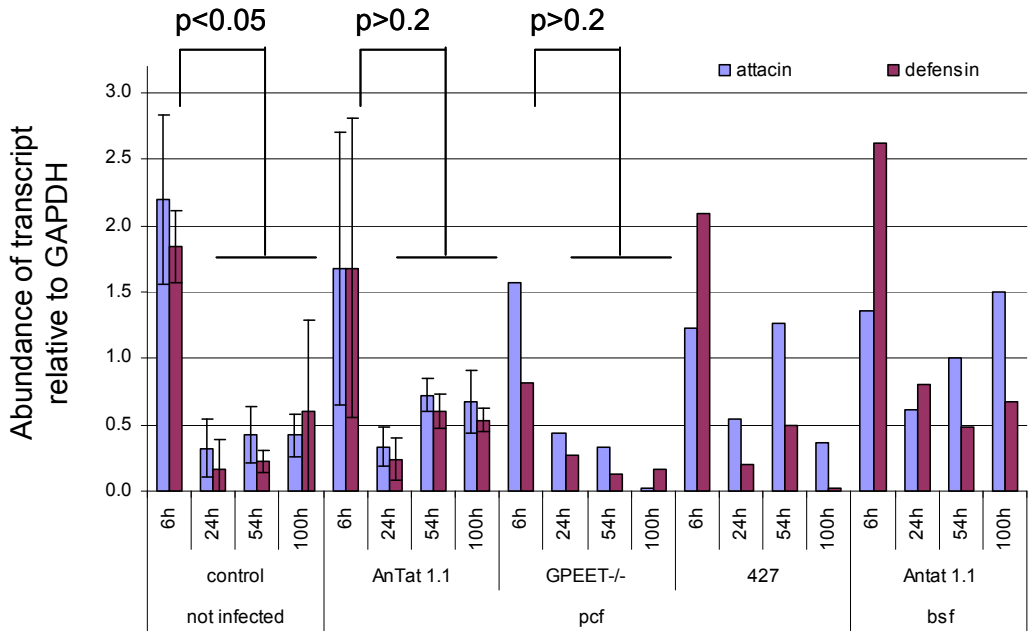

Fig. 5.4: Teneral tsetse flies were infected with procyclic forms of AnTat 1.1, GPEET-/-, and strain 427 as well with bloodstream forms (bsf) of AnTat1.1. Control group (unchallenged flies) and infected groups were dissected 6, 24, 54, and 100 hours after infection. Standard deviation is given for control and AnTat 1.1 (pcf) where 3 different experiments were conducted. With GPEET-/- infected flies two experiments were conducted. Attacin and defensin transcription levels were compared within and in-between groups using Students T-test, smallest p-values resulted between 6 hours and the remaining time points (indicated in the figure).

Three independent experiments were carried out. In all of them the control and AnTat 1.1 pcf infected group was included, in two of them GPEET-/- infected flies were present and in one experiment strain 427 pcf and AnTat 1.1 bloodstream forms infected flies were included. The fat bodies of the flies were dissected 6, 24, 54, and 100 hours post infection. The mRNA expression levels of the infected tsetse flies and the control flies from these experiments are summarized in Fig. 5.4.

Two main outcomes can be described: first, we could not see a trypanosome infection specific increase of the transcript levels of defensin and attacin in any group. For each dissection day, T-tests showed no significant difference of the attacin and defensin mRNA level in the control group compared to the infected groups ($p > 0.1$). Second, the mRNA levels of defensin and attacin in the flies dissected 6 hours after the infection/ first blood meal compared to the later time points were significantly increased in the control flies ($p < 0.05$) but not in infected flies ($p < 0.2$) with the procyclic forms AnTat 1.1 and GPEET-/- (Fig. 5.4). In the remaining groups (strain 427pcf and AnTat 1.1 bsf), transcript levels were similarly remarkably increased 6 hours after infection. The result shows that the initial blood meal leads to an increased immune response in all groups even in the non-infected control group.

Bloodstream form infection

The natural route of infection is when tsetse flies feed on an infected animal and take up bloodstream trypanosomes which then have to adapt to the midgut environment. Two strains of bloodstream forms (AnTat 1.1 and STIB 247) were used to infect tsetse flies. The dissection schedule was changed in order to extend the exposure time to 2, 4, 7, and 12 days after infection (Fig 5.5). Compared to the control group, the two infected groups showed no increased attacin and defensin mRNA levels. Contrary to Hao's hypothesis (Hao et al. 2001), that trypanosomes elicit an immune response when they differentiate into procyclic forms, our results show no up-regulation at any time of dissection, even though trypanosomes differentiated into procyclic forms in the midgut (Fig. 5.1B).

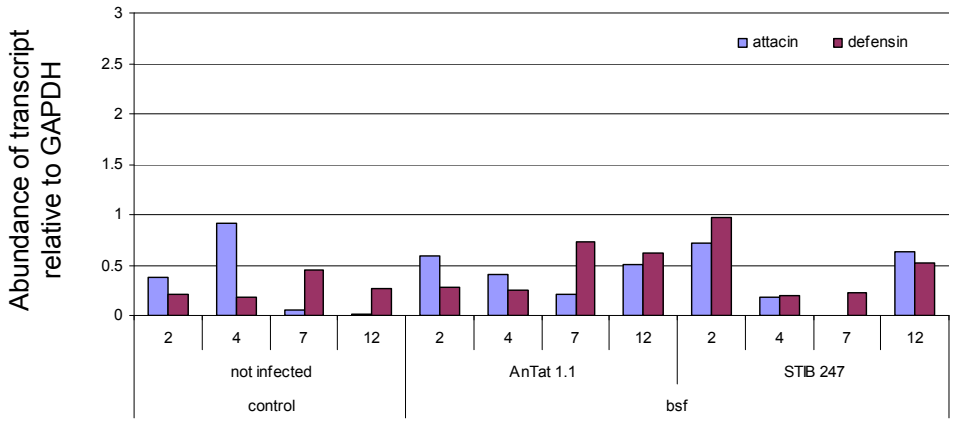

Fig. 5.5: Teneral tsetse flies infected with bloodstream forms (bsf) of AnTat 1.1 and STIB 247 were dissected 2, 4, 7, and 12 days after infection. Same y-axis scale was used as in Fig. 5.4.

All the experiments done so far revealed no trypanosome specific up-regulation of attacin and defensin mRNA and our results showed that either the trypanosome strains we used are non-immunogenic or our flies do not respond to a trypanosome infection. In a next experiment we used Ytat 1.1 that was shown to unleash an immune response (Hao et al. 2001). Additionally, Δprocyclin was used to infect tsetse flies. The procyclin null-mutant Δprocyclin was shown to successfully infect the midgut and was even able to infect the salivary glands but to a very low rate and was overgrown by wild type trypanosomes when co-infected (Vassella et al. 2009, see chapter 6, page 91). This showed that procyclins might be important for the competition with different strains under natural conditions and for the efficient colonisation of the salivary glands.

The presence of EP and GPEET on the surface of AnTat 1.1 and Ytat 1.1 confirmed the state of early procyclic forms (Fig. 5.1A). Δprocyclin was differentiated from bloodstream to procyclic forms to yield early procyclic trypanosomes and cultivated in SDM-79 supplemented with glycerol (Vassella et al. 2000). Flies were infected with these trypanosomes and dissected after day 4, 7, 12, and 18 (Fig. 5.6A). In all groups the attacin and defensin mRNA levels was comparable to the one of the control group. We could confirm this result in two further independent experiments for days 2, 4, and 7 (data not shown).

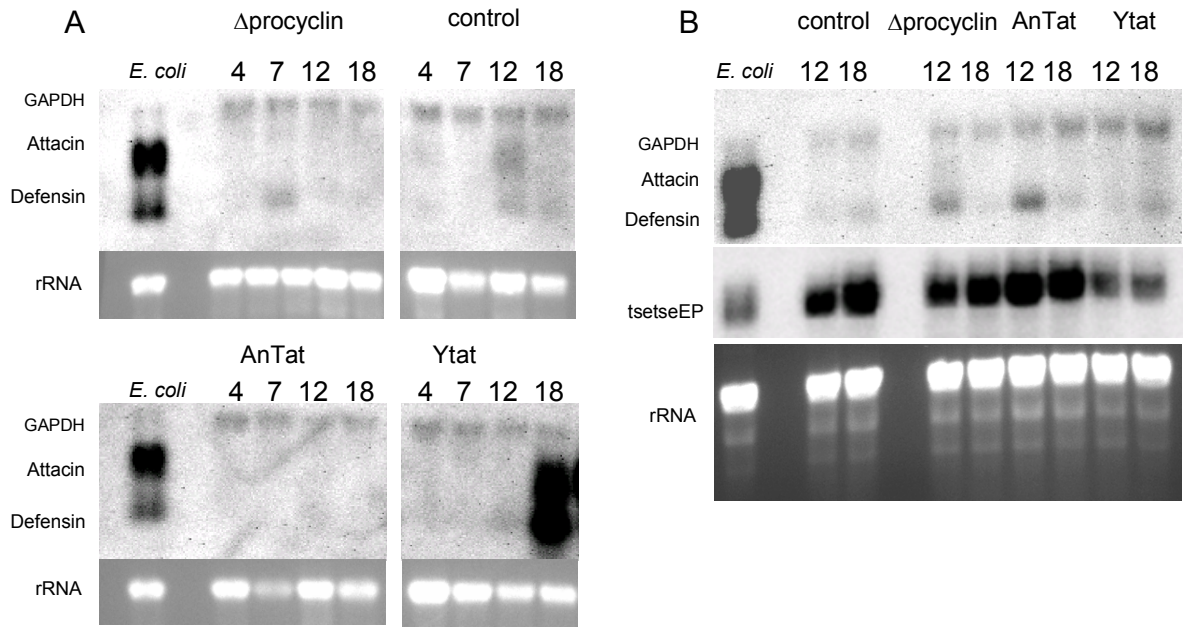

Fig. 5.6: Teneral tsetse flies infected with the procyclic forms AnTat 1.1, Δprocyclin and Ytat 1.1. Control flies received uninfected blood. A) RNA was isolated from the abdomen of flies from the first batch after 4, 7, 12, and 18 days. GAPDH and rRNA are given for loading control. B) The RNA from days 12 and 18 of the second batch of flies was used for Northern blot. The blot was first hybridised with GmmEP and after stripping with GAPDH, attacin, and defensin DIG probe.

The sample of the Ytat 1.1 infected group dissected at day 18 showed an increased up-regulation of attacin and defensin. In this experiment we used the whole abdomen of one or two flies. Blood meal, plate, and silicon membrane were kept as sterile as possible. However, an activation of the immune response due to an experiment-unrelated event can not be completely excluded. Co-infection of flies with an unknown microbe that elicit an immune response might be problematic in studies on insect immune regulation. A replicate of the samples from the days 12 and 18 was analysed (Fig. 5.6B). The attacin and defensin mRNA abundance of the Ytat 1.1 infected fly at day 18 could not been confirmed even though the flies derived from the same experiment. The result of the second analysis confirms that attacin and defensin were not increased due to a trypanosome infection. We could not see a trypanosome infection-specific up-regulation of these mRNAs with any trypanosome strain at any time of dissection.

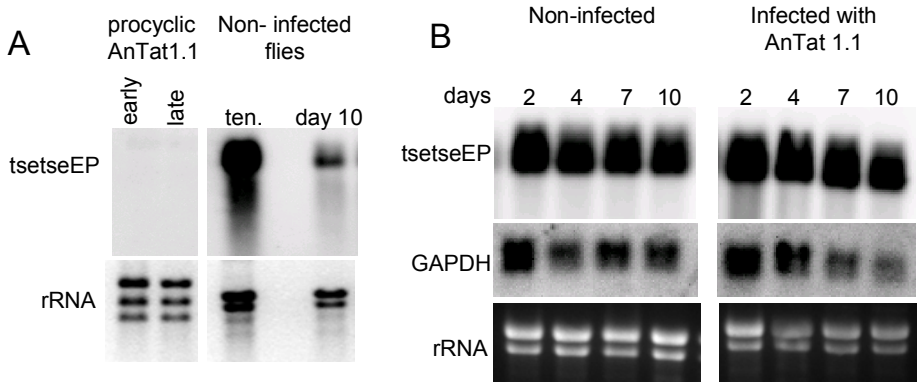

Fig. 5.7: Northern blot analysis of TsetseEP. A) Total RNA from early and late procyclic AnTat 1.1, and from teneral tsetse flies (ten.) were hybridised. Ethidium bromide stained rRNA were loading controls. B) Total RNA of non-infected and AnTat 1.1 bloodstream infected tsetse flies were extracted 2, 4, 7, and 12 days after the first blood meal/ the infective feed. Ethidium bromide stained rRNA and DIG hybridisation of GAPDH were used as RNA loading controls.

TsetseEP

Teneral *G. m. morsitans* showed high tsetseEP mRNA abundance which confirms the results of Chandra et al. (2004) (Fig. 5.7A). TsetseEP transcript was shown to be continuously expressed during a period of 10 days in un-challenged tsetse flies but showed a weak increase in trypanosome infected flies (Fig. 5.7B). The regulation of the tsetseEP mRNA in flies 12 and 18 days (Fig. 5.6B) as well 15 and 40 hours (Fig. 5.3, 'midgut') after infection showed not a clear pattern since the control groups demonstrated similar mRNA abundance like in the treated groups. One exception revealed the Ytat 1.1 infected flies that show a lower tsetseEP activation compared to the control flies.

Discussion

Our results show that trypanosome infection does not up-regulate attacin and defensin in the tsetse flies, none of the wild type or the knock-out strains tested showed a specific activation of these transcripts. We could show that their mRNAs were similarly unaffected upon infection with bloodstream or procyclic forms. These results are in disagreement with a

number of publications that reported an up-regulation of attacin and defensin after trypanosome infection (Hu et al. 2008, Aksoy et al. 2008, Hao et al. 2001). In the most prominent publication on tsetse innate immunity, Hao et al. (2001) showed that the transcripts of these AMPs are activated after infection with procyclic trypanosomes. The up-regulation of attacin and defensin was apparent after six days and detectable for 20 days in infected flies. Interestingly, self-cured tsetse flies showed an increased mRNA level still ten days after infection (Hao et al 2001). The up-regulation of these AMP transcripts could not always be confirmed by others. A cDNA subtraction study of transcripts isolated from the fat bodies of non-infected and self-cured tsetse flies showed no increased mRNA level of attacin and defensin eight to ten days after infection (Lehane et al. 2008). Also attacin peptides were detectable only in the initial phase of the infection but not later, even though the flies were confirmed to be infected with trypanosomes (Boulanger et al. 2002).

Challenging flies with bacteria by micro-injection resulted in a very strong and fast up-regulation of attacin and defensin mRNA. The hybridisation method employed, gave a clear signal showing that the DIG labelled probes detected the right transcripts. The fast and strong up-regulation a few hours after challenge confirms published data (Wang et al. 2008, Hu et al. 2008, Hu and Aksoy 2006, Hao et al. 2001). *Per os* infection of *E. coli* did also show a fast up-regulation of attacin and defensin transcripts and demonstrated that signalling between midgut and fat body is functioning, as shown by others (Hao et al. 2001, 2003, Wang et al. 2008). However, it is unknown how the ingested bacteria in the midgut can elicit an immune response in the fat body. Diffusible cytokine-like molecules or reactive oxygen species (ROS) produced in the midgut or proventriculus epithelium might induce a systemic immune response in the fat body (Hao et al. 2001). This crosstalk was also observed in immune challenged Drosophila where the mRNA levels of AMP's are up-regulated in the fat body within a few hours (Lemaitre et al. 1997).

We showed that transcript levels of attacin and defensin were mainly increased after the first blood meal whether or not tsetse flies were infected. It appears that the first blood meal leads to the up-regulation of these AMP transcripts as it was shown elsewhere (Nayduch and Aksoy 2007). This phenomenon, oral tolerance, is very common and is well described in other animals (Bauer et al. 2006). The up-regulation of attacin and defensin mRNA was seen only after the first blood meal but not after later blood meals and was significant in the non-infected flies only but not in infected flies (Fig. 5.4). When the transcript abundance of the

time point of 24 to 100 hours after infection/ feeding were combined and tested against the ones after 6 hours we calculated a 10-fold higher significance for the control flies ($p < 0.001$) than for the infected flies ($p < 0.04$). It can be hypothesised that the presence of trypanosome leads to a decreased AMP transcript activation after the first blood meal. This could explain why the mRNA level of attacin was lower in flies challenged with a bacteria/ trypanosome mixture compared to flies infected with bacteria only (Fig. 5.3). Down-regulation of attacin was also described in tsetse midgut tissue but not in the fat body ten days after trypanosome infection (Wang et al. 2008) and no up-regulation of AMPs either was found in midgut tissue of infected tsetse flies (Lehane et al. 2003). Proventriculus tissue did express AMPs after challenge with bacteria but not with trypanosomes (Hao et al. 2003). This suggests that trypanosomes might repress the activation of attacin in their proximity (Wang et al. 2008) which would make sense since attacins were reported to be trypanocidal (Hu and Aksoy 2005). A possible down-regulation of AMP transcript due to trypanosomes was reported only in midgut tissue up to now. We collected fat body and showed that AMP-transcripts may also be down-regulated in a more distant tissue when trypanosomes are in the midgut. Might trypanosomes in the midgut reduce the AMP activation in the fat body in some flies? Fly experiments showed that knocking-down of attacin by RNAi in infected tsetse flies led to an increased infection rate (Hu and Aksoy 2006). It seems that trypanosomes are susceptible to attacin and hence would or should have developed strategies to evade either the production of or the interaction with attacin. Up to now a possible immune suppressive effect of trypanosomes on the tsetse fly's immune system was inadequately discussed.

Due to the absence of a tsetse immune response after trypanosome challenge in our study we were unable to test two recently published hypotheses. Hao et al. (2001) could see an immune response when flies were infected with procyclic forms but not with bloodstream forms and hypothesised that attacin and defensin are activated when trypanosomes differentiate into procyclic forms. The authors used distinct trypanosome strains: for the infection with bloodstream forms, *T. b. brucei* RUMP 503, which can infect the salivary glands, was used and for infection with procyclic forms, *T. b. rhodesiense* strain YTat1.1 that can not infect the glands, was used. The different recognition pattern of the tsetse immune system might be strain rather than stage specific; surprisingly they never tested their hypothesis with RUMP 503. We conducted several experiments with bloodstream trypanosomes in order to see an up-regulation of the fly's immune system when trypanosomes start to express procyclins. Because the transcripts of the two AMPs were not up-regulated in tsetse flies infected with

procyclic forms in our study, we could not test this hypothesis. The second hypothesis was made by Hu et al. (2008) who detected that GPEET-negative trypanosomes did not elicit an immune response (non-immunogenic) whereas GPEET-positive trypanosomes were immunogenic. The authors used trypanosomes that down-regulated GPEET in a dense culture. In the midgut, GPEET is completely down-regulated at latest eight days after infection (Fig. 5.1B) but tsetse flies showed an up-regulated attacin and defensin transcript level six days after infection only (Hao et al. 2001). If GPEET would elicit an immune response, it rather surprises that these AMP transcripts are activated when GPEET is being down-regulated. We infected flies with GPEET-/- and Δprocyclin, where the genes are knocked-out. AMP transcript abundance with wild type trypanosomes were similarly low as with the knock-out mutants, which did not allow to test if the presence or absence of GPEET correlates with immunogenicity.

Trypanosomes in the midgut do not influence survival of tsetse flies. In different studies the survival of the infected tsetse flies was reduced after 30 to 60 days compared to non-infected flies (Nitcheman 1988, Maudlin et al. 1998). After this time trypanosomes might not only be restricted to the midgut anymore and the development of metacyclic trypanosomes in the salivary glands could be responsible for the reduced survival of the flies. The reduction of survival was insignificant but might underestimate the real situation since many flies may have cleared the infection. The impact of trypanosome infection on tsetse fly survival is difficult to measure and only a few publications are available. This is different in mosquito-*Plasmodium* interaction where more work was done, but the influence of an infection on the survival is not clear either. This is surprising since *Plasmodium*, unlike trypanosomes, penetrates the gut epithelium, damage tissues, and invades the haemolymph and could hence directly influence the fitness of the mosquitoes. It seems, however, to be very difficult to test how *Plasmodium* influences mosquito's fitness under experimental conditions (Ferguson and Read 2002).

Trypanosomes are restricted to gut and salivary glands and influence the survival rate of tsetse flies only moderately. Furthermore, tsetse flies in the field are only rarely infected with *Trypanosoma brucei* as only about 0.1% have infected salivary glands, even though the prevalence in host animals is higher (Mattioli et al. 1990, Njiokou et al. 2004), suggesting tsetse flies take up frequently infected blood. The resistance to trypanosomes in the tsetse midgut might be based on two lines. The first line includes general defences such as proteolytic enzymes, the PM, and ROS leading to the elimination of the infection in a large

proportion even in teneral flies which do not have a completed PM in less than five days. The second line might act thereafter and includes the specific up-regulation of a whole set of genes, including AMPs (Lehane et al. 2008, 2003). The activation of the innate immune system (the second line) might occur in the vanishing small tsetse population where the first line defence failed.
A comparison of the larval deposition interval (measured as a parameter for fecundity) in female tsetse flies infected with non-immunogenic and immunogenic trypanosomes demonstrated that flies with up-regulated AMP transcripts had a significantly reduced fecundity (Hu et al. 2008). It seems that reduction of the fecundity is due to the activation of the immune response but not due to the presence of the parasite in the midgut. In tsetse flies, AMP activation was suggested to play an important role in the resistance to trypanosomes (Hao et al. 2001). Why should a tsetse fly up-regulate AMPs and risk a significant loss in fitness to fight a parasite that is harmless for its survival? Possibly, the activation of AMPs upon trypanosomes might be dependent on the combination of parasite strain and vector origin. In a focus, where tsetse flies and trypanosomes co-evolved, the activation of AMPs might play an inferior role hinting that not all tsetse fly-trypanosome combination lead to an increase of AMP transcripts.

Depending on the origin of tsetse flies different infection rates were observed. Less than 10% midgut infection rate was obtained with *G. m. morsitans* from the Yale tsetse colony (Wang et al. 2008, Hu et al. 2008) compared to about 50% midgut infection rate with *G. m. morsitans* obtained from the Bratislava tsetse colony (this study, data not shown) using the very same strain Ytat 1.1. The colonies possibly share a similar origin (Zimbabwe) but inbreeding effects might lead to different tsetse fly stocks. The difference was mainly reflected in the diverse infection rates but could also be manifested in a diverse activation pattern of the AMPs.
The immunity could be influenced by other co-infection, either by suppressing or boosting an immune response. Employing diagnostic PCR we could detect the salivary gland hypertrophic virus (SGHV) in the *G. m. morsitans* from the Bratislava colony (an original colony from the Agriculture and Biotechnology Laboratory of the International Atomic Energy Agency (IAEA) in Seibersdorf, Austria). The sequence of one positive PCR product clearly identified SGHV, described in *G. palidipes* (published on NCBI gb|EF568108.1|) and showed a 99% similarity (data not shown) (Abd-Alla et al. 2007). SGHV is transmitted via the milk glands to its progeny (Sang et al. 1996) or via the feeding under colony conditions horizontally to other flies and was described to increase mortality in colonies (Abd-Alla et al. 2007). It has to be

shown if SGHV influences the tsetse fly's innate immunity and if tsetse flies of other colonies might be infected with SGHV.

The high infection rate obtained with our flies indicates high susceptibility whereas the flies from the Yale colony would be considered as refractory. Interestingly, *G. m. morsitans* from the Yale colony showed a low attacin mRNA level in teneral flies compared to *G. p. pallidipes* and *G. palpalis* and was hence described as susceptible and the other two species as refractory (Nayduch and Aksoy 2007). Normal blood-feeding elevated the transcript level in the 'refractory' flies. This led to the hypothesis that 'refractory' flies do not need to specifically up-regulate attacin mRNA upon trypanosomes infection (Nayduch and Aksoy 2007) because their steady state of the mRNA is high already. Unfortunately, the 'refractory' flies were not infected to show first their refractoriness to trypanosomes and second to confirm the author's hypothesis. Our results do show increased AMP transcripts regardless if the blood contains trypanosomes or not and we noticed also a high steady state level of attacin and defensin abundance in teneral tsetse flies (data not shown in this chapter, see appendix, page 118) like it was shown for the two 'refractory' fly species (Nayduch and Aksoy 2007). Accordingly, our *G. m. morsitans* from the Bratislava colony would be considered as 'refractory' in terms of the AMP mRNA level in young flies and hence would not up-regulate the immune response upon trypanosome infection what we finally observed.

We conclude that trypanosomes do not necessarily unleash an immune response when colonising the tsetse fly's midgut.

Acknowledgement

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6. Major surface glycoprotein of insect forms of *Trypanosoma brucei* are not essential for cyclical transmission by tsetse

Erik Vassella[*], Michael Oberle[‡], Simon Urwyler[*], Christina Kunz Renggli[‡], Erwin Studer[*], Andrew Hemphill[§], Cristina Fragoso[*], Peter Bütikofer[°], Reto Brun[‡] and Isabel Roditi[*]

[*]Institut für Zellbiologie, Universität Bern, Switzerland

[‡]Swiss Tropical Institute, Basel, Switzerland

[§]Institut für Parasitologie, Universität Bern, Switzerland

[°]Institut für Biochemie und Molekulare Medizin, Universität Bern, Switzerland

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Major Surface Glycoproteins of Insect Forms of *Trypanosoma brucei* Are Not Essential for Cyclical Transmission by Tsetse

Erik Vassella[1¤a], Michael Oberle[2☯], Simon Urwyler[1☯¤b], Christina Kunz Renggli[2], Erwin Studer[1], Andrew Hemphill[3], Cristina Fragoso[1], Peter Bütikofer[4], Reto Brun[2], Isabel Roditi[1]*

1 Institut für Zellbiologie, Universität Bern, Bern, Switzerland, 2 Swiss Tropical Institute, Basel, Switzerland, 3 Institut für Parasitologie, Universität Bern, Bern, Switzerland, 4 Institut für Biochemie und Molekulare Medizin, Universität Bern, Bern, Switzerland

Abstract

Procyclic forms of *Trypanosoma brucei* reside in the midgut of tsetse flies where they are covered by several million copies of glycosylphosphatidylinositol-anchored proteins known as procyclins. It has been proposed that procyclins protect parasites against proteases and/or participate in tropism, directing them from the midgut to the salivary glands. There are four different procyclin genes, each subject to elaborate levels of regulation. To determine if procyclins are essential for survival and transmission of *T. brucei*, all four genes were deleted and parasite fitness was compared in vitro and in vivo. When co-cultured in vitro, the null mutant and wild type trypanosomes (tagged with cyan fluorescent protein) maintained a near-constant equilibrium. In contrast, when flies were infected with the same mixture, the null mutant was rapidly overgrown in the midgut, reflecting a reduction in fitness in vivo. Although the null mutant is patently defective in competition with procyclin-positive parasites, on its own it can complete the life cycle and generate infectious metacyclic forms. The procyclic form of *T. brucei* thus differs strikingly from the bloodstream form, which does not tolerate any perturbation of its variant surface glycoprotein coat, and from other parasites such as *Plasmodium berghei*, which requires the circumsporozoite protein for successful transmission to a new host.

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* E-mail: isabel.roditi@izb.unibe.ch

¤a Current address: Institut für Pathologie, Universität Bern, Bern, Switzerland

¤b Current address: Institut für Mikrobiologie, ETH Zürich, Zürich, Switzerland

Introduction

Two diseases that are prevalent in sub-Saharan Africa, human sleeping sickness and Nagana, a disease of domestic animals, are caused by African trypanosomes. The spread of these diseases is determined by tsetse flies (*Glossina spp.*) capable of transmitting trypanosomes from one mammalian host to the next [1]. Successful transmission of *Trypanosoma brucei* first involves the establishment of an infection in the insect midgut. This is followed by a maturation phase in which the parasites migrate to the salivary glands and finally give rise to metacyclic forms that can be transmitted to a new mammalian host.

Unlike many other unicellular parasites, *T. brucei* remains extra cellular throughout its life cycle and survives in the circulation of the mammalian host and the protease-rich environment of the fly midgut by expressing specific coats of glycosylphosphatidyl-inositol (GPI)-anchored surface glycoproteins [2–4]. In the mammalian host, bloodstream forms of the parasite are covered by a coat consisting of variant surface glycoprotein (VSG). The VSG coat acts as a physical barrier that prevents access to underlying molecules and destruction of the parasite, at the cost of provoking a humoral immune response against itself. The process of antigenic variation, involving the periodic recruitment and expression of a new VSG gene from a repertoire of several hundred genes, prevents lysis by antibodies directed against previous coats. An intact VSG coat appears to be essential for the parasite, both in vivo and in culture, since perturbation of GPI anchor synthesis [5] or down-regulation of the VSG mRNA by RNA interference [6] is lethal.

When a tsetse fly feeds on an infected host, bloodstream forms are exposed to a multitude of proteases in the insect's midgut. Certain proteases, such as trypsin, kill slender (proliferating) bloodstream forms, but promote the differentiation of stumpy (cell-cycle arrested) bloodstream forms to the next stage of the life cycle, the procyclic form [7]. Synchronous differentiation of the stumpy form to the procyclic form can be induced in culture, either by proteases [7] or, more commonly, by the addition of citrate or cis-aconitate to the medium [8,9]. As the trypanosome differentiates, a membrane-anchored metalloprotease (MSP B) cleaves VSG from the surface, making way for a new stage-specific coat of several million copies of procyclins within the space of a few hours [10,11]. The two classes of procyclins, EP and GPEET, are characterised by internal dipeptide and pentapeptide repeats, respectively. These repeats are resistant to cleavage by MSP-B and tsetse midgut proteases, although the

procyclin N-termini are removed by the latter [12,13], for reasons that are unclear [14]. Expression of different forms of procyclin is regulated temporally. A few hours after differentiation is induced, trypanosomes express all three EP isoforms (EP1, EP2, EP3) and GPEET [15]. This is followed by a surge of GPEET synthesis, making it the predominant component of the coat of early procyclic forms [12,15]. In vivo, GPEET is repressed after a few days and replaced by N-glycosylated forms of EP, EP1 and EP3, in late procyclic forms [12]. In culture, trypanosomes can be kept as early procyclic forms when glycerol is present in the medium and induced to differentiate to late procyclic forms when glycerol is removed [16]. EP procyclins are also expressed by the mesocyclic form in the anterior midgut and by trypomastigotes in the proventriculus [17], but not by the epimastigote form that colonises the salivary glands or the metacyclic form [18]. Epimastigote forms express a family of GPI-anchored proteins known as brucei alanine-rich proteins (BARPs) [4] while metacyclic forms are covered by VSG [19].

In contrast to the VSG coat of bloodstream forms, procyclins are not essential for procyclic culture forms. We previously obtained an EP/GPEET null mutant by deleting the genes from the bloodstream form and then triggering the knockout to differentiate in culture [20]. After shedding the VSG coat, the null mutant required almost two months before it was able to proliferate normally. Analysis of this mutant revealed that, in the absence of procyclin polypeptide precursors, free GPI anchors were on the surface, forming a glycocalyx. Since both the wild type parental strain and the mutant were very poorly infectious for tsetse, we were unable to draw any conclusions about the function of procyclins in vivo. Several mutants have been generated in procyclic forms and tested in tsetse, but none of these gives a definitive answer about the role of procyclins in the fly. One mutant (Nour 6C) that lacked all EP genes, but still retained a single GPEET gene, was almost an order of magnitude less efficient than the wild type at establishing heavy midgut infections in flies [21]. The phenotype was partially restored by reintroduction of either EP1 or EP2, suggesting that they play a protective role. Mutants defective in GPI anchor biosynthesis showed different phenotypes. Mutants lacking the GPI transamidase (encoded by *GPI8*) [22], or GlcNAc-phosphatidyl-inositol de-N-acetylase (encoded by *GPI12*), [23] no longer synthesised membrane-bound EP or GPEET and were also extremely inefficient at establishing midgut infections. This could be due to a lack of GPI-anchored proteins other than procyclins, however. In contrast, deletion of the gene encoding another GPI biosynthetic enzyme, GPI10, had little effect on the ability of procyclic forms to infect flies [5].

In addition to any functions they might have in the first phase of infection, another possibility is that procyclins are required in order for *T. brucei* to leave the midgut and migrate to the salivary glands. It has not been possible to test this hypothesis previously as none of the mutants described above could complete the life cycle in the fly (a common problem when procyclic forms are cultured for any length of time). In order to study the function of procyclins throughout the life cycle we deleted all procyclin genes from a fly-transmissible strain. A surprising outcome of these experiments was that trypanosomes without a procyclin coat are still transmissible by tsetse, albeit with a substantial reduction in the prevalence and intensity of salivary gland infections.

Results

GPEET is not required for the establishment of infection

The procyclin mutants described to date [14,20,21,24] were generated in laboratory-adapted stocks that had either been passaged in rodents or maintained in culture for an extended period of time. For unknown reasons, these trypanosomes lose their ability to complete the life cycle in the fly. We therefore decided to generate a new null mutant in a fly-transmissible clone, AnTat 1.1. The basic arrangement of procyclin genes is the same as in *T. brucei* 427 [20]. Both stocks have procyclin loci on chromosomes 6 and 10. In Antat 1.1, the locus on chromosome 6 is homozygous for the gene pair GPEET and EP3-3, while the two EP genes on chromosome 10 are heterozygous (Figure 1). The first knockout of the procyclin locus on chromosome 6 was generated in bloodstream forms. Bloodstream forms of pleomorphic stocks are notoriously difficult to transfect and maintain in culture, however, so subsequent knockouts were generated in procyclic forms. To maintain their competence to complete the life cycle, trypanosome clones were transmitted through tsetse and mice after each round of transfection, and triggered to differentiate to procyclic forms in vitro. Deletion of the procyclin locus from the second copy of chromosome 6 gave rise to the mutant ΔGPEET that lacked the GPEET gene and the adjacent EP3-3 gene (Figure 1).

Since GPEET is expressed abundantly for the first few days of an infection, and then repressed thereafter [16], we analysed the ability of bloodstream forms of the mutant to establish infections when given to teneral flies as part of their first blood meal. The flies were dissected 11–14 days post infection and graded as described previously [21]. These experiments revealed only slight differences in the prevalence and intensity of midgut infections, with the wild type being able to establish heavy infections in 42.5% of flies and ΔGPEET in 32.3% (Figure 2); these differences were

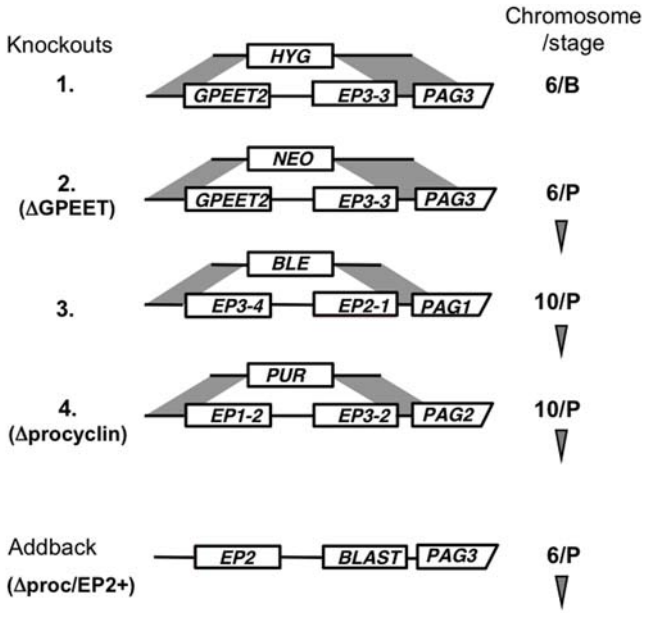

Figure 1. Schematic depiction of the procyclin loci in AnTat 1.1 and lineage of the knockouts [15]. GPEET2 is a variant that has 5 rather than 6 copies of the pentapeptide repeat. Deletion constructs containing antibiotic-resistance genes were designed to delete tandemly linked procyclin genes by homologous recombination (see Materials and Methods). In the addback Δproc/EP2+, the neomycin-resistance gene was replaced by EP2 procyclin and the blasticidin resistance gene. The truncated 3′ UTR (ΔLII) downstream of EP2 ensures high levels of expression in procyclic forms [36,40]. B: bloodstream form; P: procyclic form. Arrowhead: transmitted through tsetse and mice.
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not statistically significant. Non-teneral flies (flies that have taken a blood meal) are more refractory to infection. This is probably a closer reflection of the situation in the wild and might potentiate differences that are not detectable when conditions are optimised for high infection rates in teneral flies. Of the flies exposed to trypanosomes in their second blood meal, ,7 times fewer flies were able to establish heavy midgut infections, but this applied to both the wild type and ΔGPEET (Figure 2). In the standardised procedure that we use for transmission experiments, the flies normally receive an inoculum of about $4x10^4$ trypanosomes (assuming that they consume ,20 ml blood), which is considerably more than from an infected animal. However, reducing the inoculum given to teneral flies by a factor of 20 did not reveal any difference between ΔGPEET and the wild type (data not shown).

A procyclin null mutant is fly-transmissible

Since no obvious phenotype was observed in trypanosomes lacking GPEET, apart from a slightly lower prevalence of heavy midgut infections, we proceeded to delete the remaining procyclin genes. After two further rounds of transfection many drug-resistant clones were isolated, but only one clone of more than 12 that were examined had deleted both of the remaining two copies of EP (Figure 1 and Supplemental Figure S1). This is reminiscent of our earliest attempts to generate procyclin knockouts, which also suggested that retention of a procyclin gene was advantageous for procyclic forms [21]. Procyclic forms of the null mutant

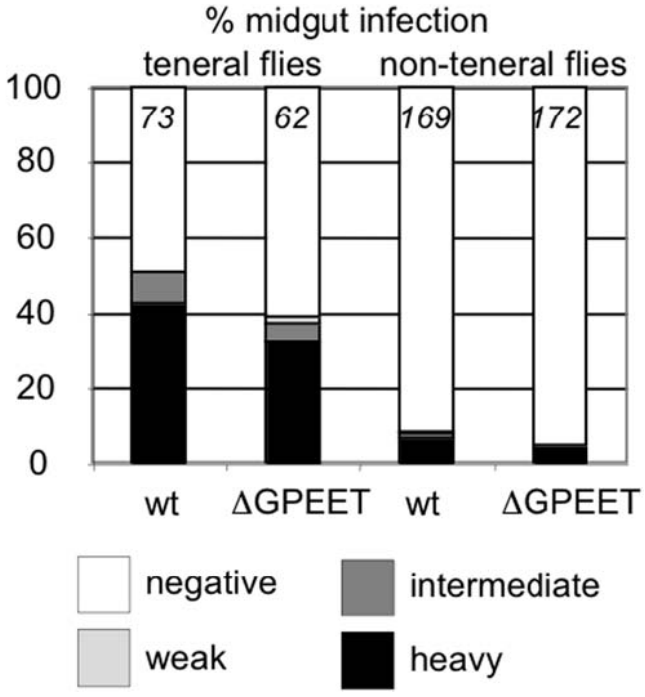

Figure 2. Comparison of the prevalence of midgut infections in tsetse flies exposed to stumpy bloodstream forms of wild type AnTat 1.1 (wt) or ΔGPEET. Infections were classified as described [21]. Teneral flies were infected with trypanosomes in their first blood meal after hatching. Non-teneral flies were infected with the second blood meal when they were 4–6 days old. Infection prevalence and intensity were assessed 11–14 days post infection. The number of flies per experimental group is indicated at the top of each column. Statistical analysis showed no significant differences between the wild type and the mutant in the prevalence or intensities of midgut infections.

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(Δprocyclin clone#6) were used to infect teneral flies. To our astonishment, Δprocyclin was able to complete the life cycle in tsetse and infect mice. The resulting bloodstream forms were triggered to differentiate to procyclic forms and subjected to immunofluorescence analysis with anti-EP and anti-GPEET antibodies (Figure 3A) and Southern blot analysis (Supplemental Figure S1). Both confirmed that the trypanosomes that survived transmission were indeed procyclin null mutants. Procyclic forms were also subjected to [^{3}H]ethanolamine-labelling and fractionated according to standard procedures that separate free GPI anchors and GPI-anchored proteins (Figure 3B). Both early procyclic forms and late procyclic forms of Δprocyclin expressed increased amounts of free GPIs, as shown previously for the EP/GPEET null mutant [20], and neither stage expressed any detectable GPI-anchored proteins as alternatives to procyclins (Figure 3B). Unlike the EP/GPEET mutant, which was generated entirely in the bloodstream form [20], Δprocyclin did not show defects in differentiation from the bloodstream to the procyclic form, nor did it undergo growth arrest shortly after differentiation (data not shown).

To obtain quantitative data on the efficiency of transmission, teneral flies were infected with stumpy bloodstream forms of the wild type or Δprocyclin and monitored for the establishment of midgut and salivary gland infections (Figure 4). Surprisingly, after 11–14 days 18.9% of flies infected with Δprocyclin exhibited heavy midgut infections, compared to 32.8% of flies infected with the wild type (Figure 4; $p = 0.01$). This is in striking contrast to the EP null mutant Nour 6C which was 5–10 times less efficient at establishing heavy infections than its wild type parent, despite expressing high levels of GPEET [21]. We have previously shown that overexpression of either EP1 or EP2 by the Nour 6C mutant promotes its survival in the midgut [21]. To investigate whether procyclins can rescue the phenotype of Δprocyclin, an addback (Δproc/EP2+) was constructed by integrating an EP2 coding region into the procyclin locus on chromosome 6. When stumpy bloodstream forms of Δproc/EP2+ were used to infect flies, they established heavy midgut infections at the same rate as the wild type (Figure 4).

Fitness of the mutants in competition experiments

The experiments described above clearly show that, even without procyclins, trypanosomes can establish midgut infections reasonably successfully. In the long term, however, variants with even a slight competitive disadvantage would be lost from the population. In order to gauge the influence of procyclin genes on fitness, a tagged form of AnTat 1.1 expressing cyan fluorescent protein (CFP#5) was used as a reference in competition experiments [25]. Stumpy bloodstream forms of CFP#5 were mixed with equal numbers of wild type AnTat 1.1, ΔGPEET or Δprocyclin and induced to differentiate by the addition of cis-aconitate. The percentage of CFP-positive cells in each culture was monitored over a period of two weeks (Figure 5A). A control culture containing CFP#5 alone became 100% positive within two days of triggering differentiation and remained so throughout the experiment. When CFP#5 was combined with Δprocyclin, the ratio of the two cell types remained reasonably constant over 14 days. In contrast, CFP#5 was overgrown by the wild type and ΔGPEET with the same kinetics and was barely detectable at the last time point. These experiments indicate that Δprocyclin has a similar level of fitness to CFP#5 in culture, but both are less robust than the wild type and ΔGPEET.

In parallel, the same mixtures of bloodstream forms were used to infect teneral flies and midgut infections were analysed (Figure 5B). Wild type AnTat 1.1 and ΔGPEET behaved in the

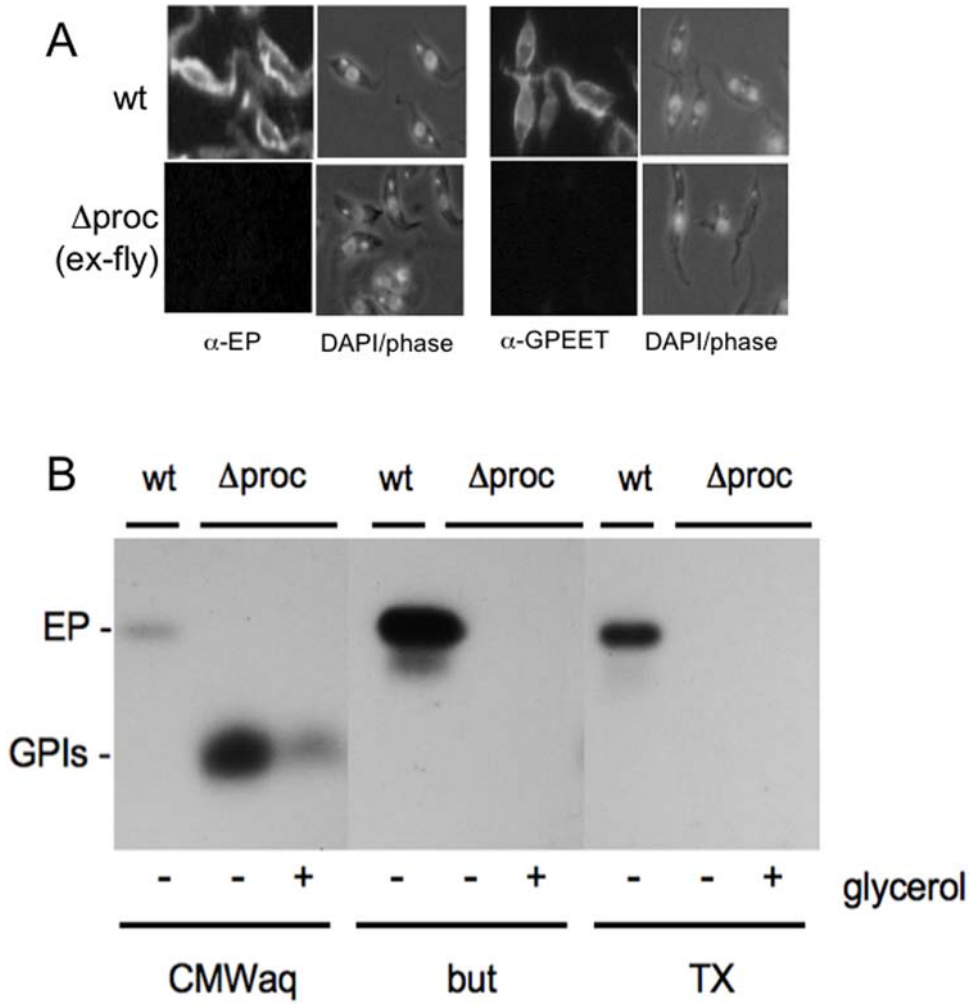


Figure 3. A. Immunofluorescence analysis of procyclic forms of trypanosomes after cyclical transmission through tsetse flies (ex-fly). Procyclic forms of the wild type (wt) and Δprocyclin (Δproc) were labelled with the monoclonal antibody TBRP1/346 against EP or with a rabbit polyclonal antiserum against GPEET. Right panels: DAPI images (dark field or phase) from the same fields. B. Analysis of GPI-anchored molecules. Wild-type trypanosomes (wt) and Δprocyclin (Δproc), cultured as early procyclic forms (+glycerol) or late procyclic forms (2glycerol), were labelled with [^{3}H]ethanolamine as described [20]. The delipidated pellets were extracted with CMW to solubilise free GPIs, followed by 9% butan-1-ol (but) and 0.1% Triton X-100 (TX) to solubilise procyclins. The CMW extracts were dried and partitioned between water and butan-1-ol; the aqueous phase contains free GPIs (CMWaq). All fractions were analyzed by SDS-PAGE and fluorography. The positions of EP procyclin (EP) and free GPIs (GPIs) are indicated.
doi:10.1371/journal.pone.0004493.g003

same way as they did in culture, progressively overgrowing CFP#5 over a period of two weeks. Thus there is no indication that ΔGPEET was any less fit than wild type AnTat 1.1 in vivo.In contrast to the situation in culture, however, Δprocyclin was unable to maintain its equilibrium with CFP#5 in the fly and was competed out within one cycle of infection.

Experiments were also performed with the addback Δproc/ EP2+, which was fitter than Δprocyclin at the beginning of fly infection, but overgrown by CFP#5 by day 7 (data not shown).

Reduced salivary gland infections and adherence to epithelial cells

The initial transmission experiments performed to obtain bloodstream forms of each mutant indicated that procyclins were not required for maturation, but gave no information on the efficiency of this process. To study this more precisely, teneral flies were infected with bloodstream forms of wild type AnTat1.1, ΔGPEET, Δprocyclin or Δproc/EP2+. Several indep-endent experiments were performed, with similar results. In the experiment depicted in Figure 6A, both midgut and salivary gland infections were assessed 28–35 days after infection, enabling us to determine the transmission index (percentage of flies with midgut infections also giving rise to mature salivary gland infections). Under the conditions used, wild type AnTat 1.1 produces salivary gland infections in a high proportion of infected flies (25% in this experiment, with a transmission index (TI) of 42.8%). In general, the glands were heavily infected and electron micrographs showed the flagella of epimastigote forms in tight association with the salivary gland epithelia (Figure 7A, B) as has been documented previously [26]. ΔGPEET produced salivary gland infections in fewer flies (10.7% infection, TI = 20.5%); the difference to the wild type was slightly significant statistically ($p = 0.021$). In contrast, Δprocyclin produced a lower prevalence of salivary gland infections that was highly significant (2.3% infection, $p < 0.0001$) compared to wild type and there were at least 10 times fewer trypanosomes in the glands. These established less intimate contact with gland tissue, and in the rare instances that parasites adhered to microvilli, they did not appear to attach

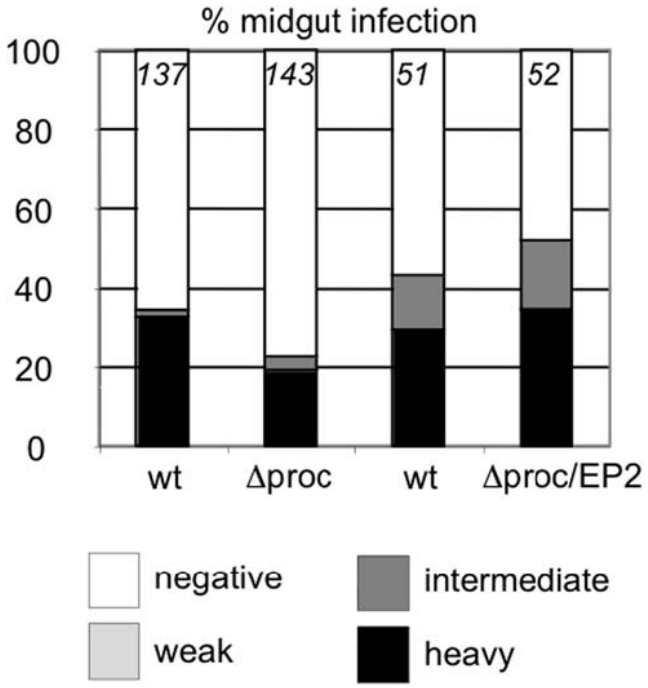


Figure 4. Comparison of midgut infection rates in flies infected with stumpy bloodstream forms of wild type AnTat 1.1 (wt), Δprocyclin (Δproc) or the addback mutant Δproc/EP2+. Midgut infection rates were determined 11–14 days post infection and graded as described in the legend to Figure 2. The number of flies per experimental group is indicated at the top of each column.
doi:10.1371/journal.pone.0004493.g004

tightly to epithelial cell surface structures (Fig 7C–F). The lower prevalence of mature infections by Δprocyclin was not due to a decrease in midgut infections after 4 weeks (no significant difference in total midgut infection prevalence), and is reflected by a substantially lower TI of 4.6%. Sequential transmissions of Δprocyclin through tsetse did not increase the prevalence or intensity of salivary gland infections, which could have been the case if a sub-population had acquired additional mutations or otherwise adapted itself to the lack of procyclins (data not shown).

To ascertain if there was a link between EP procyclin expression and colonisation of the salivary glands, infections were performed with Δproc/EP2+. Compared with Δprocyclin, however, the prevalence of salivary gland infections increased only marginally, to 3.9% infection (TI = 7.5%), and there was no obvious increase in the intensity of infection. One possible interpretation of these results is that trypanosomes might require more, or different procyclins in order to penetrate the proventriculus and gain access to the salivary glands. We can conclude, however, that procyclins are not essential for full cyclical transmission by the tsetse fly.

It could be hypothesised that multiple resistance genes carried by the null mutant and the addback might contribute to the reduction in maturation rates and/or the intensity of salivary gland infections. Although we felt this to be unlikely, given that the parasites used in transmission experiments were not exposed to antibiotics, we performed a control experiment with a null mutant for procyclin-associated genes (allPAGko) [25] that carries the identical set of resistance genes inserted downstream of the procyclin genes. In contrast to Δprocyclin, however, there was no significant difference in the prevalence and transmission index of this mutant compared to wild type AnTat 1.1, and the majority of salivary gland infections were of heavy or intermediate intensity (Figure 6B).

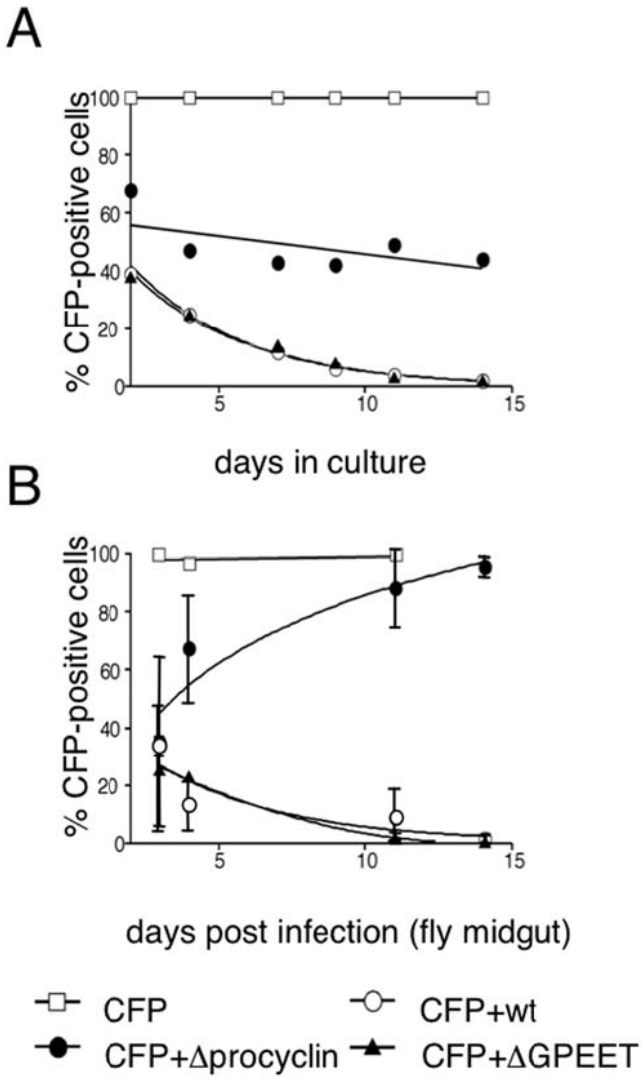


Figure 5. A. Competition with CFP-tagged trypanosomes in culture. Stumpy forms of wild type AnTat 1.1 (wt), ΔGPEET or Δprocyclin (Δproc) were mixed with an equal number of stumpy forms of CFP#5, which expresses cyan fluorescence protein from the procyclin promoter. The mixed cultures were triggered to differentiate by the addition of cisaconitate and incubated at 27uC in SDM-79 supplemented with 10% FBS and 20 mM glycerol. The percentage of CFP-positive cells was determined by observing the cells under a fluorescence microscope. B. Mixed infections with CFP#5. Stumpy forms of the cell mixtures described above were used to infect tsetse flies and the percentage of CFP-positive cells was determined from cells isolated from the midgut of infected flies. As a control for CFP expression, a pure culture of CFP#5 was analysed in parallel.
doi:10.1371/journal.pone.0004493.g005

Discussion

The sequential deletion of pairs of procyclin genes from a fly-transmissible strain of *T. brucei* resulted in the unexpected finding that the null mutant, Δprocyclin, was still able to complete the entire life cycle in tsetse and mammals. Δprocyclin was able to establish heavy infections in the fly midgut at approximately two-thirds of the prevalence of its wild type parent and was able to produce mature salivary gland infections and be transmitted by

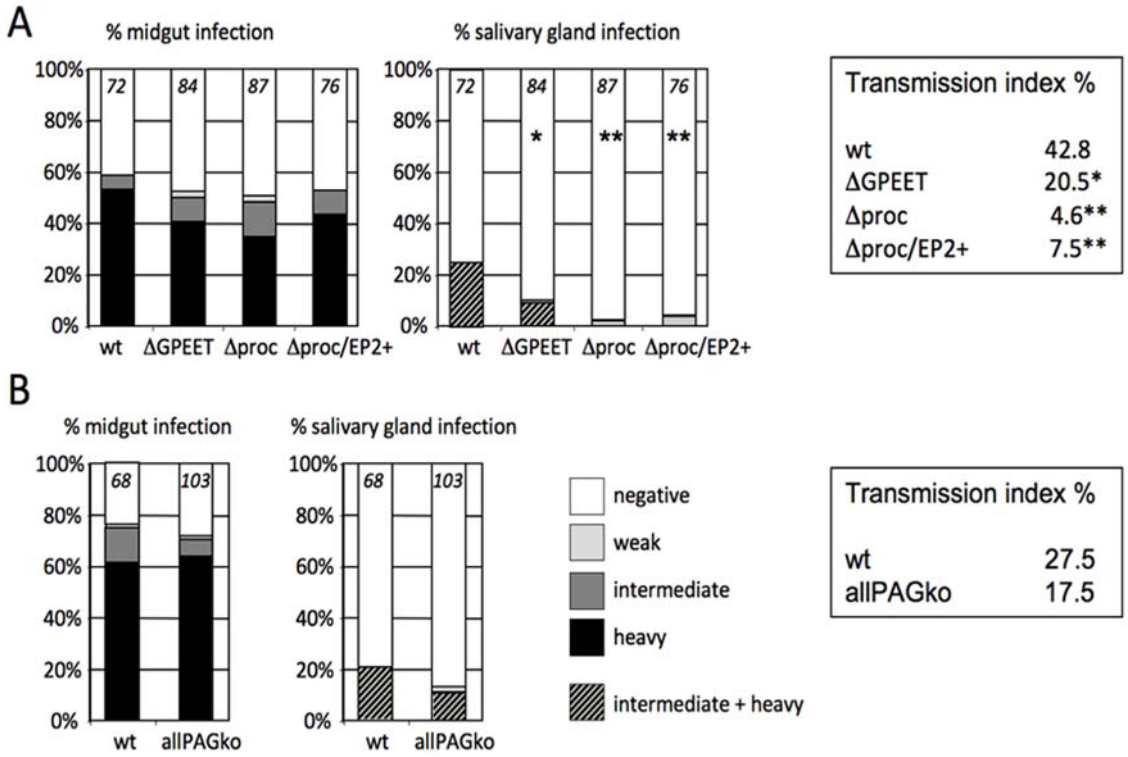


Figure 6. Maturation rates and transmission indices. A. Teneral flies were infected with stumpy bloodstream forms of wild type AnTat 1.1, ΔGPEET, Δprocyclin or Δproc/EP2+. Flies were dissected 28–35 days post infection and graded for the prevalence and intensity of midgut and salivary gland infections. Significant differences between the prevalence and transmission index obtained with wild type AnTat1.1 and various mutants are indicated by asterisks; $p<0.05$ (*) and $p<0.001$ (**). No significant differences were observed when the mutants were compared to each other. B. Multiple resistance genes do not reduce the intensity of salivary gland infections. Teneral flies were infected with early procyclic forms of AnTat 1.1 or allPAGko clone 1 [25], which carries the same resistance genes as Δprocyclin. Midgut and salivary gland infections were categorised as described above. The number of flies per experimental group is indicated. doi:10.1371/journal.pone.0004493.g006

flies to mice. In addition, bloodstream forms of the mutant were capable of differentiating to the procyclic form without a detectable growth phenotype.

In this study we have formally proven for the first time that procyclins are not essential for the establishment of an infection in the ectoperitrophic space. The null mutant expresses free GPIs, however, and these may take over the function(s) of procyclins [20,27]. The observation that Δprocyclin is notably more robust than other procyclin mutants that we have generated previously is most probably because of the experimental strategy that was used. It was necessary to do this because prolonged passage in rodents or in culture tends to select for parasites that lose the ability to differentiate and/or complete the life cycle. Deletion mutants were therefore subjected to fly transmission after each round of transfection, selecting for parasites that could adapt to a progressively thinner protein coat. This is in contrast to the EP/GPEET null mutant generated in bloodstream forms, which makes the transition from a full VSG coat to no glycoprotein coat when the parasite differentiates to the procyclic form [20] or to the Nour 6C mutant [21], which first becomes negative for procyclins when GPEET is repressed a few days post infection in the fly midgut [16].

The expression of GPEET mRNA is elaborately regulated by its 39 UTR, both in the fly and in response to carbohydrate sources and mitochondrial activity in culture [16,28]. In a wild type infection, down-regulation of GPEET correlates with the time that the trypanosome crosses the peritrophic matrix [16]. Nevertheless, trypanosomes lacking GPEET were able to establish midgut infections as efficiently as the wild type, in both teneral and non-teneral *G. m. morsitans*, even when a low infective dose was used. They were also equally competitive in mixed infections with the CFP-tagged wild type, which has the full complement of procyclins. The function of GPEET may only become apparent in other species of Glossina or under less favourable conditions for transmission. We have found that a mutant that constitutively expresses GPEET is transmitted very efficiently (unpublished data). The GPEET expressed by late procyclic forms and epimastigote forms was not phosphorylated, however, which is consistent with reports that the activity of the kinase is restricted to early procyclic forms [29].

On its own, the procyclin null mutant could colonise the midgut at rates and intensities comparable to the parental strain, and its reduced fitness only became apparent in mixed infections. This contrasts with a deletion mutant lacking all procyclin-associated genes, which could compete as effectively as the wild type in mix infections [25]. There are two possible interpretations of these results. Midgut infections are reported to plateau at a constant number of ~$2x10^5$ parasites [30,31]. The simplest explanation is that a fitter strain would replicate faster and occupy the available space before Δprocyclin would be able to do so. This implies that a coat of free GPIs cannot completely substitute for procyclins and may leave other surface molecules vulnerable to attack by proteases. Another possibility is that trypanosomes ex-pressing procyclins might elicit an anti-microbial response that affects parasites that are not covered by the same coat. What the nature of this response might be is unknown at present, but it does not

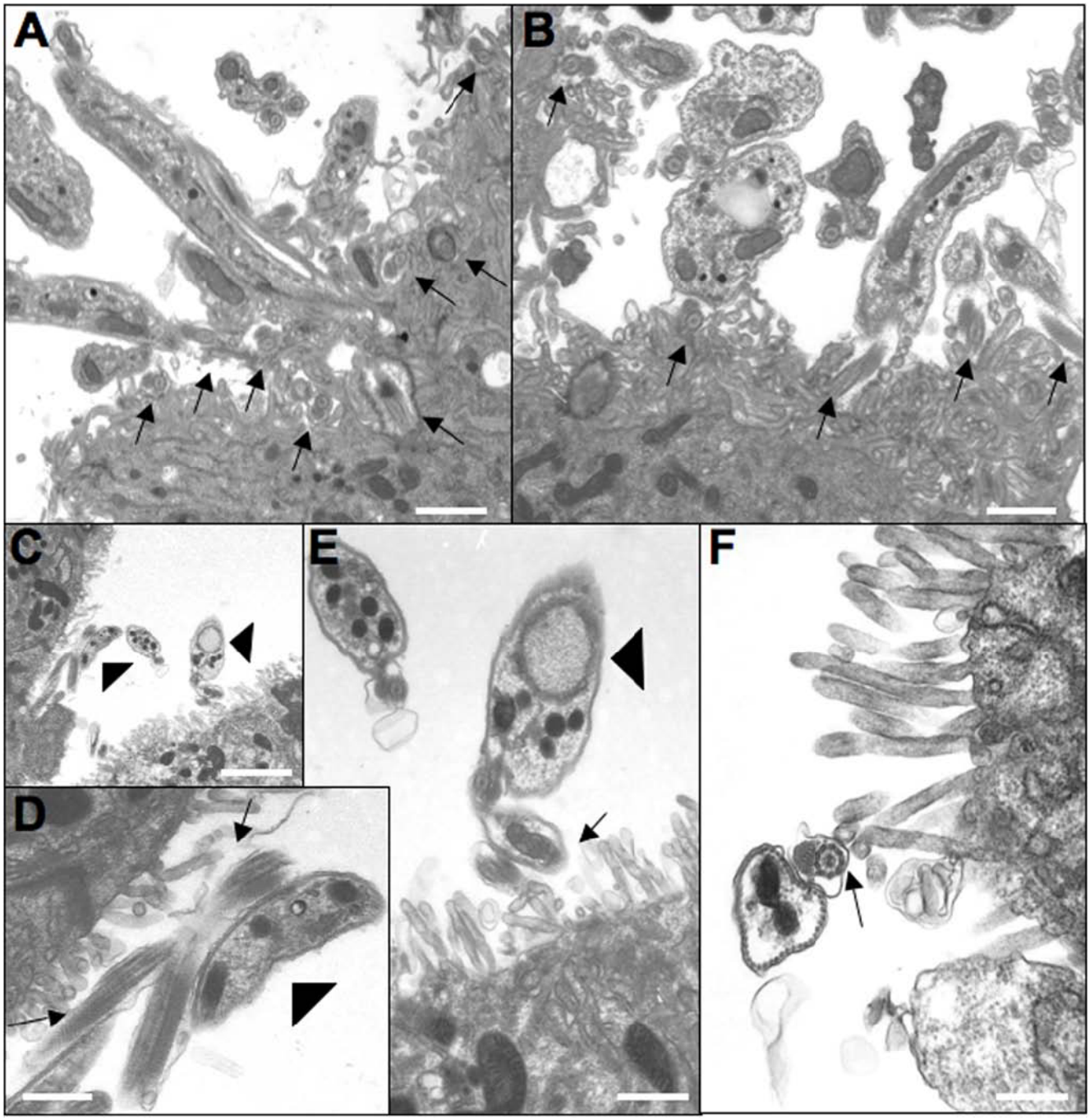


Figure 7. Representative images from sections of salivary glands. (A, B) Salivary glands isolated from flies infected with wild type AnTat1.1. Note that the flagella (marked with arrows) are in very close contact with the epithelial cells and often appear completely entangled with surface microvilli of the salivary gland. (C–F) Salivary glands from flies infected with Δprocyclin. (D) and (E) are higher magnification views of those areas in C marked by arrowheads. Arrows mark the flagella adhering to the epithelia. Note the much lower density of parasites, and the much less pronounced interaction of Δprocyclin with the epithelium compared to wild type AnTat1.1. Bars in (A) = 1.1 μm; (B) =0.8 μm; (C) =2.4 μm; (D) =0.6 μm; (E) = 0.6 μm; (F) = 0.44 μm.
doi:10.1371/journal.pone.0004493.g007

seem to involve an increase in defensin or attacin transcripts (M. Oberle, unpublished data).

The null mutant was able to produce mature infections, demonstrating that procyclins are not required to direct the trypanosomes to the salivary glands. The prevalence of salivary the surface architecture of gland infections and transmission index were about 10-fold lower than with the wild type, however, and the number of trypanosomes was also reduced by more than one order of magnitude. Since procyclins are not normally detected on the surface of epimastigote forms [18], the lack of procyclins or the expression of free GPIs on the surface of midgut forms [20] might exert an indirect effect. Perturbation of the parasite, for example by exposing surface molecules that are normally shrouded by procyclins, might alter interactions with host

molecules. This, in turn, could conceivably reduce migration and/or adherence of epimastigote forms to the salivary gland epithelia. These hypotheses are extremely difficult to test, however, given the paucity of differentiation markers and the very low numbers of epimastigote forms in glands infected with Δprocyclin. The finding that the maturation rate could not be restored by the reintroduction of a single procyclin gene into the null mutant might be due to a requirement for multiple procyclin isoforms, in order for the parasite to migrate efficiently, or for appropriately regulated expression. Another possibility is that trypanosomes that have abundant free GPIs on their surface may have difficulty readjusting to attaching GPI anchors to proteins such as BARP, which might be needed for successful colonisation of the salivary glands. In line with this, the sequential deletion of procyclin genes resulted in progressively worse maturation rates and transmission indices (Figure 6A).

In conclusion, although procyclins are not essential, trypanosomes that express them are clearly at a competitive advantage and would probably eradicate procyclin-negative cells within one cycle of transmission. It is conceivable that a primordial trypanosome might originally have been covered by a glycocalyx of free GPIs and only later have acquired surface protein moieties that enhanced its survival and transmission. Intriguingly, a protein containing an extended EP repeat, tsetseEP, is also expressed in the midgut of tsetse flies [32] although the differences in codon usage makes it improbable that the parasite recently acquired this gene by horizontal transmission.

Materials and Methods

Trypanosomes

T. b. brucei AnTat 1.1 [33], and derivatives thereof, were used in this study. Bloodstream forms were cultured in HMI-9 containing 1.1% methylcellulose and supplemented with 10% fetal bovine serum (FBS) [34] or harvested from the blood of female NMRI mice or Wistar rats (Charles River Laboratories, France) that were immunosuppressed with cyclophosphamide (200 mg/kg body weight). Short stumpy bloodstream forms were harvested from mice 5–6 days post infection. Procyclic forms were cultured in SDM-79 [35] supplemented with 10% FBS and 20 mM glycerol. Bloodstream forms were triggered to differentiate to procyclic forms by transferring them to SDM-79 containing 20 mM glycerol, adding 6 mM cis-aconitate to the culture medium and lowering the incubation temperature to 27°C [8].

Constructs, deletion and addback mutants

The constructs pCorleone-hyg, pCorleone-neo [20], pKOP, conferring resistance to bleomycin [21], and pKO-PAC, conferring resistance to puromycin [20], have been described previously. These were used sequentially to generate pairs of procyclin genes and replace them by an antibiotic resistance gene. The bicistronic construct pCorleone-EP2DLII-blast is based on pCorleone-CAT/ GPEET [16], but in this case the first open reading frame encodes EP2 procyclin (previously EPβ, [21]) and the intergenic region between both open reading frames is derived from pGAPRONE [36]. The blasticidin resistance gene was amplified from pHD 887 [37] (courtesy of C. Clayton, Heidelberg) using the primer pair 5´-GCTAGCTAGCATGGCC-AAGCCT-3´ and 5´-CCATCGATACTCACAGCGACTA-3´ and cloned between the NheI and ClaI sites from the newly derived construct, thereby replacing the phleomycin-resistance gene. Stable transformation of bloodstream forms and procyclic forms were performed as described [16,34]. In each case, clones were analysed for correct integration of the construct before being transmitted through tsetse and mice. The resulting bloodstream forms were triggered to differentiate to procyclic forms in culture and DNA was isolated and reanalysed before the next knockout was performed.

Infection of tsetse flies

Pupae of *Glossina morsitans morsitans* were obtained from the International Atomic Agency (Vienna), the Department of Entomology, Slovak Academy of Science (Bratislava) or the Institute of Tropical Medicine, Antwerp. Teneral flies were infected with trypanosomes during the first blood meal as described [21]. Non-teneral flies were infected with the second blood meal after emergence. The blood meal consisted either of $2x10^6$short stumpy bloodstream forms in defibrinated horse blood or, alternatively, of $2x10^6$ procyclic forms in SDM-79 supplemented with washed horse red blood cells. Infected flies were fed three blood meals per week through artificial membranes. Flies were analyzed for midgut infections by dissection of their midguts and for mature infections by the presence of metacyclic forms in saliva from flies extruded onto slides. Midgut infections were scored quantitatively as light, intermediate or heavy by criteria defined by Ruepp et al. [21]. To complete cyclical transmission, flies were fed on anesthetized NMRI mice, which were subsequently monitored for parasitaemia. The two tailed Fisher's exact test was used for all statistical analyses (Daan G Uitenbroek, ''SISA-Binomial.'' 1997;http://www.quantitativeskills.com/sisa/distributions/binomial.htmæ).

Immunofluorescence and transmission electron microscopy

Immunofluorescence. Procyclic culture forms were fixed with 2% formaldehyde at 4°C. Rabbit polyclonal anti-GPEET antiserum (K1; [21,38] was used at a dilution of 1:500. The monoclonal antibody TRBP1/346, which recognises the first 20 amino acids of EP procyclins [39], was diluted 1:1000. Alexa 488-conjugated anti-rabbit (Molecular Probes) and TRITC-conjugated anti-mouse (Sigma) secondary antibodies were used at 1:500.

For transmission electron microscopy, salivary glands were isolated from infected flies and fixed for 4 hrs at 4°C in a solution containing 0.1 M cacodylate buffer, pH 7.4 and 2% glutaraldehyde. Subsequent treatments were performed exactly as described [21]. All preparations were observed using a transmission electron microscope (model 600, Philips Technologies, Cheshire, CT) operating at 60 kV.

Ethanolamine labelling

Early procyclic forms (cultured in SDM-79 supplemented with 20 mM glycerol) or late procyclic forms (cultured in SDM-79 without glycerol) were labelled with [^{3}H]ethanolamine for 18 h as described [20].The delipidated pellets were extracted with CMW to solubilise free GPIs, followed by 9% butan-1-ol (but) and 0.1% Triton X-100 (TX) to solubilise procyclins. The CMW extracts were dried and partitioned between water and butan-1-ol; the aqueous phase contains free GPIs (CMWaq). All fractions were analyzed by SDS-PAGE and fluorography.

Competition experiments

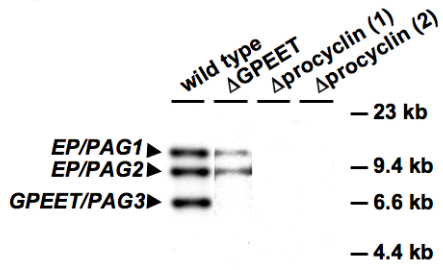
CFP#5, a clone of AnTat 1.1 tagged with cyan fluorescent protein [25] was used as a reference in co-culture experiments and mixed infections. Stumpy bloodstream forms of CFP#5 were mixed 1:1 with either wild type AnTat1.1, ΔGPEET or Δprocyclin and triggered to differentiate in culture or used to infect tsetse as described above. Procyclic forms were used for mixed infections as described previously [25]. Midgut forms were isolated from

infected flies and fixed with 4% formaldehyde in order to preserve fluorescence. The percentage of CFP-positive cells (pooled from 5– 10 infected flies) was determined by fluorescence microscopy.

Supporting Information

Figure S1 Southern blot analysis of deletion mutants Found at: doi:10.1371/journal.pone.0004493.s001 (0.17 MB TIF)

Suppl. Fig. 1



Southern blot analysis of the wild type, GPEET null mutant and procyclin null mutant before (1) and after cyclical transmission (2) through tsetse flies. Genomic DNA was digested with *Pst*I, which separates the *EP/PAG1* and the *EP/PAG2* loci from the two copies of *GPEET/PAG3*. A *GPEET*-specific probe was used for hybridization under conditions that allow cross-hybridization to the EP genes.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: EV MO SU AH PB IR. Performed the experiments: EV MO SU CKR ES AH CF. Analyzed the data: EV MO SU AH PB RB IR. Wrote the paper: EV IR.

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7. Further discussion

Cost of defence

Trypanosomes face two lines of defence when they infect a midgut. A first line of defence efficiently clears the infection in a large proportion of tsetse flies in less than five days whereas in a second line specific genes of the innate immune system or putative immune-related genes are activated. What genes are involved and specifically regulated upon trypanosome infection is complex as shown by others (Lehane et al. 2003, 2008), but I doubt that attacin and defensin play a major role in eliminating trypanosomes from the tsetse midgut.

From the literature it is not clear if midgut infection has an impact on fly fitness. Up to now survival of non-infected flies was compared to flies infected with *T. congolense* or *T. brucei* (Maudlin et al. 1998, Nitcheman 1988). In general, the infected groups showed an increased mortality after 30 – 60 days only compared to the non- infected groups, of which the midgut or the salivary gland infection rate is not known. This is unsatisfactory since infected flies may have cleared the infection or established a mature infection which might influence their survival. In one study the activation of attacin transcripts was shown to lengthen the deposition time of pupae and was interpreted to reduce fecundity of female flies (Hu et al. 2008). None of these studies showed a direct influence of midgut infections on survival and whether clearance of the parasite improves fitness of the tsetse flies.

I conducted an experiment to address this question. Ten days after infection with bloodstream form trypanosomes the flies were split in a group that starved (no blood meal anymore, high stress) and that was fed (continued blood meal every second day, low stress). A non-infected control group was treated identically in parallel (Fig. 7.1 and Appendix 2, p 117). It is not surprising that starving flies with a midgut infection have a high mortality rate since the parasites compete for nutrients in the midgut. However, it is very interesting that starving flies that cleared their infection showed an identical mortality. This predicts that fitness costs for trypanosome clearance are high. Flies that were fed (no stress) showed no significant difference between non-infected, infected and cured flies. This predicts that the advantage of clearing the infection was not obvious in this experiment. The sample size was too small and experiment duration too short to see a difference between cured, infected, and non-infected flies in the fed group. It is still not clear if, under optimal conditions, parasite clearance improves survival compared to infected flies or if clearance has a preventive character to avoid salivary gland infection which could negatively influence survival. Realistically, it

would make sense that cured flies have an advantage over infected ones which could result in an increased survival under optimal conditions (no stress). Maybe this advantage could also appear in flies under 'medium' stress condition.

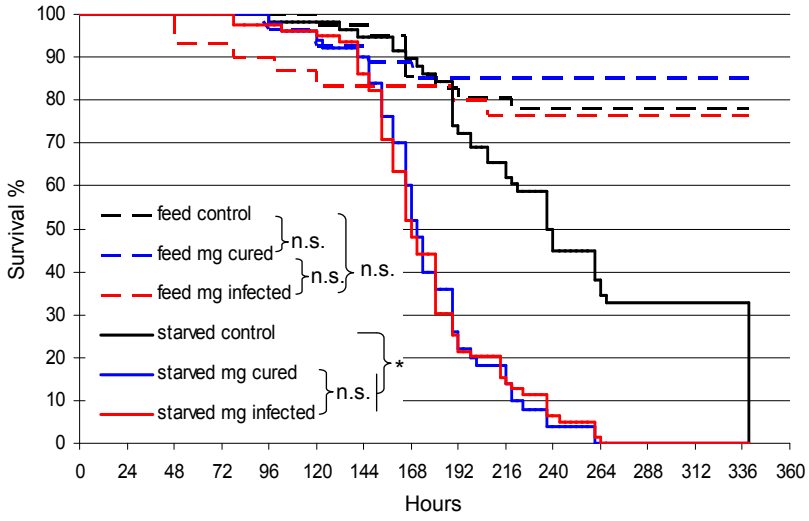

Fig. 7.1: Survival rate of infected tsetse flies with confirmed midgut infection (red lines), cured midgut (blue lines), and control flies that were not infected (black lines). Flies were either fed every second day (dotted lines) or starved (solid lines). The experiment started 10 days after infection/ first blood meal at hour 0. Dead flies were dissected to assess their infection status. 27 to 79 flies per group were used and midgut infection rate was 60% for the fed flies and 61% for the starved flies (see Appendix 2, p 117). Logrank test for equality of survival function (using STATA) was used to analyse if the difference of survival between the groups were significant ($p < 0.0001$ *) or not significant (n.s.).

It was hypothesised that procyclins might activate AMP transcripts but this would be dependent on the origin of the tsetse fly (see chapter 5). If surface proteins are involved in gene activation of the innate immunity, Δprocyclin might not activate the whole range of proteins due to the absence of the procyclin coat. This would allow its establishment in the midgut with an infection rate comparable to wild type trypanosomes because of reduced defence activation. In co-infection experiments in tsetse flies Δprocyclin was overgrown by wild type trypanosomes, which can be explained by a higher replication rate (what was shown in *in vitro* experiments) but also that Δprocyclin cannot cope with the 'full' immune response

activated by the wild type trypanosomes. To avoid the labour intensive search for differences in specific gene regulation after infection with Δprocyclins and wild type trypanosomes, one could conduct the same experiment described above but infect flies with Δprocyclin instead. This would give us indications if clearance of Δprocyclin in the midgut would have similar costs of fitness as the clearance of wild type trypanosomes.

The very similar mortality of cured and infected flies in the starved group predicts that infected flies do not activate a defence or activate it insufficiently, which would explain why infected (permissive) flies do not die faster than flies that cleared the infection (non-permissive) under starvation. Failure of defence activation would explain why we could not see an up-regulated mRNA of attacin and defensin in infected tsetse flies.

The composition of non-permissive and permissive genotypes of a tsetse population might be influence by prevalence of infected flies and availability of hosts (food sources). High prevalence in tsetse flies would result in an increased mortality of the infected flies (Nitcheman 1988) and in a possible decrease in fecundity (Hu et al. 2008) as long as flies don't suffer food stress. This would select non-permissive flies which lead to a decrease of the infection rate in tsetse flies. This fluctuation of tsetse genotype might also influence the prevalence in mammalian hosts.

Transmission rate and efficiency of salivary glands infection

Δprocyclin resulted in poor salivary gland infection rates with a very low the abundance of trypanosomes. All the other 'transmission' steps (the infection of the midgut, transmission from an infective fly to mouse, as well as differentiation from bloodstream forms to procyclic forms *in vitro*) were accomplished without problems. This observation hints that migration to the salivary glands might not only act as a bottleneck that influences the relative frequency of tags (chapter 3 and 4) but also as a barrier that prevent colonisation by trypanosomes with too many deleterious mutations. This would also explain why trypanosomes cultured for an extended time in medium have lost transmissibility. Strain 427 has become a well adapted strain in many laboratories and is therefore a prominent example that shows loss of salivary gland infectivity but can still infect midguts at high rates. An ancient 427 stock was recently shown to be transmissible (Peacock et al. 2008) what hints that its transmissibility may have been lost in several laboratories independently. Tagged AnTat 1.1 showed a significant decrease of the diversity after a culture time of only 100 days (chapter 4). It would be

interesting to test if the reduction of detectable tags would also have an effect on tsetse fly infection rate and if tsetse fly infectivity would decrease continuously with culture time or collapse suddenly after a certain time in culture. Knowing this, we could better interpret fly infection experiments with knock-out strains.

Migration bottleneck – possible relevance for drug resistance in the field

Mutations in the adenosine transporter gene that can provide resistance to melarsoprol were found in *T. b. gambiense* and *T. b. rhodesiense* isolated from relapsed patients (Matovu et al. 2001). This suggests that such resistances can develop due to treatment failure in patients, but if and how efficiently such resistant *T. brucei spp* can be transmitted by tsetse flies is not known. In *T. b. gambiense* relapsed patients the trypanosome density in the blood may be very low, hampering transmission by tsetse flies (Pépin and Mpia 2005). The situation might be different in *T. b. rhodesiense* patients but the small numbers of cases (only a few hundred are recorded per year) decrease the likelihood for fly-human contact. The likelihood of selecting drug-resistant trypanosomes in animals is much bigger and fly-cattle contact also happens more frequently. Drug-resistance in *T. congolense* was shown to be induced in cattle treated with sub-curative doses (Afewerk et al. 2000) and its transmission by tsetse flies was shown under laboratory conditions (Van den Bossche et al. 2006). This predicts that drug-resistant trypanosomes keep their transmissibility and can be spread by tsetse flies and infect other mammalian hosts.

What happens to these resistant trypanosomes in the field, where selective pressure of a drug may be absent, and they have to compete with non-resistant trypanosomes in a mammalian host? When tsetse flies take a blood meal, resistant trypanosomes (even if they are largely overgrown by non-resistant trypanosomes) can establish in the midgut and infect the salivary glands. Due to the 'race for space' during the migration, resistant trypanosomes could abound the salivary glands in some of the flies. The occurrence of dominance in the salivary glands is not always connected to the abundance in the midgut and might be an important mechanism to ensure a high diversity in trypanosome populations. This mechanism allows genotypes at low frequency to be maintained in the population of trypanosomes. The presence of drug-resistant or drug-tolerant genotypes in a trypanosome population can therefore be maintained by such transmission bottlenecks in the field. This could explain why drug-resistant *T.*

congolense maintained their resistance during several fly passages but not during an equal number of syringe passages though mice (Nyeko et al. 1989).

The passage of *Plasmodium ssp* through the mosquito was also shown to be a bottleneck (Zollner et al. 2006). Resistance against chloroquine (CQR) was found in *P. falciparum* all over the world a few years after introduction of this drug and chloroquine is therefore used against *P. vivax* only. The slow return of chloroquine-sensitive (CQS) genotypes in some malaria regions was explained by either mis-diagnosed *falciparum* cases followed by chloroquine treatment or by a possible cross-resistance of piperaquine (the actual used drug against *P. falciparum*). Both possibilities might keep the selection for CQR genotypes but do not conclusively explain why CQS genotypes returned so slowly (Chen et al. 2008).

Plasmodium might face a similar bottleneck during mosquito passage as trypanosomes do in the tsetse flies. Possibly, the frequency of CQR genotypes decreased so slowly because transmission through mosquitoes also acted as a stabilising mechanism for diversity in *Plasmodium* populations.

VSG switch does not lead to the elimination of tags – a simple model to explain why

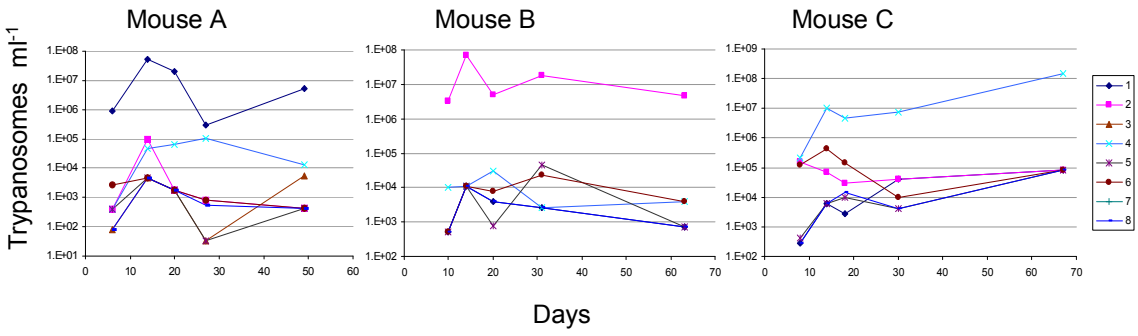

Fig. 7.2: Parasitaemia of each 'tag-population' in the three mouse experiments.

Fig. 7.2 shows the parasitaemia of each 'tag-population' in the three mouse infection experiments (see chapter 3 and Appendix 1, p 115). Minor tags fluctuate similarly to dominant tags but some order of magnitude lower. A very small difference in relative frequency in the initial inoculum is enough that the most dominant tag increased and

dominated the parasitaemia throughout the mouse infection (e.g. in mouse C tags 2 and 4 show a 1.3-fold difference only).

VSG switching did not disturb this pattern, predicting that not only the dominant 'tag-population' but, surprisingly, also the minor 'tag-populations' switched frequently enough to escape the immune response. Here I want to discuss the uniform and the homology-dependent switching pattern, both explained by Lythgoe et al (2007). Uniform switching pattern is present when trypanosomes switch to any new variant at the same rate (Lythgoe et al. 2007). If trypanosomes would switch uniformly the dominant 'tag-population' would elicit a huge diversity of specific antibodies within a very short time. The dominant 'populations' would possibly cope with the resulting activation of the high diversity of specific antibodies but most probably not the minor 'tag-populations'. A homology-dependent switching pattern is present when switching depends on the degree of sequence homology between the new and the actual VSG variant, which results in a hierarchical expression pattern. This switching model is widely accepted and suggested to happen most probably in nature (Lythgoe et al. 2007). A hierarchical VSG expression would ensure that large number of trypanosomes switch to the same VSG-variant, and would first assure the leading position of the dominant 'tag-population' and second the continuous existence of the minor 'tag-populations' as long as all the trypanosomes possess the same hierarchy (see Appendix 2 for further explanations).

I could show this in a simulation using the parasitaemias of the 'tag-populations' from the first week (Appendix 1, Table 9.21, p. 115). The simulation was done with a repertoire of only five different VSG variants a, b, c, d, and e, which were expressed in each 'tag-population' at frequencies of 0.748, 0.2, 0.05, 0.002, and 0.001, respectively. The trypanosome population increased with 1.15 generations $hour^{-1}$ (6 hours for one generation) and decreased with 0.5 generations $hour^{-1}$ when $> 10^7$ trypanosomes expressed one VSG variant (negative growth due to the developed specific immune response). Each parasitaemia peak showed the highest abundance of one VSG variant (Fig. 7.3). Trypanosomes with the dominant tag (tag 1, 2, and 4 in experiments A, B, and C, respectively) showed the highest parasitaemia throughout the simulation for each VSG variant. This pattern confirms what we have observed in Fig. 7.2 and chapter 3.

Simulation of hierarchically independent patterns (= in each 'tag-population', VSG variants have different frequencies) resulted in a different outcome than what we observed. I changed the frequency of the VSG variants in mouse C: Decreasing only variant 'a' to a frequency of 0.56 in the 'tag-4-population' but leaving the frequency in the 'tag-2-population' the two tags co-dominated the parasitaemia in the first peak which does not confirm our result (not

shown). This simulation shows that a very small difference in VSG frequency (1.33 fold) is enough to disturb the observed dominance of one 'tag-populations' when starting parasitaemias are very similar (as 'tag-2-' and 'tag-4-populations' in mouse C are). I hypothesise i) that similar VSGs were expressed at a time in each 'tag-population' and ii) that the frequencies of VSGs expressed were similar among all 'tag-populations'. As discussed in chapter 3, the similar (or identical?) hierarchy of VSG switching in the different 'tag-populations' can be explained by their clonal origin.

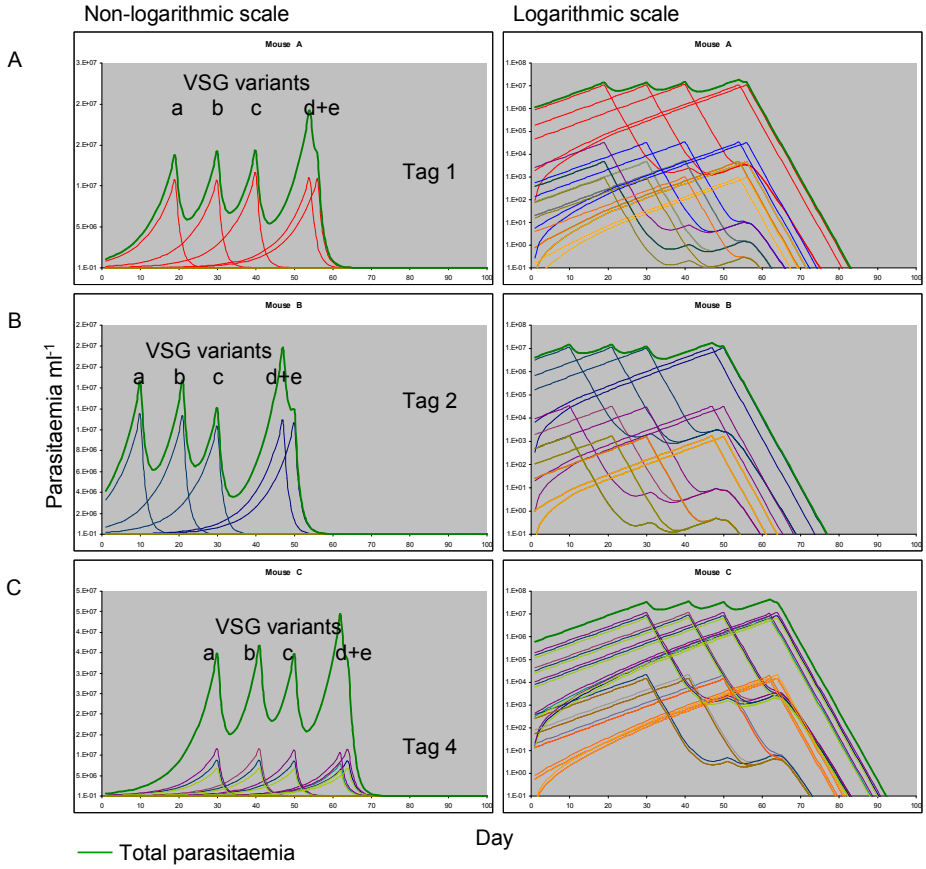

Fig. 7.3: Simulation of VSG switching in the three experiments during mouse infection. The two graphs in each row show the same results on a non-log and a log scale.

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8. Conclusion

When Mary and David Bruce discovered in 1894 that nagana was a disease caused by the protozoan parasite *Trypanosoma*, and that this disease was the same as the 'tsetse fly disease' described some 40 years earlier by David Livingston (in 1858), they were not aware of the complex life cycle that takes place in the tsetse fly. They believed that trypanosomes were transmitted mechanically between hosts in a drop of blood in or on the proboscis of the tsetse flies. Robert Koch (1905) conducted a study in Africa in 1904 where he sampled and dissected tsetse flies. He found that the flies released a drop at the tip of the proboscis when the bulb was squeezed. These drops did not contain blood and were rarely found to be crowded with trypanosomes. Further dissections of tsetse flies revealed that trypanosomes differentiate via several stages in the fly to the mammalian infective stage that were injected into new hosts (Koch 1905). This was later confirmed with some very first transmission experiments by Kleine (1909) who infected different *Glossina* species with different trypanosome isolates and showed, first, that the cyclical transmission needs several weeks and, second that not all combinations of tsetse fly species and trypanosome isolates were equally successful (Kleine 1909, Kleine and Fischer 1913). These scientists from the very early hours of sleeping sickness research already detected that transmission through the tsetse fly is surprisingly inefficient and highly complex.

Since then, investigations to shed light on the interaction revealed a very complex crosstalk between tsetse fly and trypanosomes. Specific gene regulation of the parasite in the insect stage is known, and can be specifically manipulated, and more and more genes have been discovered that are expressed specifically by tsetse flies upon trypanosome infection. Despite all these achievements the transmission through the tsetse flies remains obscure and reports contradict each other, which does sometimes not help to understand the whole complexity. With this thesis I also wanted to participate in this area of science and, typically in science, experiments conducted in different laboratories with different materials sometimes lead to different results and interpretations.

G. m. morsitans from the colony in Bratislava infected with different trypanosomes stocks and stages (bloodstream or procyclic forms) showed no specific up-regulation of attacin and defensin mRNA. Also infections with trypanosome mutants lacking specific procyclins or

having no procyclins, did not lead to a different regulation of these AMPs than wild type trypanosomes did. Challenge with bacteria showed that the flies we used can induce an immune response. This outcome contradicts what is published already and, to some extent, what is accepted widely. I questioned why a tsetse fly should up-regulate an immune response against its midgut infection since a gain of fitness is not seen conclusively in flies that cleared their infection. It is more likely that clearing the parasites from the midgut prevents salivary gland infections where trypanosomes might provoke a reduction in fitness. Experiments regarding the reproductive cycle of tsetse flies have not been performed so far and I realised that this is missing, in particular in combination with trypanosome infection: How is the reproductive behaviour of tsetse flies altered, what effect does an infection of the mother have on her offspring and how is the fitness and resistance to trypanosomes changed in the offspring? Experiments to address these questions need thousands of flies in several replicates. The high costs for the production of tsetse flies (labour intensive, work space and blood supply) can certainly explain the lack of such experiments.

My conclusion is that not all tsetse flies do elicit an attacin and defensin response, and that these two AMPs are not in all circumstances key players in the defence to *Trypanosoma* infections. It is possible that up-regulation of these AMPs happens in some tsetse flies and that this is dependent on the origin of the flies. Differences between tsetse flies can be seen, for example, in the different infection rates of flies originating form different colonies.

The flies we use are fairly permissive since we achieve infection rates of about 50% in the midgut and about 15 to 20% in the salivary glands. These rates are high and also very consistent over several independent experiments. Despite the susceptibility of our flies, trypanosome populations that infect the midgut are severely reduced to a few hundred individuals during the initial phase and may recover afterwards to establish in the midgut. A further reduction of the trypanosome population happens during the migration into the salivary glands. The diversity of the trypanosome population, measured as the relative frequency of inserted tags, is affected differently during the cycle through the fly: while diversity and relative frequency of the tags do not change in the midgut, they are reduced in the salivary glands and the relative frequency is profoundly altered.

The subsequent monitoring of the trypanosome population in a mouse infected by one tsetse fly revealed that many tags were undetectable in the salivary glands. This means that only a few trypanosomes successfully migrated, which led to the altered relative frequency of tags in the salivary glands. Interestingly, the number of different tags remained, and did so also during a long infection period in the mouse. The monitoring of the mouse infection indeed

helped to better understand what happens in the tsetse flies. I consider the migration bottleneck as a substantial population-controlling passage which is central for the evolution of trypanosomes and their population structure. We have seen that this passage very often prevents mutant trypanosomes from effectively colonising the salivary glands (Δprocyclin as well as several PSSA2 mutants, Fragoso et al. in preparation) but also trypanosomes that were cultured for extended periods have lost the ability to be transmitted through the fly. In this work it remains unclear if and to what extent migration of trypanosomes is hampered by an immune response and what genes might be activated specifically.

The finding that 'naked' trypanosomes can pass through tsetse flies raises the question of the function of procyclins. Clearly, trypanosomes with a procyclin coat show a significantly higher transmission rate and therefore have a better chance to be transmitted. Could procyclins have been acquired during an evolutionary process to gain competitive advantages over trypanosomes that had no procyclins in the past? Certainly, this hypothesis is fascinating, but very difficult to test. The ability of trypanosomes without procyclins to infect the salivary glands is by far not an indication of such an evolutionary process. It can be argued that *T. brucei* and *T. congolense* differentiated form a common ancestor. It might really be that each descendent evolved independently distinct insect-stage specific surface coats with similar functions whereas their common ancestor had no or a very basic form of such a coat. If acquiring a procyclin coat increased the selective advantage, it might be controversial when such a coat causes the production of attacin in the tsetse fly, which is suggested to be trypanocidal. In fact, it would make more sense if procyclins would either prevent the release of these AMPs or make the trypanosomes resistant to them.

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9. Appendices

Appendix 1

Flow sheet of the clone AnTat 1.1

Prior and after *in vitro* cloning of AnTat 1.1, trypanosomes were transmitted through tsetse flies (*G. m. morsitans* from Bratislava, Slovakia) and mice (NMRI from RCC, Ittingen, Switzerland). The complete flow sheet is given in Fig. 9.1.

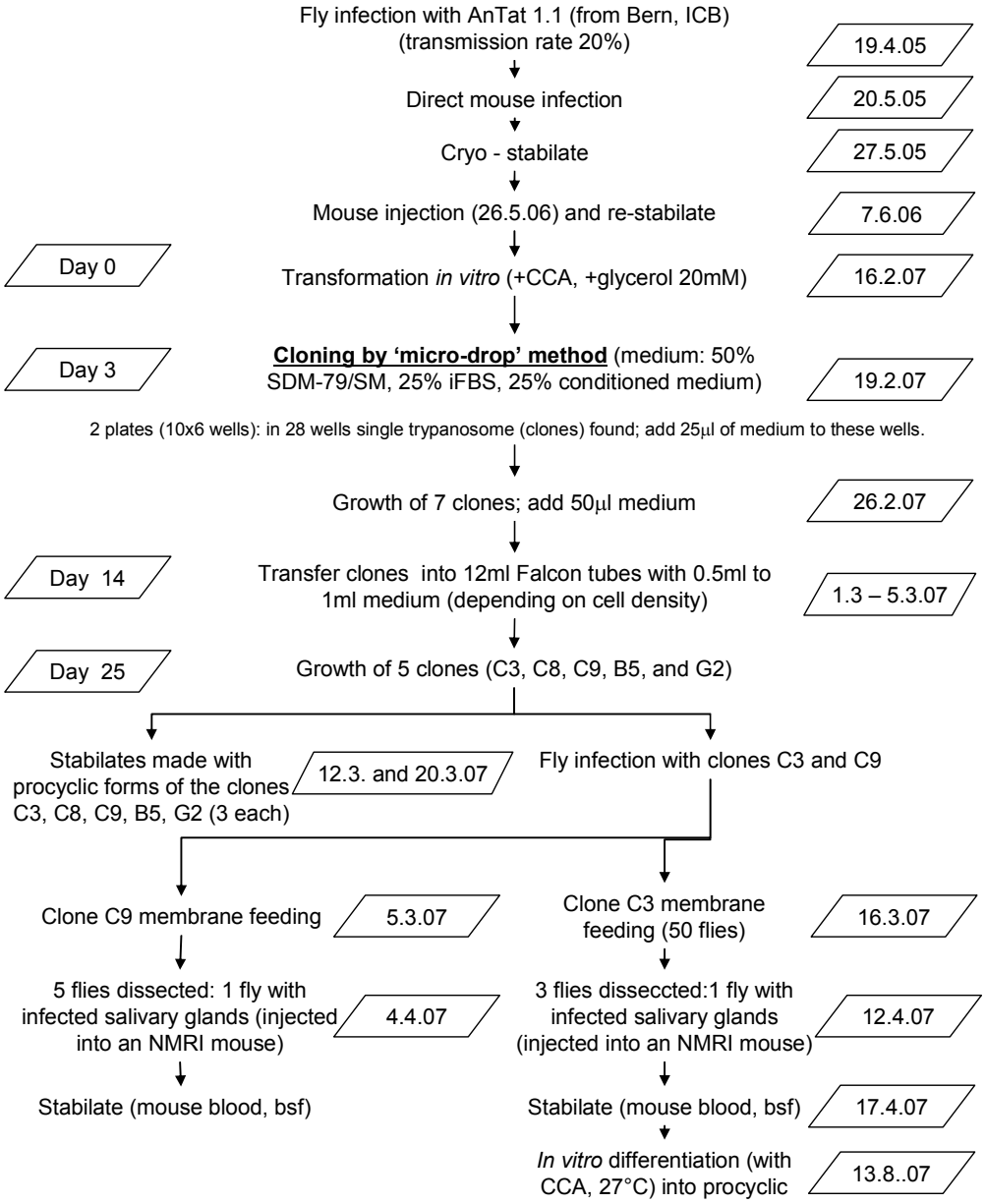

Fig. 9.1: Flow sheet of the cloned AnTat 1.1.

Parasitaemia of each 'tag-population'

A large number of tags were maintained during mouse infection and none of the tags that were injected by the tsetse fly disappeared. With the frequency of each tag I calculated the parasitaemia of each 'tag-population'. For tags that were not detectable in some of the samples minimal frequency averages were taken as an estimate of their minimal abundance (Table 9.1). Fig. 7.2 (chapter 7) shows the parasitaemia of each 'tag-population'.

Mouse experiment A

	Frequency analysed by pyro-sequencing						Frequencies with calculated minimal frequency					Calculated parasitaemia of each 'tag-population'				
	ID 6	ID 14	ID 20	ID 27	ID 49	average frequency	ID 6	ID 14	ID 20	ID 27	ID 49	ID 6	ID 14	ID 20	ID 27	ID 49
Tag 1	9.96E-01	9.97E-01	9.97E-01	7.29E-01	9.96E-01	9.43E-01	9.96E-01	9.97E-01	9.97E-01	7.29E-01	9.96E-01	9.23E+05	5.41E+07	2.09E+07	2.93E+05	5.04E+06
Tag 2	4.27E-04	1.71E-03	0	1.98E-03	0	8.25E-04	4.27E-04	1.71E-03	8.55E-05	1.98E-03	8.55E-05	3.96E+02	9.29E+04	1.80E+03	7.96E+02	4.32E+02
Tag 3	0	0	0	0	1.05E-03	2.10E-04	8.55E-05	8.55E-05	8.55E-05	8.55E-05	1.05E-03	7.92E+01	4.63E+03	1.80E+03	3.43E+01	5.30E+03
Tag 4	4.27E-04	8.57E-04	3.18E-03	2.66E-01	2.62E-03	5.47E-02	4.27E-04	8.57E-04	3.18E-03	2.66E-01	2.62E-03	3.96E+02	4.65E+04	6.68E+04	1.07E+05	1.33E+04
Tag 5	4.27E-04	0	0	0	0	8.55E-05	4.27E-04	8.55E-05	8.55E-05	8.55E-05	8.55E-05	3.96E+02	4.63E+03	1.80E+03	3.43E+01	4.32E+02
Tag 6	2.99E-03	0	0	1.98E-03	0	9.95E-04	2.99E-03	8.55E-05	8.55E-05	1.98E-03	8.55E-05	2.77E+03	4.63E+03	1.80E+03	7.96E+02	4.32E+02
Tag 7	0	0	0	0	0											
Tag 8	0	0	0	1.32E-03	0	2.64E-04	8.55E-05	8.55E-05	8.55E-05	1.32E-03	8.55E-05	7.92E+01	4.63E+03	1.80E+03	5.31E+02	4.32E+02
seqs.	2340	1167	1257	1514	1908											
				minimal frequency average		8.55E-05				**Total parasitaemia**		9.27E+05	5.42E+07	2.10E+07	4.02E+05	5.06E+06

Mouse experiment B

	Frequency analysed by pyro-sequencing						Frequencies with calculated minimal frequency					Calculated parasitaemia of each 'tag-population'				
	ID 10	ID 14	ID 20	ID 31	ID 63	average frequency	ID 10	ID 14	ID 20	ID 31	ID 63	ID 10	ID 14	ID 20	ID 31	ID 63
Tag 1	0	0	7.61E-04	0	0	1.52E-04	1.52E-04	1.52E-04	7.61E-04	1.52E-04	1.52E-04	5.24E+02	1.10E+04	3.93E+03	2.71E+03	7.13E+02
Tag 2	9.97E-01	1.00E+00	9.91E-01	9.96E-01	9.98E-01	9.96E-01	9.97E-01	1.00E+00	9.91E-01	9.96E-01	9.98E-01	3.43E+06	7.20E+07	5.12E+06	1.77E+07	4.68E+06
Tag 3	0	0	0	0	0											
Tag 4	2.90E-03	0	6.09E-03	0	8.43E-04	1.97E-03	2.90E-03	1.52E-04	6.09E-03	1.52E-04	8.43E-04	9.98E+03	1.10E+04	3.14E+04	2.71E+03	3.95E+03
Tag 5	0	0	0	2.61E-03	0	5.21E-04	1.52E-04	1.52E-04	1.52E-04	2.61E-03	1.52E-04	5.24E+02	1.10E+04	7.86E+02	4.64E+04	7.13E+02
Tag 6	0	0	1.52E-03	1.30E-03	8.43E-04	7.34E-04	1.52E-04	1.52E-04	1.52E-03	1.30E-03	8.43E-04	5.24E+02	1.10E+04	7.86E+03	2.32E+04	3.95E+03
Tag 7	0	0	0	0	0											
Tag 8	0	0	7.61E-04	0	0	1.52E-04	1.52E-04	1.52E-04	7.61E-04	1.52E-04	1.52E-04	5.24E+02	1.10E+04	3.93E+03	2.71E+03	7.13E+02
seqs.	1035	1828	1314	1535	1186											
				minimal frequency average		1.52E-04				**Total parasitaemia**		3.44E+06	7.20E+07	5.16E+06	1.78E+07	4.69E+06

Mouse experiment C

	Frequency analysed by pyro-sequencing						Frequencies with calculated minimal frequency					Calculated parasitaemia of each 'tag-population'				
	ID 8	ID 14	ID 18	ID 30	ID 67	average frequency	ID 8	ID 14	ID 18	ID 30	ID 67	ID 8	ID 14	ID 18	ID 30	ID 67
Tag 1	0	0	0	5.05E-03	0	1.01E-03	5.47E-04	5.47E-04	5.47E-04	5.05E-03	5.47E-04	2.74E+02	5.86E+03	2.69E+03	3.91E+04	8.51E+04
Tag 2	3.22E-01	6.78E-03	5.81E-03	5.05E-03	0	6.80E-02	3.22E-01	6.78E-03	5.81E-03	5.05E-03	5.47E-04	1.61E+05	7.26E+04	2.86E+04	3.91E+04	8.51E+04
Tag 3	0	0	0	0	0											
Tag 4	4.27E-01	9.54E-01	9.59E-01	9.89E-01	1.00E+00	8.66E-01	4.27E-01	9.54E-01	9.59E-01	9.89E-01	1.00E+00	2.14E+05	1.02E+07	4.72E+06	7.66E+06	1.55E+08
Tag 5	8.01E-04	0	1.94E-03	0	0	5.47E-04	8.01E-04	5.47E-04	1.94E-03	5.47E-04	5.47E-04	4.01E+02	5.86E+03	9.52E+03	4.24E+03	8.51E+04
Tag 6	2.50E-01	3.90E-02	3.00E-02	1.26E-03	0	6.41E-02	2.50E-01	3.90E-02	3.00E-02	1.26E-03	5.47E-04	1.25E+05	4.18E+05	1.48E+05	9.78E+03	8.51E+04
Tag 7	0	0	0	0	0											
Tag 8	0	0	2.90E-03	0	0	5.81E-04	5.47E-04	5.47E-04	2.90E-03	5.47E-04	5.47E-04	2.74E+02	5.86E+03	1.43E+04	4.24E+03	8.51E+04
seqs.	1248	1180	1033	792	1366											
				minimal frequency average		5.47E-04				**Total parasitaemia**		5.00E+05	1.07E+07	4.92E+06	7.75E+06	1.55E+08

Table 9.1: Raw data of the frequency of the tags in each mouse sample (the five rows on the left) were analysed by pyro-sequencing. The minimal frequency average was calculated and used to estimate the minimal frequency for tags undetectable in some of the samples (the five rows in the middle). Parasitaemia of each 'tag-population' was then calculated for each sample time (frequency x total parasitaemia) in the five rows on the right.

Frequency independent synchronous switching

The average highest and lowest tag-parasitaemia in the mice experiments (chapter 3) were about $2x10^7$ and $9x10^3$ trypanosomes ml^{-1}, respectively, that co-infected a mouse. A switch rate of $>2x10^{-3}$ switches $cell^{-1}$ $generation^{-1}$ was estimated for fly transmissible trypanosome strains (Turner 1999). This predicts that the dominant population would produce about $5x10^3$ trypanosomes with a new VSG in one generation whereas the minor population would achieve only about 45. Hypothesising that all trypanosomes, independent of their tag, would express identical variants of VSG would more likely explain how the 'tag-populations' with different frequencies co-exist. As long as trypanosomes with minor tags express what the trypanosomes with dominant tags do, they can less likely be eliminated since they are similarly selected by the immune response.

Simulation of the VSG switch

Simulation was done in Excel.

Appendix 2

Survival of infected tsetse flies

Tsetse flies were infected with STIB 247 bloodstream forms ($2x10^6$ ml^{-1}) and fed every other day; a control group (non-infected flies) was also maintained in parallel. After ten days each group was split in flies that starved (no nutrition anymore, high stress) and that were fed every second day (no stress). Dead flies were collected every six hours and midguts of infected flies were immediately dissected to classify infected and cleared tsetse flies (Fig. 7.1). Tsetse flies that were infected showed the lowest survival irrespectively if they kept or cleared the infection in the midgut ($p = 0.963$, logrank-test for equality of survival function was calculated with STATA). These flies sowed a significantly lower survival than the starved control flies ($p < 0.0001$, logrank-test). Whether or not they cleared the midgut infection, flies that were fed (no stress) showed no significant difference to each other as well as to the fed control flies ($p > 0.4$, logrank-test, see Table 9.2).
Both groups, fed and starved infected flies sowed a similar midgut infection rate of 60 and 61%, respectively, which demonstrates that trypanosomes did not die before starving flies died. It is, however, interesting that clearing the midgut infection does not improve the fly's survival under stress situation (Fig. 7.1).

		No. Flies	50% survival (in hours)	midgut infection rate
fed	control	41	>339	60%
	mg cured	27		
	mg infected	30		
starved	control	58	237	61%
	mg cured	50	171	
	mg infected	79	168	

Logrank test for equality of survival function

fed	mg indected	mg cured	p = 0.4
	mg cured	control	p = 0.5
	mg indected	control	p = 0.7
starved	mg indected	mg cured	p = 0.963
	mg cured	control	p < 0.0001
	mg indected	control	p < 0.0001

Table 9.2**:** Number of flies used and survival time (50% survival) is given on the left. Logrank-test for equality of survival function (STATA) was used to analyse significance between groups (right table).

Appendix 3

Possible mating related immune response of female tsetse

As a side effect we became aware of immune stimulation in teneral females flies (Fig. 9.2). During pilot experiments we became aware of up-regulated attacin and defensin transcripts in female teneral flies. They were not exposed to a blood meal or any infection and they showed no abnormalities that could hint at injuries. The flies hatched in one cage where male and female were together and were then separated prior to the experiment. Between hatching and male/ female separation the flies may have had the opportunity to mate. It was shown in *Drosophila* that AMPs were up-regulated after mating by a male seminal peptide, the sex-peptide, transferred during copulation. It was identified to be the major agent eliciting transcription of AMP genes (Kubli 2008, Peng et al. 2005). It is very probable that such a peptide is also present in male tsetse fly's seminal fluid.

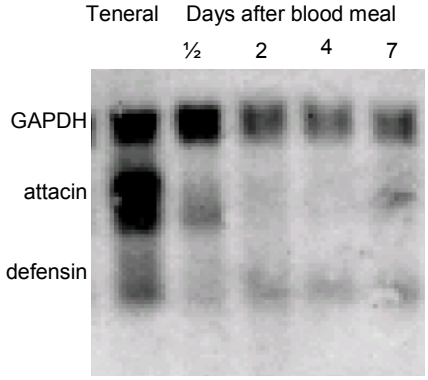

Fig. 9.2: Female tsetse flies received a non-infected blood meal and were dissected 12 hours (½), and 2, 4, and 7 days later. To quantify the transcript level of the flies before feeding teneral female tsetse flies were also included in the analysis.

200 pupae were separated in tubes to prevent that hatching flies have contact with the opposite sex. Hatched female flies were collected and split into four groups for the following experiment: two groups with teneral flies of which one was exposed to males and two groups with fed flies of which one was exposed to males. Flies were dissected 6 hours, 1, 2, and 3 days after the start of the experiment. Total RNA was extracted from isolated fat body, blotted

on a membrane and hybridised with DIG labelled probes of attacin, defensin, and GAPDH described in 'materials and methods' in chapter 5 (Fig. 9.3, upper panel). The results do not show a clear pattern of up-regulation of AMPs in female tsetse flies. In *Drosophila* mating was reported to up-regulate mainly Metchnikowin, an AMP that is regulated by both pathways Toll and Imd (Peng et al. 2005). Possibly we should also include other AMPs in such a study. Comparing the averages of transcript level in each group hints at a slight tendency that both attacin and defensin are increased in teneral mated female flies (Fig. 9.3, lower panel).

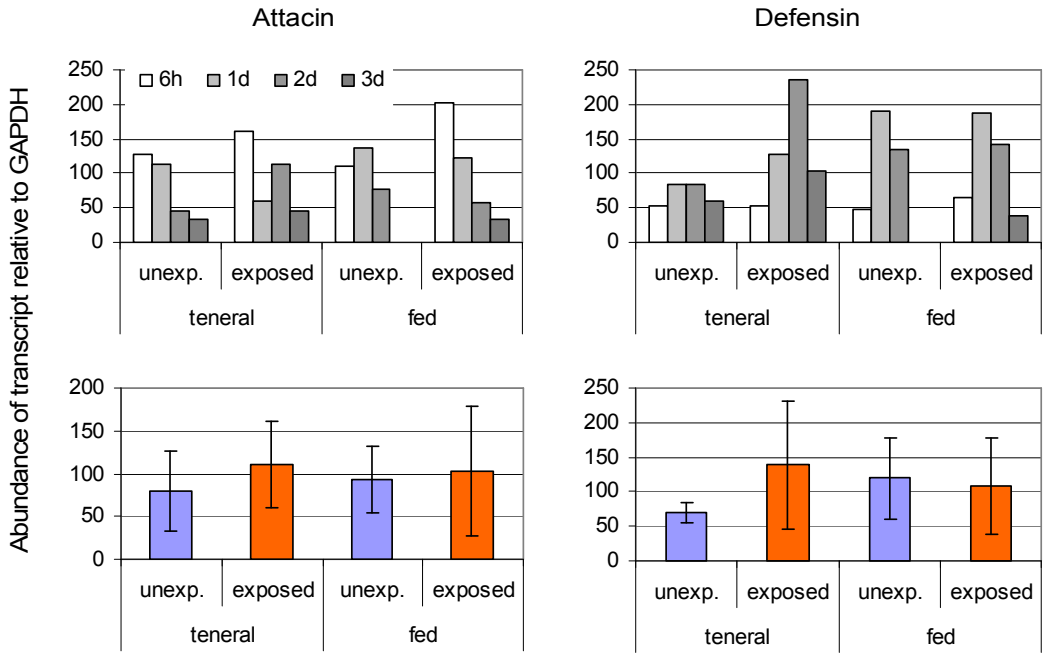

Fig. 9.3: Transcript of attacin and defensin in the fat body of female tsetse flies exposed to males (exposed) or not (unexposed), fed or not (teneral) after 6 hours, 1, 2, and 3 days (upper panel). The averages of the experiments were calculated in the lower panel (as well as the standard deviation). No data are available due to RNA degradation for the fed, unexposed group of flies sampled at day 3.

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11. Curriculum Vitae

Name:	Michael Oberle	Civil status:	married
Date of birth:	03.01.1975	Children:	two (2007; 2010)
Citizenship:	Full – Reuenthal, Switzerland		

EDUCATION

- 2009 – present Advanced training as FAMH specialist in Microbiology, Cantonal Hospital Aarau, Switzerland
- 2005 – 2009 PhD, Swiss Tropical Institute, University Basel and Cell Biology Institute, University Bern, Switzerland
- 2001 – 2002 Master of Science, Swiss Tropical Institute in Basel, University Basel
- 1996 – 2001 Biology I (botany and zoology), University in Basel, Switzerland
- 1992 – 1996 High school in Aarau, Switzerland
- 1982 – 1992 Primary and secondary school in Lenzburg, Switzerland

EMPLOYMENT HISTORY

- 2009 – present Assistant, cand.FAMH, Microbiology, Zentrum für Labormedizin, Cantonal Hospital of Aarau, AG
- 2002 – 2005 Academic Technical Assistant, Swiss Tropical Institute, Basel
- 2000 – 2002 Tutor at the private school 'Interlink', Olten, Switzerland
- 1999 Technical assistant at the Institute of Zoology, Basel

GRANTS

- Roche Research Foundation (1 year)
- Novartis Foundation for Medical Research (1 year)
- Emilia Guggenheim – Schnurr Stiftung, Basel (Roche Genome Sequencer FLX system for high-throughput pyrosequencing technology, 2008)
- Reisefond der Universität Basel (Meeting in Woods Hole, USA, 2007)
- Basler Stiftung für experimentelle Biologie (education and training at Yale School of public Health, Yale University, New Haven, USA, 2005)

PUBLICATIONS

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MEETINGS (POSTER OR PRESENTATION)

- Swiss Trypansomatid Meeting, Leysin, Switzerland (5.2.-7.2.2009),
- Swiss Trypansomatid Meeting, Leysin, Switzerland (31.1. - 2.2.2008)
- 9th Annual EANETT conference, Kunduchi (Dar es Salaam), Tanzania (24.-26.10.2007)
- Kinetoplastid Molecular Cell Biology Meeting in Woods Hole, USA (23.-26.4.2007)
- Symposium: Host-Pathogen Interaction: The Role of Innate Immunity, Bern, Switzerland (15.6.2007)
- Swiss Trypansomatid Meeting, Leysin, Switzerland (8.-10.2.2007)
- 8th Annual EANETT workshop, Kampala, Uganda (18.-20.9.2006)
- SSTMP Students Meeting 2005, Ascona, Switzerland (3.-4.11.2005)
- Swiss Trypansomatid Meeting, Leysin, Switzerland (27.-29.1.2005)
- Swiss Trypansomatid Meeting, Leysin, Switzerland (29.-31.1.2004
- 5th Annual EANETT workshop, Bagamoyo, Tanzania (20.-22.10.2003)
- Swiss Trypansomatid Meeting, Leysin, Switzerland (30.1-2.2.2003)
- Swiss Trypansomatid Meeting, Leysin, Switzerland (30.1-2.2.2002)

I visited lectures and courses given by following lecturers:

Reto Brun, Christian Burri, Ingrid Felger, Tim Albert, Marcel Tanner, Niggi Weiss, Gerd Pluschke, H-P Beck