Aus dem Institut für Parasitologie und Tropenveterinärmedizin
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Tsetse (Diptera: Glossinidae) Bloodmeal Analysis by PCR and Species Differentiation by MALDI TOF MS as Contributions to Rational Vector Control

Inaugural-Dissertation
zur Erlangung des Grades eines
PhD of Biomedical Sciences
an der
Freien Universität Berlin

vorgelegt von

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Tierärztin aus Berlin

Berlin 2012

Journal-Nr.: 3620

Gedruckt mit Genehmigung des Fachbereichs Veterinärmedizin

der Freien Universität Berlin

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Deskriptoren (nach CAB-Thesaurus):
trypanosomiasis, Trypanosoma, Trypanosoma vivax, Trypanosoma congolense, haematophagous insects, Glossina, host preferences, polymerase chain reaction, MALDI-TOF, risk assessment, vector control, Mali

Tag der Promotion: 06.03.2013

Bibliografische Information der *Deutschen Nationalbibliothek*
Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über <http://dnb.ddb.de> abrufbar.

ISBN: 978-3-86387-391-2
Zugl.: Berlin, Freie Univ., Diss., 2013
Dissertation, Freie Universität Berlin
D 188

Choriner Str. 85 - 10119 Berlin
verlag@menschundbuch.de – www.menschundbuch.de

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List of Abbrevations

A	fly abundance per trap per day
AAT	African Animal Trypanosomoses
BLASTN	Basic Local Alignment Search Tool for Nucleotides
(F)BM	(frakturierte) Blutmahlzeiten
BMZ	German Federal Ministry for Economic Cooperation and Development
BVL	German Federal Office of Consumer Protection and Food Safety
CI	confidence interval
CCI	composite correlation index
CIRDES	Centre International de Recherche-Développement sur l'Elevage en zone Subhumide
cyt b	*cytochrome b*
d	day
DAAD	German Academic Exchange Service
DALY	disability-adjusted life years
DNA	deoxyribonucleic acid
DNS	Desoxyribonukleinsaeure
DRS	Dahlem Research School
ELISA	enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
FTA	free to air
G	*Glossina*
Gpg	*Glossina palpalis gambiensis*
Gt	*Glossina tachinoides*
HAT	Human African Trypanosomoses
HP	host preference
IAEA	International Atomic Energy Agency
ILRI	International Livestock Resaerch Institute
IPP	intact protein profiling
IR	trypanosome infection rate in tsetse
MALDI TOF-MS	matrix-assisted laser desorption/ionisation time-of-flight-mass spectrometry
MSP	main spectra
NCBI	National Center for Biotechnology Information
P	probability of an infective tsetse bite
PATTEC	Pan African Tsetse and Trypanosomiasis Eradication Campaign
PCR	Polymerase Chain Reaction
R	infective tsetse bites per bovine per day = tsetse challenge
RFLP	restriction fragment length polymorphism
RS(F)	repas sanguins (fracturés)
RTC	real time classification (software tool)
s.l.	*sensu lato*
spp	species
T	*Trypanosoma*
Tbg	*Trypanosoma brucei gambiense*

Tbr	*Trypanosoma brucei rhodesiense*
Tc	*Trypanosoma congolense*
TTRI	Tsetse & Trypanosomiasis Reseach Institute
Tv	*Trypanosoma vivax*
WHO	World Health Organisation

PREFACE
Introduction and Study Objectives

"You must keep in mind, gentlemen, that HAT is an exceptional disease, that if it is neglected, or forgotten, it can turn into the horrid fire that nearly burnt down your Africa and its tribes during this century, that danger of revivescence will persist as long as there are *Trypanosoma gambiense* and tsetse flies. Those who have not lived recent and yet bygone past have no idea of the danger because it has become very difficult today to show them a classical HAT patient (...)." (Pierre Richet (1904-1983), 1964 in Courtin et al., 2008)

By the year 2000, Human African Trypanosomoses (HAT) or sleeping sickness cases were spiking at a never before seen prevalence of 500 000 cases per year (Cattand et al., 2001). Tsetse fly transmitted trypanosomoses affect not only people but also their livestock, where it is called African Animal Trypanosomoses (AAT) or Nagana. HAT and AAT cause a longstanding wasting disease with severe power loss and debilitation before death if untreated. Consequent economic and social burdens are substantial, eventually preventing affected communities from overcoming poverty (Boelaert et al., 2010). But how could an almost effaced disease like HAT, coined "residual trypanosomosis" back then (Richet, 1962), rise again to become such a threat to human development?

In the 1960's sleeping sickness was not considered a significant public health problem any longer (Molyneux et al., 2010). Unaware of the unseen danger, Richet's warning was disregarded with known consequences. Trypanosomosis reservoirs in people and animals have built up for at least 700 years (de Raad, 2005) and remained occult under persistent disease management. The ongoing collapse of former colonial disease surveillance followed by civil unrest and ceasing control measures caused steady resurgence resulting in a significant increase of HAT case numbers over the years, climaxing by the year 2000 (Malvy and Chappuis, 2011). AAT on the other hand, has existed long before trypanosomes jumped the species barrier to men (Stevens et al., 1998). While African game animals have developed resistance, AAT outbreaks have been rampaging amongst farm animals in Africa from the beginning of agricultural development until today (Steverding, 2008).

As tsetse-transmitted diseases with zoonotic potential, control efforts need to be aimed at the vector and the pathogen in human and animal carriers. But African trypanosomoses affect people and their livestock in rural and peri-urban parts of subsaharan Africa where medical and veterinary health care is not always accessible. On these grounds the WHO developed a *Global plan to combat neglected tropical diseases 2008-2015 (WHO, 2007)* with the core aims being the strengthening of veterinary public health structures and improving vector control.

Rational control strategies rely on the definition of disease hot spots and the identification of tsetse habitats. Trypanosomosis risk assessment combines data sets allowing a better understanding of a disease scenario (Symeonakis et al., 2007) revealing the most important trypanosomosis drivers in a specific region. One core component of trypanosome transmission is the host preference of local tsetse (Davis et al., 2011) which is determined by analysis of the flies' gut contents. Regular risk models (Milligan and Baker, 1988; Rogers, 1988) are bulky and data acquisition often is very laborious and thus expensive. A simplified formula with host preference as the core component next to apparent density and fly infection rate as the tsetse challenge (Snow and Tarimo, 1983) could simplify the decision making process before any vector intervention, rendering risk assessment feasible for small-scale projects at the community-level.
However, bloodmeal analysis of tsetse is the most challenging data to obtain under field conditions when compared to fly numbers and fly infection rate. Besides, serologically well-established methods often bear problems as cross-reactivity and lengthy laboratory analysis

(Rurangirwa et al., 1986; Clausen et al., 1998). Enhancing specificity and simplifying the process by applying PCR would therefore improve current methods. Species-specific PCR of the mitochondrial *cyt b* region has been developed to identify vertebrate DNA for forensic questions (Parson et al., 2000). Recently, several *cyt b* primers were applied for bloodmeal identification in biting midges (Bartsch et al., 2009), which proved as a reliable method to track feeding patterns in insects.
In Sikasso, southeast Mali, mainly cotton is cultivated by the use of Zebu cattle, whose draught power is constrained by AAT and multiple drug resistances (Geerts et al., 2001). In the course of a pilot project dealing with best bet strategies to contain AAT in cattle of the Sikasso region (Mungube et al., 2012), a field study was conducted collecting entomological data on tsetse at cattle watering sites in and around the villages.

The aim of this field study is testing a simplified tsetse challenge formula for suitability with the corresponding AAT herd prevalence by applying collected entomological data that include refined bloodmeal analysis by PCR.

Another important component of rational vector control is the identification of fragmented tsetse populations, currently done by microsatellite DNA markers, in order to assess the risk of fly re-invasion of cleared areas (Solano et al., 2010). Recent developments in the field of proteomic analyses allow accurate microbial identification much more rapidly than by PCR (Hortin, 2006). However, tsetse flies have not been specified by mass spectrometry yet. Successful species identification would be the prerequisite for high-throughput tsetse diagnostics, perhaps up to the population-level and for identifying trypanosome species and incorporated bloodmeals.

Based on these perceptions, the objective of this work is to further improve tsetse laboratory analysis contributing to rational vector control through tsetse species identification by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI TOF MS).

This cumulative thesis is structured as follows:

Chapter 1 provides a review on the current state of knowledge of HAT and AAT history, disease mechanisms, tsetse biology, vector control means, trypanosomosis risk assessment, laboratory methods for analysing tsetse bloodmeals and species identification by MALDI TOF.
Chapter 2 applies an adapted tsetse challenge formula in 2 villages of southeast Mali drawing on entomological data of tsetse that were trapped at cattle watering points. Their incorporated bloodmeals were analysed by species-specific *cyt b* PCR and the calculated tsetse challenge is checked for accuracy with the AAT prevalence of proximate cattle herds.
Chapter 3 reveals unexpectedly high proportions of multiple host feeding and trypanosome infection rates of *Glossina (G.) tachinoides* compared to *G. palpalis gambiensis* of the Sikasso region discussing possible impacts on trypanosomosis transmission.
Chapter 4 deals with the establishment of a reference database for 5 tsetse species by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI TOF MS).
Chapter 5 contains a summarising discussion on the results of the previous chapters and provides a perspective on how the achievements of this work can be applied for rational vector control.

CHAPTER 1
Literature Review

1.1 Relevance of Human African Trypanosomosis (HAT) and African Animal Trypanosomosis (AAT)

HAT-impact is counted in DALY's (disability-adjusted life years), the sum of years of potential life lost due to premature mortality and the years of productive life lost. The DALY's due to HAT amount to 1609 in Africa for the year 2004, ranking second amongst the other neglected tropical diseases (WHO, 2008). However, the cost per adverted DALY is ranging between 12 and 24 US$ which is obviously less than the averaging 2000 US$ in the case of Dengue, another vector-borne disease (Conteh et al., 2010). HAT containment is therefore deemed feasible within the next years provided present containment efforts will not seize again (Nimmo, 2010).
Damage through AAT in livestock can be estimated best through pecuniary losses because large-scale veterinary public health records do not exist. AAT is responsible for an estimated loss of US$ 4.5 billion annually through decreased animal work power, meat and milk production loss and the expenditure on trypanocidal drugs (Kristjanson and de Leeuw, 1999; Eisler et al., 2003). Containment costs for AAT are difficult to estimate because case numbers are immense and unlike HAT there is no gold standard treatment procedure. Disease control plans require laborious studies of the local conditions in order to choose the most successful method (Van den Bossche, 2001). Contrary to HAT, AAT eradication is not considered a realistic goal in the near future due to poor co-ordination of available control means (Torr et al., 2005).

Disease containment measures have been frequently applied on the continent throughout the decades, either by colonial institutions, international organisations or African governments. The foreign powers' organisation allowed large scale control missions with considerable success: the French colonial administration organised mobile teams that performed a stringent HAT case detection and treatment policy (Courtin et al., 2008), while British authorities concentrated on vector control (Hide, 1999). On the other hand, colonisation certainly triggered HAT and AAT outbreaks and its spreading (Steverding, 2008). Political conflicts, missionary interventions, large-scale cattle trade and the forced placement of plantation workers caused migrations, which greatly contributed to trypanosomosis dissemination (de Raad, 2005; Maudlin, 2006).

Until today, high disease prevalence is linked to historic foci where outbreaks are triggered by violent conflict and poverty (Fevre et al., 2004). The ongoing war-like conflicts in the Democratic Republic of the Congo where 90% of today's cases origin is a current proof of those mechanisms (Tong et al., 2011). Correspondingly, recent AAT outbreaks in Uganda and Sudan are associated with political unrest and long distance cattle movements (Okoth, 1999; Salim et al., 2011). Modern challenges in Trypanosomosis management include climate change (Moore et al., 2012) and extensive land use due to demographic pressure (Van den Bossche et al., 2010), greatly affecting tsetse distribution.
Obviously, the triggers of human and animal trypanosomoses are cross-linked: poor public health services, climatic changes and socio-economical pressure (Courtin et al., 2008; Tabachnick, 2010). Both HAT and AAT transmission rely on the interaction between human and animal hosts, the tsetse fly (*Glossina* spp.) as vectors, and the pathogen *Trypanosoma* spp. Thus, the apprehension of HAT and AAT features is the key to developing precise containment measures; best to be achieved by studying the past and applying learned lessons in the future (Molyneux et al., 2010). "Neither the past nor the future is best served by misunderstanding or by ignorance." (Rogers et al., 1994)

1.2 Epidemiology of Human African Trypanosomoses (HAT)

The prevalence of HAT or sleeping sickness before the colonial era is often a subject of assumptions but there are reports that the disease has been endemic in Africa since the Middle Ages. A report, written in 1373 by the Arabian historian Ibn Khaldun, mentions HAT symptoms in a Malian emperor describing the long-lasting lethargy before his death of "a disease that frequently befalls the inhabitants" (Steverding, 2008). First European reports about sleeping sickness derive from slave traders of the 18th century who learned from Arabian slave traders that swollen lymph glands on the back of the slaves' necks were signs for sleeping sickness (Cox, 2004). It took two more centuries before sleeping sickness trypanosomes and the tsetse fly were linked.

The development of microscopes enabled Sir David Bruce to identify *Trypanosoma (T.) brucei* as the causative agent for Nagana in 1895 before he provided evidence for tsetse flies as vectors in 1902 (de Raad, 2005). Robert Michael Forde discovered the first human-related trypanosomes in 1901 but believed they were helminths. In 1902, Joseph Everett Dutton eventually identified them as trypanosomes and named them *T. gambiense,* since they were isolated from a steamboat captain from The Gambia. But it was the Italian Aldo Castellani who postulated first that *T. gambiense* actually caused sleeping sickness (Cox, 2004; Steverding, 2008). In 1909 cyclical transmission was proven by Friedrich Karl Kleine by showing the development of *T. brucei* in tsetse flies after Robert Koch recognised *T.* spp. in the flies during his studies in present day Tanzania (Wenk, 2003). A second human pathogen was found in 1910 when John William Watson Stephens and Harold Benjamin Fantham inspected an isolate from a patient in former North Rhodesia. The increased virulence in contrast to the previously identified West-African trypanosomes made them believe to have found a new species, *T. rhodesiense* (Stephens and Fantham, 1910).

Today, the agent that is causing chronic sleeping sickness is classified as *Trypanosoma brucei gambiense (Tbg)* or West African sleeping sickness and the one responsible for the acute form as *Trypanosoma brucei rhodesiense (Tbr*) or East African sleeping sickness. Exposure to HAT occurs through the bite of an infected tsetse but other transmission-ways are deemed possible, too. Mother-to-child infection (Lindner, 2010) and mechanical infection through other blood-sucking diptera or laboratory equipment have been reported (Herwaldt, 2001), although these cases are rare exceptions as are non-endemic ones in military personal or tourists (Migchelsen et al., 2011).
Both forms develop slowly after an infectious tsetse bite but especially *Tbg*-infections persist for months or even years post-exposure before neurological symptoms begin to show, while *Tbr* is characterised by acute symptoms (Wenk, 2003). HAT occurs in two stages, the first one when the parasite develops in the patient's blood and lymph, resulting in constitutional symptoms like fever, aching joints and general indisposition. During this stage pentamidine is given in the case of *Tbg* and suramin for *Tbr*, respectively. Both drugs were discovered in the first half of the 20th century and provoke severe side-effects but they are generally better tolerated and easier to apply than drugs of the 2nd stage (Kennedy, 2008). Second stage symptoms are caused by the trypanosomes' invasion of the central nervous system leading to widely ranging symptoms as personality changes, general lethargy, increased aggression, and changed sleeping patterns, giving the disease its name. At this point, treatment becomes risky, hard to administer and is often irremediably due to the toxicity of the few available drugs (Cattand et al., 2006). Recently, a simplified regimen of combined therapy was introduced applying nifurtimox together with eflornithine, which is meanwhile considered gold-standard for late-stage treatment (Lutje, 2010). Still, resistance has been reported for all of the above (Barrett et al., 2011) and research for new potential drugs, as the promising diamidine series (Barrett, 2010; Teka et al., 2011) or halo-nitrobenzamides (Hwang et al., 2010), is needed.
HAT diagnostics and staging, both crucial for a correct treatment, are complicated because symptoms often are unclear and *Tbg* and *Tbr* are not morphologically distinguishable. Simple test kits, as the Card Agglutination Trypanosomosis Test are only available for *Tbg* (Matovu

et al., 2012) and exhaustive screenings are necessary in order to identify occult early stage patients. *Tbr* diagnostics still rely on parasite detection in the patients' blood or lymph and specific clinical signs (Kennedy, 2008). A powerful vaccination has not been developed yet which is mostly due to the trypanosomes' possession of variable surface glycoproteins leading to frequent alterations of potential target sites (La Greca and Magez, 2011).

Prevalence and distribution of West African sleeping sickness has endemic features. *Tbg*-infected patients are able to continue working for months where they will most likely have contact to susceptible tsetse populations, for instance when fishing (Kohagne et al., 2011). Therefore, transmission of West African sleeping sickness seems predominantly a man-fly cycle although *T.b.g.* is found frequently in domestic animals (Mehlitz et al., 1982; Makumyaviri et al., 1989; Noireau et al., 1989; Guedegbe et al., 1992; Simo et al., 2006; Massussi et al., 2010; Wissmann et al. 2011). On the other hand, the impact of animal reservoirs on West African sleeping sickness is rarely documented and considered subordinate to the man-fly cycle (Njiokou et al., 2010).
Tbr-patients are quickly too ill to return to tsetse-infested areas and cannot contribute alone to the transmission cycle. Wild animals, mainly bovids, form the natural reservoir for East-African sleeping sickness leading to epidemics when people penetrate into formerly unoccupied land (Bursell, 1973; Anderson et al., 2011). Another main reservoir consists of cattle, which form a domestic transmission-cycle when paired with peri-domestic tsetse, for instance around market places (Fevre et al., 2001; Hutchinson et al., 2003). The first recordings of East African sleeping sickness outbreaks begin with major epidemics in 1896 and 1906 in Uganda (Hide, 1999). The epidemics could be linked with a drastic decline in cattle numbers due to Rinderpest, which caused infected tsetse turning to man (Okoth, 1999; de Raad, 2005). Outbreaks keep reoccurring in this region every few decades (Magona, 2011) and there seems to be a trend towards overlaps of *Tbr*- and *Tbg*-transmission-cycles, leading to new containment challenges (Jannin, 2005).

Presently, the WHO and the Organisation of African Unity have turned their focus on early-case detection, improved drug-access and vector control. Results are improvements in surveillance and monitoring structures, the free access of trypanocides provided by Bayer HealthCare and Sanofi-Aventis and the establishment of the Pan African Tsetse and Trypanosome Eradication Campaign (PATTEC) (Kennedy, 2008). Recent control efforts proved to be successful when in 2009 HAT cases went under the 10. 000 case-mark for the first time in 50 years (WHO, 2012). While HAT may not be so neglected any longer, the same does not apply to AAT.

1.3 Epidemiology of African Animal Trypanosomoses (AAT)

Early veterinarian records from Egypt, dated between 3000 BC and 2000 BC, possibly mention African Animal Trypanosomosis (AAT) when describing an ailment in cattle with the advice to use a certain ointment for cure (Steverding, 2008). The long co-existence of bovids and trypanosomes in Africa of roughly 7000 years (Leak, 1998) has led to a certain immunological adaption to AAT.
For that reason, a relative trypanotolerance occurs in indigenous taurine, non-humped cattle (Clausen et al., 1993; Mattioli and Wilson, 1996) while a relative AAT tolerance has been observed in game animals, which form the natural reservoir of African trypanosomes (Mulla and Rickman, 1988). In contrast, the larger, humped *Bos indicus* or Zebu cattle remained trypano-susceptible until today because they were introduced much later, in *AD 669* (Leak, 1998). Furthermore, Fulani herdsmen traditionally avoided tsetse infested areas during their migrations and therefore bypassed trypanosome infection (Steverding, 2008).
A longstanding host-parasite-relationship as the adjustment of African game to AAT is sensitive to any changes in the eco-system. As soon as immuno-naive individuals enter the habitat, the disease scenario can easily turn from equilibrium to outbreak (Roelants et al., 1987). In the colonial era of the late 19th century for instance, foreign powers promoted large-

scale cattle farming and animal traction power. This was pushing agriculture towards regions where sufficient annual rainfall and vegetation provided suitable grounds not only for crops but also for tsetse flies (Starkey, 2000), leading to the major outbreaks at the turn of the previous century. In the course of the following trypanosomosis research Alphonse Broden and Hans Ziemann discovered the main cattle pathogens *T. congolense* and *T. vivax* in 1904 and 1905, respectively (de Raad, 2005).

Until today, the most important pathogenic agents affecting cattle are *T. congolense, T. vivax* and to a lesser extend *T. brucei s.l.,* whereby the latter is frequently found in cattle but not primarily pathogenic to them. Cattle as a reservoir must not be ignored because African livestock as camels and equines are severely affected by *T. brucei brucei,* not to mention their role in HAT epidemiology. The remaining African trypanosomes *T. simiae, T. suis, T. godfreyi* and *T. equiperdum* affect domestic and companion animals but have little importance for cattle (Coetzer, 1994).
AAT or Nagana is endemic throughout sub-saharan Africa with epidemic features depending on the pathogen, tsetse distribution and the susceptibility of cattle, whereby multiple infections frequently occur (Mekata et al., 2008; Dayo et al., 2010; Seck et al., 2010; Salim et al., 2011). *T. congolense* generally causes a chronic wasting disease while *T. vivax* leads to a rapidly fatal disease in West Africa contrary to East and Central Africa. There, *T. vivax* infection leads to a mild disease with exceptions of occasional, fatal outbreaks in Kenya (Leak, 1998).

Trypanosomes are circulating within a widely ranging host reservoir, consisting mainly of domestic and wild bovids or suids (Anderson et al., 2011). *T. congolense* mostly relies on cyclic transmission by tsetse, while *T. vivax* may also be transmitted mechanically through interrupted feeding by tabanids, *Stomoxys* spp. and also by tsetse themselves (Desquesnes and Dia, 2003).
Cattle become infected when in contact with tsetse, either at watering sites or in the savannah while grazing. The general incubation period lies between 8 and 20 days, with *T. brucei brucei* being highly variable (Coetzer, 1994). Clinical signs are often unspecific involving intermittent fever, oedema, lacrimation, a rough coat and, most important, anaemia. That is why clinical diagnosis can be difficult but is most precise when detecting anaemia, for instance through the FAMACHA© eye colour chart (Malan et al., 2001; Grace et al., 2007). More accurate diagnoses are useful for scientific research but they are barely field applicable for everyday use as the examination of fresh or stained blood films under the light microscope, the haematocrit centrifuge technique, the dark ground buffy coat technique (Paris et al., 1982), serological means (Goto et al., 2011) or by PCR (Desquesnes and Davila, 2002).

AAT treatment involves traditional applications as carving figures into the animal's skin or applying local herbs (Cheikh-Ali et al., 2011). However, most farmers rely on 2 available therapeutic options for Nagana treatment: isometamidium chloride as prophylactic treatment, while diminazene aceturate can be used as a curative and sanative. Unfortunately, chemo-resistance is wide-spread and new therapeuticals are not available (Geerts et al., 2001).
On that account, current research projects propagate rational drug use amongst farmers and para-vets (Clausen et al., 2010). The definition of situation-related best-bet strategies (Mungube et al., 2012) helps farmers with the application of effective AAT containment measures. One of these options, especially suitable for regions with high trypanosomosis prevalence, is the adoption of trypanotolerant breeds that keep losses through AAT at a minimum (Maichomo et al., 2009). Unfortunately, the majority of farmers prefer tall, humped Zebus over the smaller taurine cattle because of their seemingly superior performance in traction and milk production (Grace et al., 2009).
Partially, farmers adopt cattle spraying with insecticides as disease prevention (Bekele et al., 2010; Bouyer et al., 2011). This method may in some cases be an example of applied veterinary public health because of the animal reservoir role in HAT epidemiology (Welburn and Maudlin, 2012). In Uganda for instance, a combination of trypanocidal treatment and

insecticide spraying of domestic animals led to a drastic decline in human sleeping sickness cases (Magona, 2011).
Beyond particular project interventions though, the responsibility for AAT treatment is mostly carried by the farmers alone because of lacking administrative structures (WHO, 2007). The development of cost-efficient AAT containment strategies, either designed for farmers or for large-scale interventions, cannot be mastered without knowledge about the vector and its behaviour.

1.4 Biology and Systematics of Tsetse Flies (*Glossina* spp.)

Glossina spp. possibly have evolved on the supercontinent Gondwana more than 100 million years ago since tsetse-like fossils have been found on several continents, the oldest ones dating back approximately 35 million years (Cockerell, 1917; Krafsur, 2009). Within the suborder of flies, the *Brachycera*, tsetse are grouped in the superfamily *Pupipara* alongside louse flies due to their unique reproduction cycle involving viviparity and the production of 'milk' in pregnant females as a nutrient for the larva. Unusual for most insects is the fact that both sexes suck blood.
Tsetse size varies between a length of 0.5 cm and 1.5 cm. They differ from common flies through their characteristic posture when resting with one wing superimposed on the other, the biting apparatus pointing forward. Veins of the wing form the significant 'hatchet' shaped cell. Another significant feature is the biting apparatus, the proboscis. It consists of labium and labellum with the labrum at its end, forming a tube around the hypopharynx. The palpi shield the feeding apparatus when resting and are folded up while the proboscis is lowered onto the host's skin when feeding (Coetzer, 1994).

Within the genus *Glossina* classification is traditionally based on the flies' morphology and site of origin. Accordingly, three subgenera are described including more than 30 species and sub-species (Leak, 1998). They are grouped according to their respective habitat: the *morsitans* group inhabits savannah landscapes, the *palpalis* group river banks and the *fusca* group tropical forests.
Recent findings applying genomic means challenge traditional taxonomy in some cases as in terms of group affinity, subspecies status or evolutionary origin (Gooding and Krafsur, 2005). However, these findings are of minor importance for trypanosomosis control, whereas other tsetse features can greatly affect disease transmission and the success of vector control means.

1.4.1 Live Cycle

The mean life span of male tsetse is around 4 weeks while the one of females comes to 8 weeks (Coetzer, 1994). After hatching from the puparium and subsequent hardening of the chitin shell, tsetses seek their first bloodmeal. Then or shortly afterwards when resting mating takes place whereby females store the sperm for a livelong fertilization of their eggs. Four days old females ovulate and deposit a third stage larva after another 10 days. Every ovulation of the 4 ovarioles follows a strictly regular pattern, which can be used for age determination in females (Saunders, 1960). Within an hour after larviposition the cuticle hardens and forms the puparium. Pupae usually mature on soft ground in the shade with sufficient humidity. Their development is temperature-dependent at a rate of approximately 30 days at 24°C (Leak, 1998).
Accordingly, tsetse reproduce at a rather low rate which makes them k-strategists contrary to most other insects who are r-strategists for producing large quantities of eggs (McArthur and Wilson, 1967). This makes them particularly vulnerable to any changes in the environment affecting population mortality. If for instance the daily mortality of females reaches 3%, the respective population won't be able to survive, provided no reinvasion takes place (Hargrove, 1988). On the other hand, their longevity compared to most insects plays an important role in

disease transmission since once infected flies basically remain infected for their entire lives (Rogers et al., 1994).

1.4.2 Trypanosome Infection in Tsetse

Before a tsetse infects a vertebrate host with trypanosomosis through its bite trypanosomes undergo cyclic development in the fly. Unfed, very young flies or teneral tsetse, and starved flies are more susceptible to trypanosome infections than older flies (Kubi et al., 2006; Walshe et al., 2011).
Trypanosome development from procyclic bloodstream forms acquired from the mammalian host to infective metacyclic trypanosomes in the fly involves 7 stages (Vickerman et al., 1988). The shortest development cycle of 5-10 days is found in *T. vivax*, where the entire development takes place in the proboscis. In *T. congolense* the procyclic forms multiply in the tsetse midgut before entering the proboscis, taking roughly 15 days. The most complicated development undergo *T. brucei s.l.* involving stages in the salivary glands as well with a mean duration of 20 days (Leak, 1998).
Due to the respective development cycles, tsetse dissection and microscopic inspection of hypopharynx, midgut and salivary glands reveals the infection status of a tsetse fly (Lloyd, 1924). Although PCR is useful for differentiating various trypanosome strains (Ferreira et al., 2008; Enyaru et al., 2010) fly dissection shows satisfactory sensitivity and is predominantly used in field surveys (Ouma et al., 2000).
Not every trypanosome ingestion by the fly leads to infectious stages in the case of *T. congolense* and even more so in *T. brucei s.l.* where often only 3% of the ingested trypanosomes mature (Rogers, 1988; Peacock et al., 2012). This so-called refractoriness shows in relatively low infection rates in flies compared to the Nagana infection rate of local cattle. It especially occurs in flies of the palpalis group, involving physiological aspects in the digestive tract (Rogers, 1988).

1.4.3 Physiology

Fundamental research on tsetse metabolism, maturation and their senses has revealed vector control sites and set standards for laboratory rearing of tsetse in the past. A core component is bloodmeal processing, the only source providing energy and fluid for the flies. As blood-sucking insects, tsetse must avoid erythrocyte agglutination, so saliva is discharged when probing on a host. Saliva of tsetse contains anticoagulant, vaso-dilating enzymes and platelet aggregation inhibitors (Van Den Abbeele et al., 2007). These factors also provide a suitable precondition for trypanosome infections of the host (Caljon et al., 2006).
Meal uptake drastically increases the fly's weight hindering flight performance, which is why excess water is excreted rapidly after feeding. Further processing in the alimentary tract of tsetse decompounds the bloodmeal in order to metabolise it in a proline-based cycle as energy supply for the flight muscles and as storage in the form of fat bodies (Leak, 1998). The gut also contains vital, meal processing factors that are often anti-trypanosomal agents, such as proteases (Nayduch and Aksoy, 2007) and bacterial commensals (Wang et al., 2009; Weiss et al., 2012).
Signal transduction in tsetse is greatly based on pheromones and hormones affecting physiological functions and the communication between individuals. Sex-recognition and mating behaviour for instance, is based on the interaction between female pheromones expressed on the cuticle and male chemoreceptors on the wings and tibiae (Wall, 1989; Carlson et al., 2005). The most important example of hormonal influence on tsetse is the juvenile hormone of undeveloped flies preventing insect maturation (Clarke, 1980).
As diurnal insects, tsetse rely on a good vision being able to sense even slight movements (Turner and Invest, 1972). It has been observed repeatedly that tsetse react to visual stimuli by avoiding striped objects, having a preference for certain colour combinations, following moving objects and seeking shady locations as resting sites (Leak, 1998).

Tsetse are challenged to maintain their water balance in order to avoid desiccation. Hygro- and thermo-receptors enable tsetse to sense relative humidity and temperature. The fine adjustment to certain climatic conditions leads to significant differences in the ability to sustain heat and drought among savannah, forest and riverine tsetse (Kleynhans and Terblanche, 2011).

1.4.4 Tsetse Distribution

Excavations in Colorado and Germany revealed tsetse fossils from 30-40 million years ago, leading to the assumption that tsetse have existed almost worldwide (Cockerell, 1917; Krafsur, 2009). Today, tsetse are exceptionally present in a belt-shaped area of roughly 10 million km^2 of subsaharan Africa traversing the continent between 14 degrees North and 20 degrees South latitude (Swallow, 1998). There, tsetse find suitable climatic conditions of temperatures between 20-30°C and sufficient rain fall, following the 508 mm annual isohyet (Leak, 1998).
Each tsetse group is bound to a more or less specific landscape within the tsetse belt, where climate is the most significant criterion (Rogers et al., 1994). The *morsitans* group inhabits arid or semi-arid landscapes opposed to *fusca* group tsetse that thrive in very humid, thickly forested regions. *Palpalis* group tsetse survive in a much wider range of climatic conditions compared to the other 2 groups, tolerating semi-arid conditions as well as humid ones. But they are also vulnerable to desiccation (Kleynhans and Terblanche, 2011), resulting in a dependency on open water sources and shady vegetation as found at river galleries.
Due to the characteristic vegetation of the respective habitats, tsetse distribution, up to the level of fragmented populations, can be predicted through geographical information systems with supporting climatic data (Rogers and Williams, 1993; Bouyer et al., 2006). Yet, remotely sensed data needs to be backed up by entomological surveys because neglect of biotic factors as animal herding techniques and host abundance have led to miscalculations with deviations up to 70% from the actual fly distribution (Rogers et al., 1996).
As a species living in ecological niches, tsetses are vulnerable to any changes of their environment. Demographic growth for instance resulted in drastic land use changes, which have led to a decline of tsetse populations (Bouyer et al., 2006) or distribution shifts, respectively (Harrus and Baneth, 2005; Courtin, 2009). Although human settlements generally disturb tsetse habitat, the *palpalis* group seems less affected compared to the other groups because of their ability of adapting to peri-urban conditions (Fournet et al., 1999; de La Rocque et al., 2001). Environmental adjustment of tsetse as obligate bloodsuckers also includes their behaviour towards hosts.

1.4.5 Feeding Behaviour

Roughly every three days a tsetse fly needs a bloodmeal in order to maintain its vital functions. Tsetse feeding behaviour is closely linked to host-related components as diurnality, odour, size, shape, colour and fly-defence reactions (Colvin and Gibson, 1992). Generally, a hungry fly first detects the odour of a suitable host up to long distances before visual stimuli result in a landing response (Willemse and Takken, 1994). Landing takes place most likely on the lower body parts of the host as its feet (Torr et al., 2007a). A not too smooth, warm surface induces the probing for a bloodmeal through the insertion of the proboscis into the host's skin (Leak, 1998). If the fly does not find a capillary or if it is disturbed by defensive host movements, it will withdraw the proboscis and probe again. Interrupted feeding is more likely to occur in tsetse than in other biting insects due to their increased sensitivity to host reactions (Schofield and Torr, 2002) leaving epidemiological consequences to be explored.
Certain odour components lead to the attraction of tsetse as for instance rumen metabolites (Harraca et al., 2009) while others are repellent to them such as waterbuck-specific odours (Gikonyo et al., 2002). When approaching the host, visual stimuli gain importance as large shapes like grown cattle are preferred over the smaller ones of calves (Torr et al., 2007b).

Vertical structures seem to be avoided by *morsitans* group tsetse while *palpalis* group flies are attracted by both horizontal and vertical shapes (Tirados et al., 2011). These findings are useful for designing tsetse catching devices in order to conduct epidemiological studies.

1.5 Sampling Tsetse

Analysing tsetse catches provides indispensable information on local fly ecology and trypanosomosis risk before and after interventions. Early sampling techniques involved human catchers supplied with hand-nets. Even when refined through the use of oxen and predetermined routes, it became obvious that those so-called fly-rounds were biased because of the repellent effect of humans especially on *morsitans* group tsetse (Hargrove, 1976).
Further development included traps that mimicked oxen until cheaper and compact ones as the biconical trap were invented in the 1970's (Challier and Laveissière, 1973; Leak, 1998). Traditional traps rely on visual stimuli through the use of blue and black cotton (Green, 1990), possibly attracting tsetse that are seeking a resting or mating place (Steverding and Troscianko, 2004). Tsetse seem to be attracted most by shades of blue with a low UV reflectance (Lindh et al., 2012) but prefer landing on the black parts of the trap (Kappmeier and Nevill, 1999).
In order to attract a greater share of tsetse, the impregnation of traps with ruminant-specific odours proved to be very effective for *morsitans* group flies (Jaenson et al., 1991; Mihok et al., 2007; Harraca et al., 2009). Research on suitable chemicals for riverine tsetse revealed a promising mixture of these components (POCA) which increased fly attraction but did not have much impact on the actual catches (Rayaisse et al., 2010).
Whatever design is used, traps are capable of catching only active flies that are attracted to them for mostly unknown reasons (Vale, 1993). Consequently, catches of traps represent a fraction of a population on a certain day at a certain location, underlying various factors such as climate, trap siting, host abundance and fly physiology (Randolph et al., 1991).

1.6 Vector Control

Reducing tsetse numbers is important for trypanosomosis containment. If sustainability is maintained, vector control often is more effective than applying trypanocides to sick individuals due to the mentioned difficulties with diagnostics and drug resistances (Rogers et al., 1994; Van den Bossche and Delespaux, 2011).
Systematic vector control was first undertaken on Principe, a small island in the Gulf of Guinea, where tsetse were successfully eradicated in 1914 (deRaad, 2005). Until today total tsetse eradication is realisable in isolated habitats only (Kagbadouno et al., 2011). Hence, the term 'vector control' is more appropriate for the mainland since re-invasion from neighbouring tsetse-infested regions into cleared ones will always remain a challenge (Hargrove, 2000).
Ideally, large-scale interventions are aimed at pre-defined isolated populations on the edges of tsetse-infested areas. For lasting effects, tsetse barriers must be set at the boundaries and surveillance is to be maintained in order to prevent re-invasion (Kgori et al., 2006; Bouyer et al., 2010). Various tsetse control techniques are available, either direct ones by increasing fly mortality or by inhibiting their reproduction, or indirectly through changes of the habitat.

1.6.1 Insecticidal Application

Direct methods of tsetse control include all means of insecticides that kill the flies through contact. DDT has been widely used from the 1940's until the 1970's when accumulation in the food chain sparked public concerns. Since then less persistent pyrethroids as deltamethrin have been used and yet no resistance has occurred in tsetse, in spite of its common use for roughly 40 years (Leak, 1998).

First approaches applying insecticides to tsetse breeding sites were mistblowers carried by men in the 1950's or later by vehicles, predominately in the former British colonies (de Raad, 2005). Expensive men power and laborious track clearing prohibited spraying of large areas. Eventually, aerial application became widely applied until the 1990's, covering vast areas up to 10.000 km^2 per intervention (Allsopp, 2001).
When tsetse control became less interesting for the main donors, the high costs for annual aerial sprayings were not covered any longer. Smaller projects as community-based usage of insecticide-impregnated and optional baited traps or targets, which had been already in use in West Africa for a long time, were favoured instead (Torr et al., 2005). Impregnated cotton cloths or targets operate similar to tsetse traps but they are less voluminous. Current research is aimed at making targets even more cost-efficient through optimal size reduction (Rayaisse et al., 2011; Rayaisse et al., 2012). Pyrethroids remain effective on the targets in concentrations of 0.4% for several months under field conditions (Mangwiro et al., 1999).
Another vector control option is the treatment of cattle with pour-ons (Bauer et al., 1988; Bauer et al., 1992; Bauer et al., 1995; Bouyer et al., 2007), cost-effectively applied to the lower body parts of grown animals only (Torr et al., 2007a). Though cattle spraying has been successfull in reducing tsetse in an area of high cattle density, it didn't have much impact on another region where tsetse could retreat to thick vegetation (Hargrove et al., 2003), probably feeding on other hosts. In conclusion, insecticide treated cattle cannot serve as a tsetse barrier when their distribution is inconsistent (Rowlands et al., 2001; Torr and Vale, 2011a).
Another useful strategy for fly reduction in kraals or pigsties is the application of insecticide treated nets which are adjusted to the flies' host approaching flight altitude (Bauer et al., 2011). Further, burning cattle dung and wood, practised since the very beginnings of African agriculture, efficiently repels tsetse, reducing the abundance near the kraals up to 90% (Torr et al., 2011b).

1.6.2 Non-insecticidal Methods

Soon after tsetse and wild animals were linked with trypanosomosis at the beginning of the 20th century, bush clearing and systematic hunting of reservoir species were conducted in the British colonies until the arrival of insecticides (de Raad, 2005). Even if this indirect control method succeeded in temporary tsetse reduction, it is ethically unacceptable today.
The sterile insect technique, SIT, is based on the fact that female tsetse mated with sterile males cannot produce any offspring. In a pilot project on Unguja Island of Zanzibar, tsetse were reduced in numbers by sticky traps before SIT was able to eliminate tsetse from the island within two years (Vreysen et al., 2000). However, even if combined with tsetse-pathogenic biological agents such as fungi (Abd-Alla et al., 2012), SIT requires too many radio-sterilised males to meet the threshold of efficient population decline on the mainland (Torr et al., 2005).
Sterilizing tsetse could also be reached through artificial juvenile homones applied to traps. The persistency of the active ingredient amounted to 20 days (Langley et al., 1990) requiring further research to reduce its susceptibility to climatic conditions. Other biological agents as the salivary gland hypertrophy virus are causing considerable damage in lab reared tsetse colonies and became subject of current tsetse control research (Abd-Alla, 2011).

1.7 Trypanosomosis Risk Assessment

Various biotic and abiotic components influence trypanosomosis transmission risk. Epidemiological risk assessment evaluates and weighs those factors in order to detect trypanosomosis risk areas and also to identify rational means of control.

1.7.1 Risk Models

The most detailed risk model for African trypanosomosis by Rogers (1988) incorporates interactions of tsetse population dynamics, human and animal populations, trypanosome infection rates of both the vector and its hosts, disease transmission probabilities, climatic conditions and tsetse host preference. The obtained data proved useful for the prediction of trypanosomosis incidence and explained observed seasonal fluctuations. But in many locations the actual disease incidence deviated due to neglected local conditions (Rogers et al., 1994). Other models deal with trypanosome treatment and vector control impact (Milligan and Baker, 1988; McDermott and Coleman, 2001) or with predicting tsetse distribution under changing climatic conditions (Moore et al., 2012). All of these stochastic models require vast data input, which is not always feasible.

1.7.2 Tsetse Challenge

If a rapid appraisal of trypanosomosis risk is needed, a model requiring fewer but crucial variables may also provide valuable information although it will most likely be lacking in precision (Bett et al., 2008). Such a deterministic mini-model is the tsetse challenge: it is defined as “the number of infective bites from tsetse which a host receives in a unit of time” (Smith, 1958). Originally developed by mosquito researchers it was fitted to trypanosomosis transmission by tsetse (Snow and Tarimo, 1983). Tsetse sampling and investigation of the trypanosome infection rate in the fly provide input data. But the core component is the proportion of bloodmeals from cattle, or people respectively, enabling researchers to predict trends in trypanosomosis prevalence of a certain area.

1.8 Bloodmeal Identification

The host reservoir of a tsetse population may help to design appropriate vector control strategies and is also an essential factor in trypanosomosis risk assessment. The share of human-, or respectively cattle-derived meals, has the highest impact on increased transmission risk compared to other important factors such as the abundance of tsetse and trypanosomes (Davis et al., 2011).
Initially, native bloodmeals were examined under the microscope in order to class differing erythrocytes either with mammals or reptiles (Leak, 1998). Later, filter papers emerged as convenient storage matrix for further laboratory processing. The most recent development are DNA-preserving FTA cards which inactivate contagious pathogens in the blood (Muthukrishnan et al., 2008).

1.8.1 Serological Techniques

Early serological methods include the precipitin test and the agglutination inhibition test that gave valuable host-species-specific results. Unfortunately, they produced cross-reactions between closely related species and they required high quality anti-sera as well as relatively large tsetse meals (Leak, 1998). Nevertheless, 5 feeding patterns according to tsetse species have been set up which are still applicable today, like the fact that *palpalis* group tsetse are opportunistic feeders, biting a wide range of available hosts including humans (Weitz, 1963). Refined in sensitivity and specifity (Rurangirwa et al., 1986), bloodmeal ELISA confirmed these early findings. Besides, it became clear that tsetse feeding patterns depend not only on species-derived preferences but that especially riverine tsetse are able to rapidly adapt to changing host availabilities (Clausen et al., 1998).

1.8.2 Polymerase Chain Reaction (PCR)

Originally, species-specific identification by PCR has been developed for forensic reasons (Parson et al., 2000) but it has emerged as a very reliable technique for bloodmeal analyses of arthropods. Most suitable for species determination are segments of the highly conserved mitochondrial *cytochrome (cyt)* regions such as *cyt b* (Kocher et al., 1989; Boakye et al., 1999) or *cytochrome oxidase I* (Hebert et al., 2003) because of their presence in high numbers even in small bloodmeals and because certain regions are vertebrate-specific and do not exist in the fly.
Another advantage of *cyt b* is the vast number of more than 120 000 entries in GenBank, covering a wide range of vertebrate species. Sequencing is a very specific (Branicki et al., 2003) yet expensive approach. But when the host spectrum is unknown or as confirmation for any given results it is the method of choice. The obtained sequences are matched in data systems such as GenBank or Barcode of Life (Waugh, 2007). Disadvantages are the comparatively high costs and contamination risks that may lead to false conclusions.
When many samples are to be investigated and when the host pattern consists of a moderate number of species known to be abundant, multiplex and heteroduplex PCR's are useful (Kent, 2009). Multiplex PCR works with the use of several oligonucleotide primers to one sample, potentially being able to detect multiple blood meals. Detailed manuals are available for a moderate number of domestic species (Tobe and Linacre, 2008). Heteroduplex PCR relies on a control DNA sample, which is annealed with sample DNA, building mismatches. In the gel electrophoresis these mismatches travel slowly compared to the "correct" matches. However, cross-reactivity and primer interference may occur in multiplex PCR while heteroduplex PCR is technically challenging and methodology-caused misinterpretations are likely to happen (Kent, 2009).
Species-specific *cyt b* primers are available for a wide range of possible host species and produce sensitive results even in small bloodmeals as found in biting midges (Bartsch et al., 2009). A soon as a limited number of hosts come into question, a wide variety of universal species-specific primers are available (Verma and Singh, 2003). If many samples are to be processed and if a variety of host species are deemed possible, this method becomes too expensive although results are specific (Njiokou et al., 2004).
If closely related species with a common sequence range are to be investigated, restriction-fragment-length-polymorphism (RFLP) analysis may be applied. A universal primer produces amplificates that are digested enzymatically at species-specific target sites resulting in fragments that differ in length. Developed to detect meat of various sources of bovine origin (Gupta et al., 2008) it has been used to identify different ruminant species in lab-reared tsetse (Steuber et al., 2005). A variation of this process is the use of fluorescent dyes at the restriction sites. Digested and fluorescence-labelled products can be analysed in a database that has been uniquely designed for this purpose (Meece et al., 2005). If specific primers are unavailable and a restriction pattern library is present, this method is viable.
Real-time PCR is not only sensitive but is able to measure quantity in a blood meal. Visualisation of bloodmeal amounts in a sample is reached mostly through fluorescent resonance energy transfer, labelling the probe at its 3'- and 5' ends with a fluorescent or a quencher, respectively. The fluorescent signal is released when the probe is cleaved by a polymerase, revealing the amount of templates when comparing it with known fluorescent concentrations of internal standards (Kent, 2009).
All these methods combine higher sensitivity and more rapid sample processing compared to ELISA, making PCR-based methods the gold standard in tsetse bloodmeal analysis. Nevertheless, a problem that frequently occurs is PCR inhibition through heme, a porphyrin that is present in red blood cells (Akane et al., 1994). Fortunately, unlike other haematophagous insects, tsetse contain a respectable amount of blood enabling DNA-positive results up to 96 hours after feeding (Steuber et al., 2005). PCR was successfully used for various studies of tsetse bloodmeals in the course of epidemiological investigation in trypanosomosis foci (Njiokou et al., 2004; Simo et al., 2008; Muturi et al., 2011).

1.9 Matrix-assisted Laser Desorption/Ionisation Time-of-Flight (MALDI TOF) - based Diagnostics

As a recent development in proteomics, MALDI TOF mass spectrometry produces valuable results in a wide field of protein or nucleic acid analyses (Bonk and Humeny, 2001). Lately, it has evolved as a reliable method for microbial diagnostics for clinical purposes and research (Steensels et al., 2011). Compared to current techniques for microbial identification, MALDI TOF -based diagnostics are much more rapid than conventional diagnostic kits (El-Bouri et al., 2012). The amount of time saved through quicker and simpler processing reduces the costs per tested isolate (Cherkaoui et al., 2010).
MALDI TOF is based on the specific mass-to-charge (m/z) values of ionised peptide or protein isolates. A pulsed laser ionises the sample and an electrical field accelerates the created ion groups, during which a vacuum is maintained. The ion extraction is co-ordinated by a pulse generator, which turns high voltage electricity on and off in order to extract the respective ion groups subsequently. After having passed an extraction grid, the ions enter a flight tube where the ion packets show a spatial distribution according to their mass. A detector plate receives the ion impacts and then a software program transforms the specific time of flight into a peak scheme. The measurements are then automatically evaluated for validity and then finally compared to a peak database. Evaluating a single sample takes a few minutes, enabling a quick diagnosis.
Such mass spectra databases are provided by proteomic companies consisting of valid data from laboratories all over the world (Bohme et al., 2012a). In the veterinary field this technique is used for clinical diagnostics and food hygiene purposes (Alispahic et al., 2012; Bohme et al., 2012b; Hijazin et al., 2012; Kuhnert et al., 2012). Recent works include rapid identification of the facultative mastitis pathogen *Prototheca* up to the genotype level after creating a reference library (Murugaiyan et al., 2011). Characterisation of bacteria by MALDI is in some cases even more accurate than 16S rDNA sequence data (Sandrin, 2012).
Insect-related MALDI studies proved that distinguishing *Drosophila* spp*. (Feltens et al., 2010)* and *Culicoides* spp. (Kaufmann et al., 2011a; Kaufmann et al., 2011b) was possible up to the species and in some cases up to the sub-species level.
Rapid insect identification greatly simplifies current morphological methods because tsetse identification often requires trained entomologists using a stereo microscope (FAO, 1992). *Morsitans* group subspecies for instance, weren't differentiated as such until 1949 when researchers learned that individuals of the seemingly same species kept in a cage produced sterile offspring (Gooding and Krafsur, 2005). Tsetse speciation though may be important for trypanosomosis risk evaluation due to species-related differences in feeding behaviour and trypanosome susceptibility (Rogers, 1988). Tsetse differentiation by MALDI TOF MS could also be a basis for speciation up to the population level identifying fragmented ones and it could possibly speed up trypanosome diagnostics and bloodmeal analyses as well. Identifying fragmented populations would allow determining the risk of re-invasion from neighbouring habitat.
Besides, MALDI TOF MS has added to the knowledge of bacterial taxonomy (Cash, 2009). Perhaps, the creation of dendrograms based on the proteomic mass spectra of several tsetse species could contribute to the ongoing discussion about insect taxonomy. Especially since recent studies based on genomic analyses have come to question traditional grouping and species relations of tsetse (Petersen et al., 2007, Gariou-Papalexiou, 2007), proteomic outcomes could be put in context with the latter.
Still, MALDI TOF MS results mostly derive from ribosomal proteins (Kliem and Sauer, 2010), which are influenced by the way of how isolates are prepared. In bacteria, for instance, peak schemes differed greatly according to the medium used for cultivation (Lay, 2001). On these grounds, all results with tsetse must be seen in context with the applied methods of lab-rearing and protein extraction. The question whether MALDI TOF results can measure up to genomics in taxonomy research is yet unsolved due to a lack of comparative studies (Murray, 2010). However, taxonomy is a manmade system whose classifications always depend on the applied method. Any method can only mirror very few aspects of complex individuals, often leading to contradictory results.

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CHAPTER 2

Host Preference of Tsetse: an Important Tool to Appraise the Nagana Risk of Cattle in the Cotton Zone of Mali

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http://dx.doi.org/10.1007/s00508-010-1443-9

Published in Wiener Klinische Wochenschrift

25-32

CHAPTER 3

Double Feeding in *Glossina palpalis gambiensis* and *Glossina tachinoides* in Southeast Mali

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http://dx.doi.org/10.1111/j.1365-2915.2012.01046.x

CHAPTER 4

Identification of Tsetse (*Glossina spp.)* using Matrix-Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry

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Published in Public Library of Science, Neglected Tropical Diseases

OPEN ACCESS Freely available online

PLOS | NEGLECTED TROPICAL DISEASES

Identification of Tsetse (*Glossina* spp.) Using Matrix-Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry

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Abstract

Glossina (G.) spp. (Diptera: Glossinidae), known as tsetse flies, are vectors of African trypanosomes that cause sleeping sickness in humans and nagana in domestic livestock. Knowledge on tsetse distribution and accurate species identification help identify potential vector intervention sites. Morphological species identification of tsetse is challenging and sometimes not accurate. The matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI TOF MS) technique, already standardised for microbial identification, could become a standard method for tsetse fly diagnostics. Therefore, a unique spectra reference database was created for five lab-reared species of riverine-, savannah- and forest- type tsetse flies and incorporated with the commercial Biotyper 3.0 database. The standard formic acid/acetonitrile extraction of male and female whole insects and their body parts (head, thorax, abdomen, wings and legs) was used to obtain the flies' proteins. The computed composite correlation index and cluster analysis revealed the suitability of any tsetse body part for a rapid taxonomical identification. Phyloproteomic analysis revealed that the peak patterns of *G. brevipalpis* differed greatly from the other tsetse. This outcome was comparable to previous theories that they might be considered as a sister group to other tsetse spp. Freshly extracted samples were found to be matched at the species level. However, sex differentiation proved to be less reliable. Similarly processed samples of the common house fly *Musca domestica* (Diptera: Muscidae; strain: Lei) did not yield any match with the tsetse reference database. The inclusion of additional strains of morphologically defined wild caught flies of known origin and the availability of large-scale mass spectrometry data could facilitate rapid tsetse species identification in the future.

Citation: Hoppenheit A, Murugaiyan J, Bauer B, Steuber S, Clausen P-H, et al. (2013) Identification of Tsetse (*Glossina* spp.) Using Matrix-Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry. PLoS Negl Trop Dis 7(7): e2305. doi:10.1371/journal.pntd.0002305

Editor: Rhoel Ramos Dinglasan, Johns Hopkins Bloomberg School of Public Health, United States of America

Received December 14, 2013; **Accepted** May 24, 2013; **Published** July 11, 2013

Funding: The study was financed through non-monetary resources of the department of Parasitology and Tropical Veterinary Medicine and the Institute of Animal and Environmental Hygiene. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The trypanosomiasis infection risk of a particular area is determined by several factors, including tsetse species abundance and the sex distribution of a fly population [1]. While the sex is easily distinguishable with the bare eye, species identification can be challenging because there are 32 recognised tsetse species and subspecies [2]. Differentiation relies on morphological differences in colour, size and on minimal male genitalia variations [3]. Recent genome-based analyses revealed the subspecies status of seemingly uniform riverine *G. palpalis palpalis* individuals in Equatorial Guinea [4]. Accordingly, current tsetse specification based on morphology may not be the only way to rapidly determine the species status of *Glossina* spp.

The matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS) is an established method of identification for microorganisms [5,6,7,8,9,10,11]. The MALDI-based identification of microorganisms requires only a small portion of a microbial colony and a drop of matrix solution [12,13,14]. The intact microbial cells are mixed with matrix solution (UV observing substances like alpha-Cyano-4-hydroxy-cinnamic acid, 2,5-dihydroxybenzoic acid), dried and subjected to laser induced soft ionization. The ions are then accelerated into a vacuum tube using a high electric field and the Time of Flight (ToF) to reach the detector is recorded. The velocity of an ion is inversely proportional to its mass, thus smaller ions travel faster than heavier ones and ions with the same charge travel together. The ions hitting the detector and their time of flight are visualized as spectra. The protein composition of each organism is unique, so a species-specific MALDI signature or spectrum is expected. The species identification does not require protein sequence data; instead the acquired spectra are matched with reference spectra database using a pattern- matching algorithm [9,11]. The technique proved to be time and cost effective, as reliable as genome-based identification methods [6,9]. Recently, MALDI-based species identification has been demonstrated for higher

Author Summary

Tsetse flies are confined to tropical Africa and are carriers for trypanosomes, single-celled blood parasites. Through the bite of an infective tsetse, people and animals may contract trypanosomiasis, a degenerative disease leading to death if left untreated. Tsetse control proved effective for disease containment, but data on the flies as tsetse identification are a prerequisite for planning any control intervention. There are 32 generally accepted tsetse species and subspecies. Classical species identification relies on minor morphological differences, often challenging for field workers. In the last decade, Matrix-Assisted Laser Desorption/Ionisation (MALDI) has revolutionised microbial species identification. After a simple protein extraction, a laser-induced ionisation takes place. Then, the ions are accelerated in a vacuum tube, and their Time of Flight (ToF) to reach the detector is recorded. The protein composition of each organism is unique, and so is their MALDI signature. Comparison of the obtained signature with a database of known organisms enables rapid identification as reliable as genome-based methods. To possibly speed up tsetse diagnostics, we established a MALDI database for the identification of five defined laboratory tsetse breeds. Inclusion of wild-caught tsetse could reinforce the reference database for the identification of tsetse at the species and subspecies level.

organisms as micro-algae, *Prototheca* [15,16], the plant parasitic nematode *Anguina tritici* [17], *Drosophila* [18,19], ticks [20] biting midges (*Culicoides* spp.) [21,22,23] and fish [24]. In addition MALDI has also been utilised for differentiation of various eukaryotic cell lines [25], immune cells [26,27] and for species level classification of ancient mammalian samples [28].

Several commercial software packages designed for microbial species identification are available and include, MALDI Biotyper (Bruker Daltonics), the Axima (Shimadzu)-SARAMIS (AnagnosTec) systems (now called VITEK MS) (BioMérieux), Andromas (Andromas SAS) systems and MicrobeLynx (Waters) [7,8,29]. As far as our knowledge is concerned, reference spectra data for insects or tsetse in particular have not been included in any of these software packages. We chose the MALDI Biotyper system for creating a tsetse-specific spectra database. This system calculates the log score value, or similarity score, by considering the matching proportion of the test spectra with the database reference spectra. It also considers the consistency of peak intensities among sample and reference spectra.

The objective of this study was to investigate whether simple formic acid/acetonitrile extracts of five well known laboratory-reared tsetse breeds exhibit specific and reproducible peak patterns and if they prove to be valid for species level identification. Usually, field-collected tsetse are stored in ethanol and often parts of the insects are removed for diagnostics. Therefore, another goal was to investigate if any of the body parts (head, thorax, abdomen, legs, wings and whole insects) are useful for species prediction.

Materials and Methods

Tsetse selection and storage

To establish a tsetse database, we utilised five well-established laboratory breeds listed in table 1. They represent tsetse from three different habitats that are relevant for the transmission of trypanosomes that affect humans or animals [2]. Tsetse puparia were maintained at 26°C with a relative humidity of 75%. Two to 4 days after hatching they were sacrificed as tenerals at −18°C and then stored in ethanol (70%).

Table 1. Laboratory-reared *Glossina (G.)* spp. selected for the compilation of spectra database.

Species	Group	Tsetse Colony	Origin
G. morsitans morsitans	*Morsitans*	TTRI[1]	Kariba, Zimbabwe [37]
G. austeni	*Morsitans*	TTRI[1]	Zanzibar, Tanzania [38]
G. pallidipes	*Morsitans*	IAEA[2]	Tororo, Uganda [39]
G. palpalis gambiensis	*Palpalis*	IAEA[2]	Burkina Faso [40]
G. brevipalpis (red eye)	*Fusca*	IAEA[2]	Shimba Hills, Kenya [41]

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doi:10.1371/journal.pntd.0002305.t001

Fly dissection, protein extraction and MALDI measurement

A total of three insects each were obtained for the analysis of male and female entire individuals (table 1). Additionally, three males and females of each species were dissected representing the peak patterns of the body parts abdomen, head, legs, thorax and wings. The protein extraction was carried out as described in Murugaiyan et al. [16]. In brief, triplicates of each specimen (whole insect, head, thorax, abdomen, wings and legs) were washed with ethanol, air dried and mixed with equal volumes of 70% formic acid and 100% acetonitrile. The samples were then sonicated for 1 min on ice and the supernatants were collected for further analysis. One µl of each sample extract was spotted on to the MALDI target plate (MSP 96 target polished steel (MicroScout Target) plate Bruker Daltonics, Bremen, Germany), dried and overlaid with 1.0 µl of saturated α-cyano-4-hydroxycinnamic acid

Box 1. Key Steps in Maldi Microbial Identification and the Software Used in This Study

With the aim of creating a simple protein extraction and identification procedure for tsetse, we utilized the well-established microbial method of MALDI identification:

1. Protein extraction
2. Spot on target plate, overlaid with matrix solution and dried
3. Spectra acquisition
4. Peak picking and pattern matching with the database

The protein extraction is an essential step for creating reference spectra of multicellular organisms while direct transfer of microbial colonies from the culture plate is sufficient for microbial identification. In this study commercial software associated with MALDI MicroFlex LT (Bruker Daltonics, Bremen, Germany) are utilized to create a tsetse specific database that draws from individual mass spectrum peaks. Spectra acquisition is carried out by the software Flexcontrol 3.0 and selection is performed manually after visualisation using FlexAnalysis 3.0. software. Final tsetse reference spectra were created with the software Biotyper 3.0 that includes the manufacturer's reference database.

matrix solution. The MALDI measurements were carried out using MALDI Microflex LT (Bruker Daltonics, Bremen, Germany) on a broad range of 2000–20000 m/z (mass to charge ratio), following an external calibration with the bacterial test standard as recommended by the manufacturer. Each extract was spotted three times and each spot on the target plate was measured three times for acquiring 27 spectra per specimen. The spectra were acquired using the automated option (AutoXecute acquisition mode) in Flex control 3.0 software (Bruker Daltonics, Leipzig, Germany). (Box 1)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

In order to demonstrate the protein composition in each extract, *Glossina (G.) palpalis gambiensis* were chosen for an SDS-PAGE analysis [30]. In brief, the extracts of the whole insects and it's the body parts were precipitated in five volumes of ice-cold 100% acetone. The pellets were reconstituted with 10 µl of sample loading buffer, heated at 60°C for 5 minutes and separated using 4% stacking and 12% separating gel. The protein visualisation was carried out using Coomassie Blue staining [31].

Data analysis and creation of tsetse reference spectra

Following the visual inspection using Flex analysis 3.0 software (Bruker Daltonics, Bremen, Germany), the spectra were then loaded in Biotyper 3.0 (Bruker Daltonics, Bremen, Germany) software. The spectra were subjected to baseline subtraction (multipolygonal; signal to noise ratio 3) and smoothing (Savitzky Golay algorithm, frame size 25 Da). The composite correlation index [32], a mathematical algorithm used to assess the spectra variations within and between each set of the measurements. The Composite Correlation Index (CCI) was computed using the standard settings of mass range 3000–12000 Da, resolution 4, four intervals and autocorrelation off. The reference spectra were then created using the standard method version 1.2 settings of the software (mass error of each single spectra: 2000, desired mass error of main spectra: 200, peak frequency: 25% and desired peak number: 70). The cluster analysis (main spectra dendrogram) was calculated with "correlation" as distance measure and linkage at "complete" to evaluate the suitability of the MALDI-based differentiation of tsetse at the species level. The created main spectra were then compiled as a tsetse database.

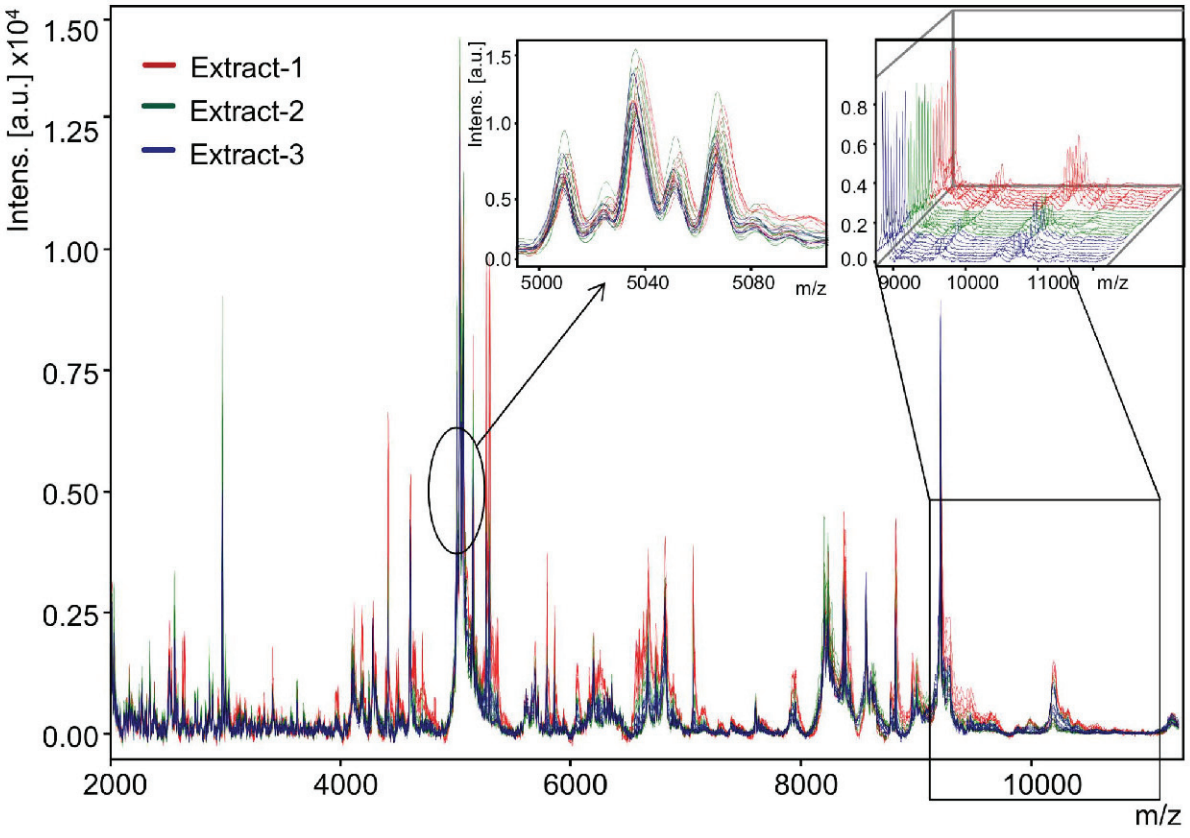

Figure 1. Spectra reproducibility among the biological and technical replicates. Overlay view of 27 spectra obtained from biological and technical replicates of *Glossina austeni* female whole insect. The masses (in Da) of the ions are shown on the *x*-axis and the *m/z* value stands for mass to charge ratio. On the y-axis, the relative intensity of the ions (a.u., arbitrary units) is shown. In the insert, zoomed m/z 5000 to 5200 displays the uniformity among the measured spectra and the stacked view m/z 9000 to 12500 provides a direct comparison of all 27 measured spectra.
doi:10.1371/journal.pntd.0002305.g001

Evaluation of the tsetse database

In order to check the suitability of the created tsetse main spectra for Biotyper-based species identification, the cross-matching status was created after matching them to the entire database. In addition, fresh extractions of the whole insect and the various insect parts were utilized in triplicates to cross-check the efficiency of the established tsetse database. For ruling out possible cross-matching with other fly species, the common house fly *Musca domestica* (Diptera: Muscidae; strain Lei) was also included in the evaluation. Identification was carried out using the Biotyper 3.0 software tool, following the manufacturer's recommendation on identification based on the calculated log score values. Values of ≥2.0 to 3.0 represent probable species level matching, while scores of ≥1.7 to 1.9 represent probable genus level matching. A score value of <1.7 stands for an unreliable identification.

Results

From each tsetse specimen a total of 27 spectra representing biological and technical replicates in the m/z range of 2000–20000 Da were acquired automatically and thus 1620 spectra from whole *Glossina* species and their body parts A–J. Visual inspection of the spectra revealed a comparable peak pattern of the biological and technical replicates; however, differences in peak intensities were observed for example as shown in figure 1.

At first look, the raw spectrum displayed consistently distinct peak patterns when comparing the two sexes of *G. palpalis gambiensis* (figure 2, samples G/H at m/z 5700, 7000 and 8000) while the three savannah species (A–F) and *G. brevipalpis* (I/J) only displayed differences in peak intensity. Occasionally observed differences as seen in the *G. pallidipes* female (sample E at 8100 m/

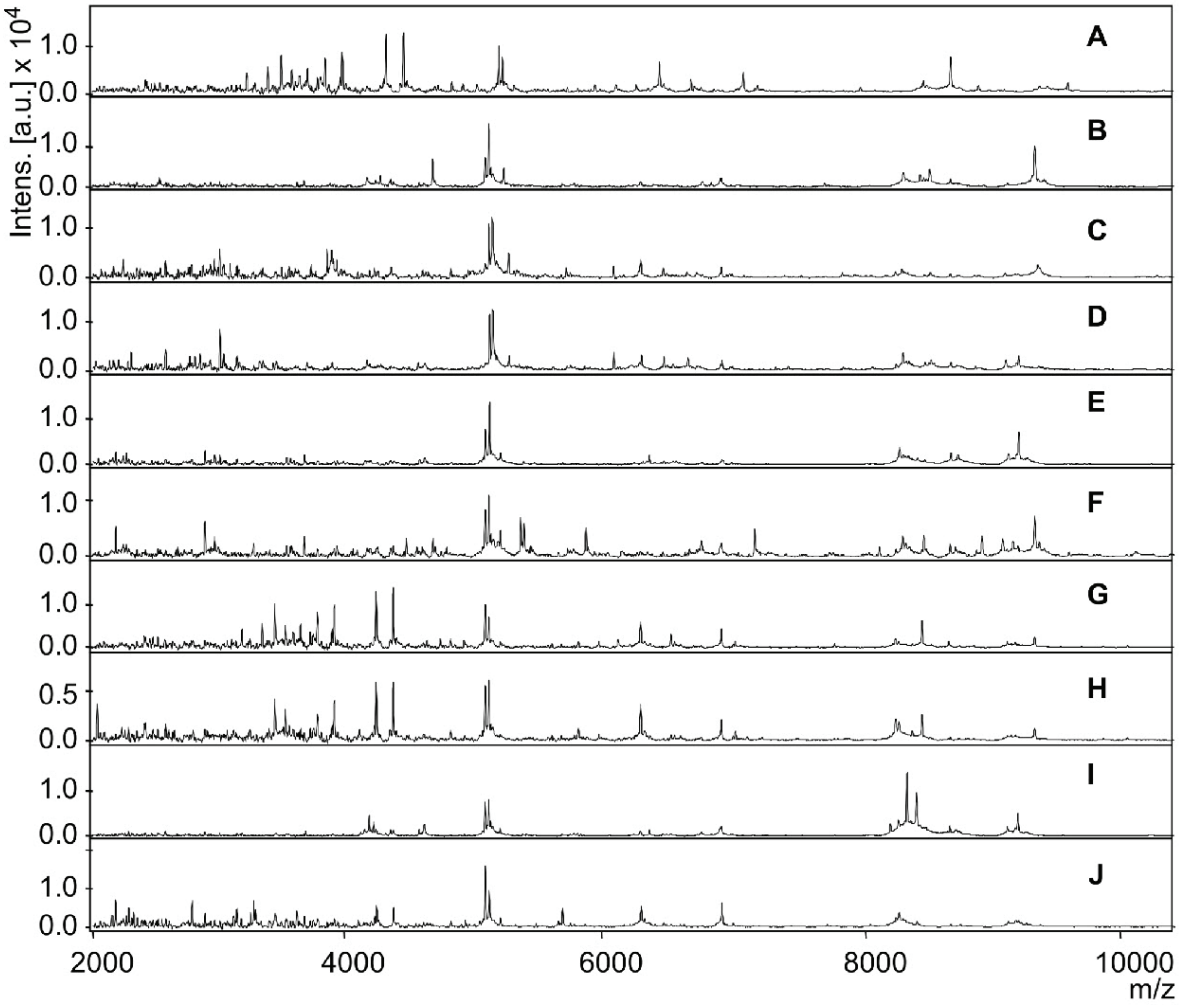

Figure 2. Representative spectra of whole insect extraction of male and female *Glossina* spp. Mass spectra peak pattern of whole insect extractions of male and female *Glossina (G.)* spp. The x-axis *m/z* value stands for mass to charge ratio and the relative intensity of the ions (a.u., arbitrary units) is shown on the y-axis. A) *G. morsitans morsitans* female, B) *G. morsitans morsitans* male, C) *G. austeni* female, D) *G. austeni* male, E) *G. pallidipes* female F) *G. pallidipes* male, G) *G. palpalis gambiensis* female, H) *G. palpalis gambiensis* male, I) *G. brevipalpis* female, and J) *G. brevipalpis* male.

doi:10.1371/journal.pntd.0002305.g002

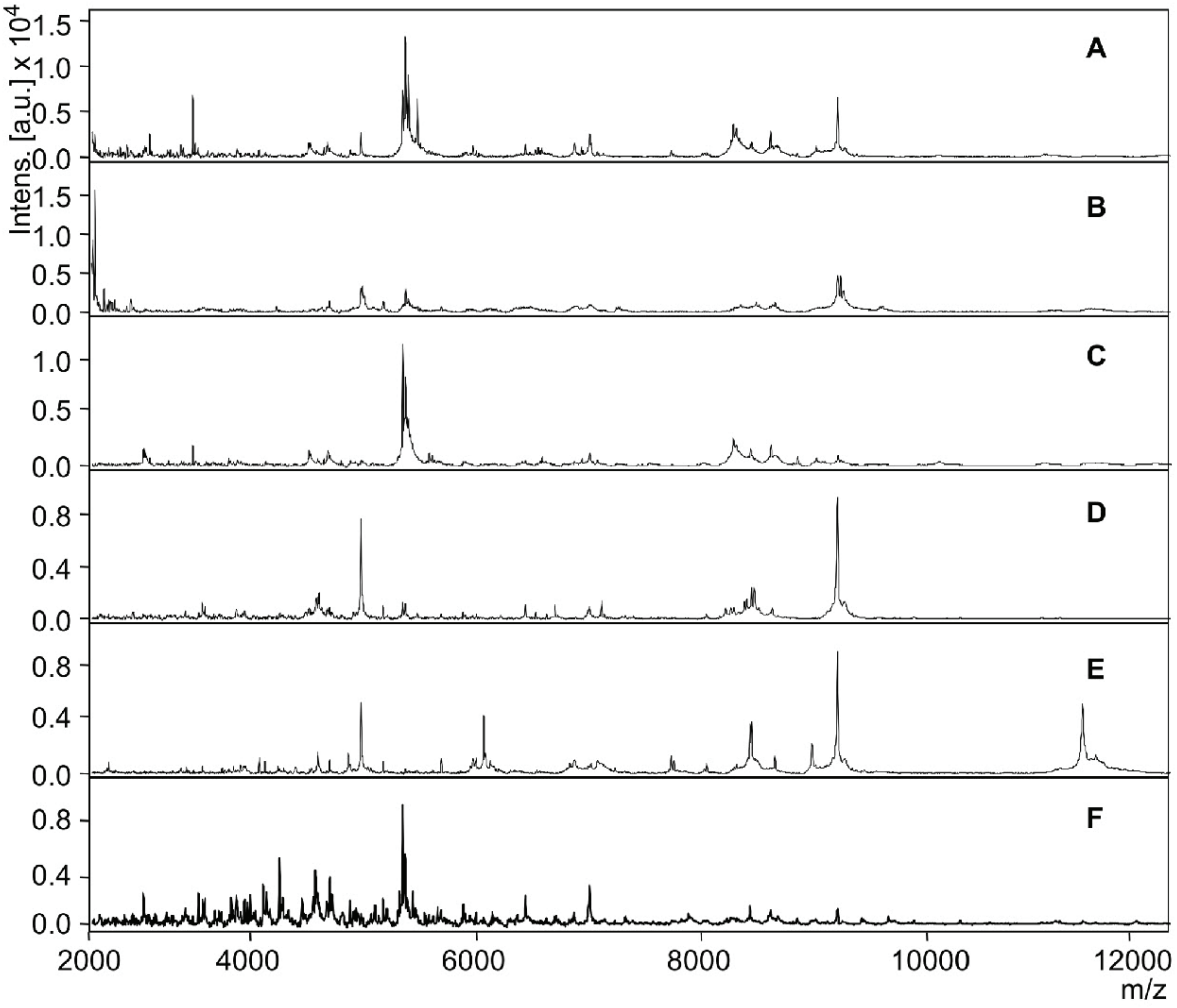

Figure 3. Representative spectra from the whole insect and different body parts of female *Glossina austeni*. Peak pattern of whole and body parts extractions of *Glossina austeni* female. The x-axis *m/z* values represent the mass to charge ratio and on the y-axis the relative intensity of the ions (a.u., arbitrary units) is shown. A) Whole insect, B) abdomen, C) head, D) legs, E) thorax and F) wings.
doi:10.1371/journal.pntd.0002305.g003

z) appeared inconsistently. However, several peaks showed to be common for *Glossina* spp. as for instance presented in figure 2 at 5000 m/z.

As shown in figure 3, the raw spectra of different body parts and the entire insects presented varying peak patterns at least in terms of peak intensities. Among the body parts, peak intensities sometimes tended to be lower in some of the leg extracts when compared to entire insects or other parts. To demonstrate the protein composition of whole insects and the different body parts, *G. palpalis gambiensis* extracts were chosen for protein separation on SDS-PAGE and visualised using a modified Coomassie staining. As shown in figure 4, the protein separation was carried out from 10 to 200 kDa. The bands out of the extracts of the dissected body parts were clearly observed in the whole insect protein extract lane. However, it should be noted that the peaks in the MALDI spectra were obtained from much smaller peptides (2–20 kDa).

Figure 5 depicts the colour-coded computed composite correlation index [31] displaying the uniqueness of the acquired spectra 1–60. A CCI value of 0.0 (dark green) represents incongruency and 1.0 (red) denotes complete congruency. The CCI was observed between 0.68 and 0.98 (individual CCI values are shown in the supplement data table S1). Very few of the spectra sets displayed some deviation among themselves, for e.g. the CCI for *G. austeni* male head was 0.68. However, this spectra set displayed a complete deviation with other body parts or other species. Despite this shortcoming, the spectra sets appeared to be suitable for the compilation of a reference spectra library.

Cross–comparison of the tsetse main spectra with the entire Bruker reference database resulted in only one clear match with a log score value of >2.3, the cut-off value representing the most probable matching at the species-level. Some isolates such as *G. austeni* female head (no. 2) appeared to resemble *G. palpalis gambiensis* male head spectra (no. 56), however, the score value was distinctly lower than the expected matching set. This clearly indicated that these spectra sets could be utilized to establish a database.

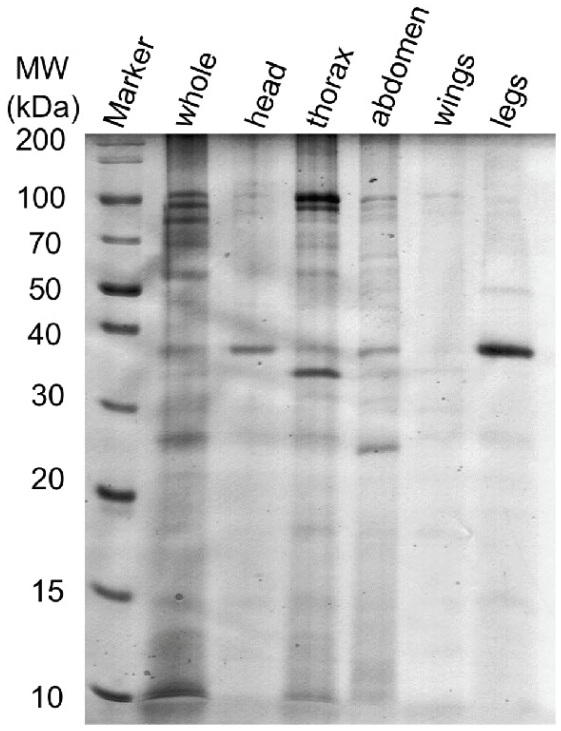

Figure 4. SDS PAGE separation of *Glossina palpalis gambiensis* protein extracts. SDS-PAGE separation of the whole insect and bodyparts of *Glossina palpalis gambiensis* extracts. 10 µg of proteins were run on 12% SDS-PAGE and stained with Coomassie brilliant blue.
doi:10.1371/journal.pntd.0002305.g004

Following these preliminary investigations, the main spectra library representing the 70 most reproducible peaks was constructed. The cluster analysis of the 10 main spectra of each species is shown in figure 6 for both sexes. Consistent clustering was observed among the extracts of *G. brevipalpis*, which always stood out as a sister group to the other species regardless of the body part. Furthermore, *G. austeni* showed inconsistent clustering, neither similar to savannah group tsetse nor to riverine *G. palpalis gambiensis* as for instance seen in the dendrogram.

The created tsetse main spectra were incorporated into the commercial Bruker system and then compared with the whole database following the manufacturer's recommendation. Accordingly, table S2 of the supplementary data describes the matching of tsetse main spectra where log score value 3.0 stands for a 100% match and lower matching probabilities were displayed as subsequent hits. The results indicate that the second hit within the acceptable cut-off value of >2.0 for some of the body part extracts matched with the correct body part but irrespective of the factors sex and species. This cross matching of body parts was predominantly observed between *G. austeni* and *G. morsitans morsitans* and among *G. palpalis* and *G. palpalis gambiensis*. Within the same species, complete deviation was observed in *G. austeni* female head with its own abdomen and legs. Similarly, *G. palpalis gambiensis* female head did not match with its legs and thorax. *G. palpalis gambiensis* male head also displayed complete deviation with *G. palpalis gambiensis* female head.

As shown in table 2 (detailed identification results are listed in supplementary table S3), the results of fresh sample identification clearly indicate that every body part and sex was correctly matched at the species level (log score value >2.0). Despite the 100% correct identification, within this high confident identification the following score inconsistencies occurred: 58% (35/60) matched with the correct body part but also with the ones of the opposite sex, 35% (21/60) matched with the correct sex but with different body parts, 16% (10/60) matched with a different body part and the opposite sex and 5% (3/60) even matched with other species. The second best matching hits indicate that about 23% (14/60) of body parts displayed lower cut-off values (log score <1.7). Among the second best hits, incorrect matching was observed among 13 samples (21%): body parts of female *G. palpalis gambiensis* (thorax, whole and abdomen), *G. pallidipes* (female thorax) and *G.austeni* (male legs). The extracts from *Musca domestica* resulted in no reliable identification.

Discussion

To establish a tsetse reference database five laboratory breeds representing epidemiologically important tsetse of the savannah type *G. morsitans morsitans, G. pallidipes and G. austeni*, a riverine type *G. palpalis gambiensis* and forest type *G. brevipalpis* were chosen for this study [2,33,34] . Earlier attempts on the identification of arthropods by MALDI were carried out after homogenisation of the samples and extraction in a mass spectrometry-compatible buffer system [18,20,21]. We used a standard formic acid/acetonitrile extraction procedure of microbial cell processing for the protein extraction from tsetse. We introduced an additional step of sonication in order to facilitate the breakage of the chitin shell for a better protein yield. This simple extraction method was chosen to accommodate the field-collected samples that are stored in ethanol and possibly dissected.

Flex analysis software revealed that the spectra of the same species appeared to be fairly comparable despite the varying peak intensities. Visual inspection of the spectra revealed differences among the body parts of the same insect. Often, the most intense peaks of body part extracts were not easily observable in the spectra of whole insect extracts. This could be due to the protein ionisation influenced by varying protein compositions/abundances of different body part extracts. Additional evaluation of the protein composition/abundance using SDS-PAGE protein separation revealed the difference in protein bands. However, the bands of the body part extracts were comparable to those of the whole insect but they varied in their intensities. This was also shown among the different sexes of the same species. As the protein separation was carried out in a higher range (10 to 200 kDa) but the MALDI spectra stemmed from a much smaller range of proteins (2 to 20 kDa), So, a direct correlation among these could not be expected. However, the compositional protein differences among the various body part extracts and the whole insect are clear. This protein compositional difference might attribute to the observed difference among the spectra from different insect body parts. Despite this variation, the technical and biological replicates appeared to influence the peak intensity while the peak pattern was almost comparable.

Among the commercially available software tools for species identification, we used Biotyper software that incorporates 4613 main reference spectra of microbial species (March 2013). The software automatically pre-process the spectra through smoothing and baseline subtraction. The peaks were picked and compared with the reference database. The results were expressed as similarity log score values between 3.0 (complete matching) to 0 (complete deviation). As a first step of the main spectra creation, the practical relation among the spectra sets was visualised by

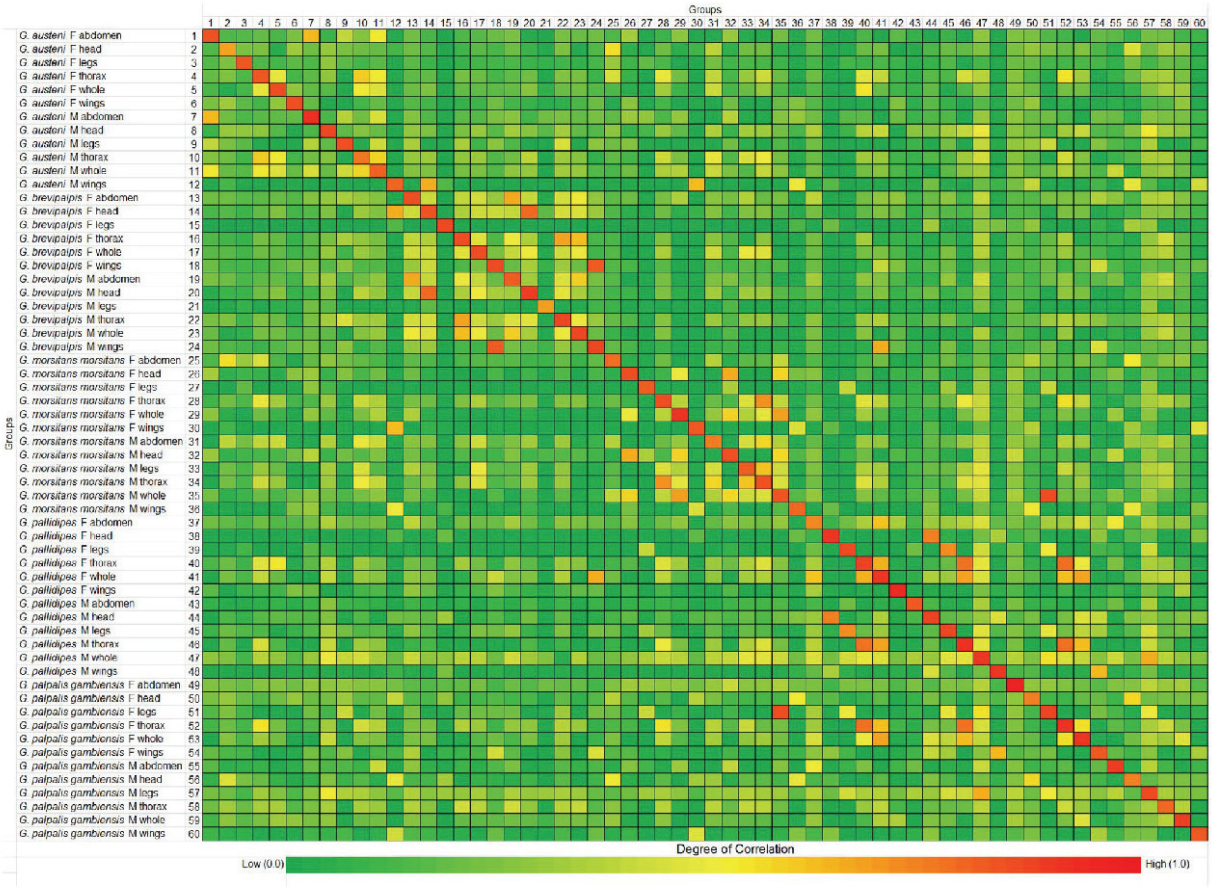

Figure 5. Composite correlation index of tsetse spectra sets. Evaluation of uniqueness among the spectra sets of 60 tsetse spectra measurements of male (M) and female (F) individuals and their body parts. Composite correlation index matrix was calculated with Biotyper 3.0 software in the mass range of 3000–12000 Da, resolution 4, 4 intervals and auto-correction off. Red indicates relatedness between the spectra sets and dark green indicates incongruence.
doi:10.1371/journal.pntd.0002305.g005

computing the composite correlation index [32]. A CCI value approaching 1 is considered to be highly significant while zero represents complete deviation. A clear distinction between the spectra sets of different body parts and the whole insect extracts was displayed in the heat-map and its corresponding value. Some

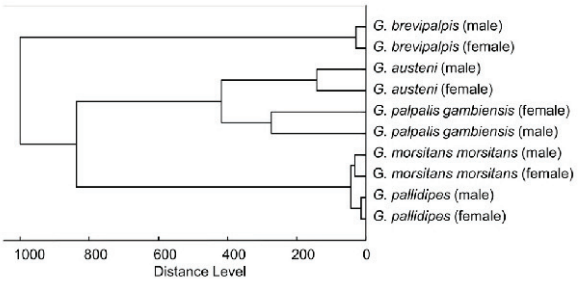

Figure 6. Score-oriented main spectra dendrogram of whole *Glossina spp.* extracts. The dendrogram was calculated by Biotyper 3.0 software with distance measure set at correlation and linkage set at complete.
doi:10.1371/journal.pntd.0002305.g006

of the spectra set displayed signs of deviation, for e.g. *G. austeni* male head. This might be due to the presence of broader peaks, which did not overlap with the corresponding spectra [32]. The heat-map and CCI values indicated that the spectra sets of different body parts and the whole insect extracts were unique and could be utilised for the creation of a spectra library. Therefore, we generated 60 main spectra for five tsetse species including male and female whole insect extracts and the corresponding body parts. These main spectra were then incorporated in the Bruker database.

The main spectra dendrogram was useful for the differentiation of the five species, picturing the similarities and differences of their mass spectra profiles. Clustering of the created tsetse main spectra revealed that they did not follow any distinct pattern with some significant exceptions. A possible explanation could be that higher organisms like insects might not cluster at the species level using MALDI measurements unless they are being standardised. However, *G. austeni* never clustered clearly with riverine nor savannah species; it seems to share mass spectra patterns with both groups reflecting the uncertainty of their phylogenetic status [35]. Very clearly though was the uniqueness of *G. brevipalpis* compared to the other species. The sister status deriving from genomic

Table 2. Matching of five lab-reared tsetse with the Bruker reference database.

Log score value*	Interpretation	Correct Species				Incorrect species
		Correct sex and body part	Correct body part but incorrect sex	Correct sex but incorrect body part	Incorrect sex and body part	
2.30–3.00	Highly probable species	44	12	4	1	3
2.00–2.29	Probable species	16	23	17	9	16
1.70–1.99	Probable genus	0	11	14	20	21
0.00–1.69	Not reliable	0	14	25	30	20

*The manufacturer's recommended cut-off values were utilised to interpret the results.

doi:10.1371/journal.pntd.0002305.t002

findings [36] could therefore be mirrored in the mass spectrum peaks of *G. brevipalpis*.

As a quality check, tsetse main spectra were cross-identified with the entire database from the manufacturer. All the tsetse main spectra matched with a log score of 3.0, indicating a clear distinction between the species. It also showed the uniqueness of the tsetse mass spectra for entire tsetse as well as every dissected body part. Among the second best matched hits, sex and species appeared to be least important while the body parts across the species matched, especially among *G. austeni* and *G. morsitans morsitans* and also in *G. pallidipes* and *G. palpalis gambiensis* extracts. The complete deviation of head extracts (*G. austeni* female, *G. palpalis gambiensis* female and male) indicates special attention when working on species identification of head samples by MALDI.

The fresh protein extracts using the same insects resulted in 100% matches with the database. No hits were achieved for similarly processed *Musca domestica* extracts, indicating the uniqueness of the created reference spectra for tsetse. Among the best hits at the species level, body parts of the same species appeared to be matched correctly but irrespective of the sex. A deviating species in the second hit might be due to the presence of shared metabolic proteins among different tsetse species. The 5% that mismatched completely and the incorrect matching among the second hits indicate that the reference database should be created for more than one body part and of both sexes for reliable identification of insects.

The overall results clearly indicate that the success in MALDI-based identification relies on the specific signature from the body parts and the whole insects. While the first hit for these lab breed tsetse appeared to be specific for species, sex and body parts, the second hit indicates that sex is the least reliable feature of MALDI identification. The complete deviation of head extracts with its own other body parts as seen among *G. austeni* and *G. palpalis gambiensis* indicate that more than one body part is needed for accurate species identification. We propose the addition of spectra from field-caught tsetse (whole insects and body parts) to extend our database for a fast and accurate identification of tsetse.

Supporting Information

Table S1 Composite Correlation Index (CCI) values of 60 spectra sets of tsetse. CCI was calculated using Biotyper 3.0 software (Bruker Daltonics, Bremen, Germany) in the mass range of 3000–12000 Da, resolution 4, 4 intervals and auto-correction off. CCI value nearing 1.0 indicates the relatedness between the spectral set and 0 indicates deviation among the spectra sets. M-male and F-female.
(XLSX)

Table S2 Cross matching values of tsetse main spectra. The created tsetse main spectra were selected in Biotyper 3.0 (Bruker Daltonics, Bremen, Germany) software and matched with the entire database. The log score value 3.0 indicates complete matching and 0 represents complete deviation. The manufacturer's recommended log score values, ≥2.0 to 3.0, ≥1.7 to 1.9 and <1.7 were utilised to interpret the identification as probable species level, genus level and no reliable identification respectively.
(XLSX)

Table S3 Identification results of freshly extracted tsetse samples. The insect proteins from whole insects and its body parts were extracted using formic acid/acetonitrile. 1.0 µl of the extracted was spotted on the target plate, air dried, 1.0 µl of saturated HCCA matrix was overlaid and dried completely. The result interpretation was carried out in accordance to the manufacturer's recommended cutoff log score values for species (≥2.0 to 3.0), genus (≥1.7 to 1.9) and the value lesser than 1.69 indicated that the samples were not reliable matched with any of the reference spectra.
(XLSX)

Acknowledgments

We would like to thank the International Atomic Energy Agency in Seibersdorf, Austria, for providing us with tsetse puparia and Dr. Marta Maia who assisted us in obtaining puparia from Tanga, Tanzania. We would also like to place our sincere thanks to Bettina Esch for her excellent technical support.

Author Contributions

Conceived and designed the experiments: PHC UR AH JM BB SS. Performed the experiments: AH JM. Analyzed the data: AH JM. Contributed reagents/materials/analysis tools: UR PHC. Wrote the paper: AH JM.

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CHAPTER 5
Summarising Discussion

The aim of this study was improving standard procedures of tsetse analysis in order to simplify relative trypanosomosis risk evaluation, applicable for rational vector control at the community level in subsaharan Africa. An introduction and literature review on human trypanosomoses, tsetse flies, and methods of analysis and control means was given in **chapter 1**.

Chapter 2 dealt with applying a simplified trypanosomosis risk model estimating the tsetse challenge (Snow and Tarimo, 1983) in a field study in the Sikasso region of southeast Mali. At the same time, bloodmeal analysis, as one of the components in the tsetse challenge formula, was improved by the use of species-specific *cytochrome b (cyt b)* primers that amplified cattle (Tobe and Linacre, 2008) and human (Matsuda et al., 2005) mitochondrial DNA. The obtained tsetse challenge values for the two villages Diassadié (0.16 infective bites per cattle per day) and Kapala (0.013 infective bites per cattle per day) corresponded with the AAT prevalence of surrounding cattle herds that were found to be 16.18% in Diassadié and 0% in Kapala. It was shown that the obtained tsetse challenges were reflecting AAT incidence in nearby villages. Rather complex risk assessment models (Milligan and Baker, 1988; Rogers, 1988) may therefore not always be required if a fairly accurate AAT risk estimation is warranted.

In a comparable study conducted in Ethiopia though, the tsetse challenge calculation failed to link the AAT prevalence to the calculated tsetse challenge (Bett et al., 2008). The authors assumed that this was due to a lack of data deriving from single villages. Perhaps rather the fact that *morsitans* group tsetse were analysed in this study may have influenced their outcome. Unlike *palpalis* group tsetse of the Sikasso region, *morsitans* populations tend to form swarms that follow bovines over long distances (FAO, 1992). Besides, a great share of the Ethiopian tsetse seemed to have fed predominantly on warthogs. Therefore the analysed tsetse possibly did not represent the AAT prevalence of the cattle herds that were screened for trypanosomosis. In our study, tsetse were present exclusively at the water courses, which made it more likely that the obtained tsetse challenge related to the monitored AAT herd prevalence of cattle close to the traps.

Looking at the monthly collection of flies from the villages around Sikasso, it appeared that *G. morsitans submorsitans* had disappeared which was observed in neighbouring Burkina Faso as well (Bauer et al. 1992; Bauer et al. 1995; Courtin et al., 2010b). Not more than 15 years ago, this savannah group species was abundant in Mali (Diarra et al., 1998). But in the study of the Sikasso region only the two riverine tsetse *Glossina palpalis gambiensis (Gpg)* and *Glossina tachinoides (Gt)* were found in low numbers of 9-152 flies per trap during the entire period of six months. This indicates an uneven distribution and decay of the tsetse populations in the region, which is in conformity with previous findings about diminishing fly populations of the same species in Burkina Faso (Bouyer et al., 2005).

Another surprise was that the 74 bloodmeals that contained vertebrate DNA almost exclusively comprised domestic cattle (n=52) and humans (n=19), with the exception of 2 reptile hosts both found at one trapping location. The presented findings differed greatly from studies of bloodmeal analysis from intact tsetse habitats where host preference patterns consisted of a great variety of game animals (Njiokou, 2004; Mekata et al., 2008; Farikou, 2010; Muturi, 2011). Traditionally, riverine tsetse are opportunistic feeders, known to prefer bloodmeals from reptiles such as monitor lizards, but they readily turn on any abundant host including humans as soon as their favoured hosts disappear (Clausen et al., 1998). The results in this study of unusually homologous feeding patterns in tsetse indicate that bloodmeal analysis may be used for an overview of the local fauna, which in our case would reveal a scarcity or even absence of game animals that used to be present in the area (Haywood, 1937).

The investigated mean infection rate (*Trypanosoma congolense 20/*279*, T. vivax 2/279)* in the flies amounted to 8.3% in Diassadié and to 5.1% Kapala. These values resemble those of tsetse in areas of traditional trypanosomoses foci (Kubi, 2007; Mekata, 2008; Farikou, 2010; Malele et al., 2011) or even outnumbered the observed 2.39% infected tsetse of a highly endemic region in Uganda (Waiswa et al., 2006). Riverine tsetse populations are known to adapt better to a changing environment than savannah species. Relic galleries or manmade habitats to provide micro-climatic conditions allowing these species to persist, albeit in low numbers (Bouyer et al., 2005). AAT will thus remain endemic in this region if cattle are not protected against the vector.

In the course of extending tsetse analysis to 4 villages east and south of Sikasso, the feeding patterns of the two species differed more than expected, which is described in **chapter 3.** *Gt* showed a higher proportion of flies that contained mixed bloodmeals of human and cattle origin. Mixed meals in field-caught tsetse have rarely been reported (Mekata, 2008) unlike publications on mosquitoes where multiple bloodmeals are commonly observed (Boreham, 1975; Meece et al., 2005; Kent, 2009).
In order to investigate the epidemiological influence of the factor multiple feeding on trypanosome infection, sex, fly age and hunger stage, a logistic regression was performed on data deriving from traps of the 4 monitored villages that caught at least 10 flies during the entire 6 months. *Gt* presented 2-fold more double-feeding flies than *Gpg,* whereby incorporated mixed meals had no relation to being infected with trypanosomes ($p=0.176$) although *Gt* were significantly more often found to be trypanosome-positive than *Gpg* ($p<0.005$). Additionally, *Gt* were more likely to feed on cattle than *Gpg* ($p<0.001$) whose favoured hosts were humans (>66%).
Mixed bloodmeals of bird and horse origin in mosquitoes could be related to a higher West Nile Virus infection rate in another study (Fall et al., 2012). A connection between mixed human-cattle meals and increased AAT incidence could not be drawn in our study, probably because only non-human pathogenic *T. congolense* infections were found in the Sikasso region. The definition “mixed host feeding” only applied to cattle and humans in this work, while individual hosts of the same species were not distinguished. But the findings indicate that multiple host feeding in *Gt* may be caused by interrupted feeding due to defensive movements. Since a higher proportion of females contained mixed bloodmeals ($p=0.049$) it cannot be excluded that some of the females abandoned their hosts prior to finishing their meals due to harassments by males (Leak, 1998).
Mixed-feeding *Gpg* on the other hand almost exclusively were fully engorged flies ($p=0.002$) indicating 2 regular subsequent meals, whose residual DNA can be found up to 96 hours after the bloodmeal uptake (Steuber et al., 2005). This theory is supported by the fact that the hunger stage had no influence on double-feeding *Gt* ($p=0.717$); neither was the factor sex significant for mixed bloodmeals in *Gpg* ($p=0.138$). However, confirmation by applying a quantifying realtime PCR to the bloodmeal samples as previously conducted in mosquitoes by Van den Hurk et al. (2007) would be necessary. Another way to explore whether *Gt* actually interrupt their meals more often than *Gpg* would be DNA finger printing of the cattle-positive samples, similar to what was done by Torr (2007).
Nevertheless, the presented results indicate that the overall vectorial capacity of *Gt* is higher than that of *Gpg* in spite of contemporary values of vectorial competence of 0.0242 for *Gt* and 0.0463 for *Gpg* (Kazadi, 1998). The term vectorial capacity comprises several environmental factors that influence disease transmission probability in a given fly population (Eldridge, 2003), while the vectorial competence only provides information on the susceptibility of a vector to a pathogen. Further, in the referred work the obtained vectorial competences are based on the susceptibility of the 2 tsetse species to *T. brucei brucei* tested in guinea pigs. In the field study presented in this work, *T. congolense* and to a minor extend *T. vivax* were the pathogens and the infected animals were bovines. This indicates that the laboratory-based vectorial competence of *Gt* and *Gpg* needs to be re-evaluated under field-conditions. Possibly, the actual vectorial competence of *Gt* may be higher than that of *Gpg* due to their

enhanced activity towards hosts to such an extent that it outshines their seemingly lower susceptibility for the pathogen.
The ratio for our investigations on AAT epidemiology in a quite disturbed and thus unusual tsetse habitat as the Sikasso region is explained by the linkage of AAT to cotton. Sikasso is part of the most fertile region of Mali, Kénédougou, where draught power is primarily provided by oxen. The trypanosomoses of the mainly trypanosusceptible zebu breeds is the major constraint for the Kénédougou region of Burkina Faso (Clausen et al., 1993) and Mali (Grace et al., 2009). This problem is exacerbated by widespread trypanocide resistance (Clausen et al., 2010; Sow et al., 2011). Therefore, a BMZ-funded pilot project was conducted to contain or reverse trypanocide resistance through a comprehensive animal health package (Mungube et al., 2012). As a part of this project, local tsetse populations were analysed in order to add to the understanding of trypanosomosis epidemiology. However, when the trapped tsetse of the villages around Sikasso were analysed, it soon became clear that this was not an undisturbed tsetse habitat with large animal reservoirs any longer.

Today, cotton replaced sorghum and millet as the mainstay of agricultural production of the Sikasso region (Delarue et al., 2009), since having been promoted as a cash crop by the Malian government and foreign organisations from the 1950's onwards (Benjaminsen, 2001). Initially, the incomes of farmers improved resulting in raised living standards in Sikasso, which attracted poor people from the countryside who were looking for employment and better education (Hussein, 2004). As a consequence from collapsing cotton prices on a global scale (Womach, 2004), the farmers' incomes became extremely low and the resulting poverty of cotton farmers compared to those of other agricultural branches became known as the 'Sikasso paradox' (Delarue, 2009).
During this development the population of peri-urban Sikasso has increased tenfold since 1960, resulting in an enhanced need for timber and firewood. In the process more than 80% of undisturbed wood- and shrub-land have made way to arable land (Brinkmann et al., 2012). Before, West African game animals were found in the densely vegetated savannah landscape of the region (Haywood, 1937). Landscape changes and demographic pressure altogether led to the disappearance of game and the destruction of suitable tsetse habitats, which contributed to the concentration of riverine tsetse at the river galleries and the outright disappearance of *Glossina morsitans submorsitans*. At the same time, better performing but trypanosusceptible Zebu cattle began to replace indigenous N'Dama breeds in order to work the fields (Diall, 2001) leading to the above described AAT scenario.

Another consequence of lacking game animals may be the high proportions of tsetse bloodmeals from humans, especially in *Gpg* (66%). Theoretically, tsetse biting predominantly humans could possibly lead to HAT re-occurrence (Harrus and Baneth, 2005), especially under the circumstances of current political unrest in Mali and neighbouring Cote d'Ivoire with resulting migrations to the Sikasso region. In Burkina Faso though, HAT cases did not resurge when refugees from Cote d'Ivoire returned even though HAT positive individuals were found (Courtin et al., 2010a). Due to the stated decline of tsetse populations in the region of southwest Burkina Faso (de La Rocque et al., 2001) fly are unlikely to reach the threshold needed for maintaining *Trypanosoma brucei* transmission cycles (Rogers, 1988). Recent reports confirmed that HAT does not occur in former historic foci of Burkina Faso and Mali (Cecchi et al., 2009) possibly due to the described impact of demographic pressure leading to declining tsetse populations.
In contrast, AAT does not require as high tsetse abundances as HAT does (Milligan and Baker, 1988) and the data of this work showed that current tsetse populations inhabiting river galleries of the Sikasso region are sufficient to maintain AAT endemicity (Mungube et al., 2012a). The ongoing development of land exploitation in southeast Mali may point towards the extinction of tsetse. But agricultural expansion is limited through climatic conditions, crop market prices and available work power, which might lead to a plateau situation, where AAT could as well remain endemic in Sikasso.

This study relies on various steps of data acquisition under field conditions, starting with the sampling of tsetse. First of all, 4 tsetse trapping locations at animal watering sites at each of the four villages were chosen through questioning farmers and paravets about their herding routes prior to the start of any interventions. Eventually, only trapping locations in the two villages of Diassadié and Kapala turned out to be representative for the monitored herds. One of the villages could not be used in the tsetse challenge study simply because it expanded over a vast area and the respective herding routes were accordingly scattered because consistently frequented watering sites were lacking. This resulted in very low tsetse catches at the four assumed watering sites in this village, unsuitable for statistical evaluation.
The remaining village did not qualify for the study because actual animal watering sites of the monitored herds deviated from the locations that were defined earlier. In other cases of this particular village, herds were watered exceptionally at wells during the dry season, leaving no herds to be linked with study trapping points although tsetse catches were consistently high at the designated watering sites. This shows that any information must be reassured by checking individual herding routes through personal questionnaires for each farmer or, if feasible, through own observations.

Once the trapping location is chosen, the exact placing of a trap affects the number of caught tsetse especially in the case of riverine flies that respond best to visual stimuli (Rayaisse et al., 2012). Traps set in vegetation openings as found at large watering sites are well seen by tsetse and tend to attract more flies than traps set in the shade (Leak, 1998). On the other hand, sunny trap locations lead to enhanced desiccation of the trapped flies, making them unsuitable for trypanosome diagnostics through tsetse dissection. Due to local conditions, some traps were set in rather small openings, barely visible from a distance of more than 30 metres, while others were placed in more open locations, well visible to tsetse from afar. Alongside occurring day-to-day changes in tsetse abundance due to fly physiology and host availability (Williams et al., 1990), this could have influenced the outcome. However, a statistical comparison of the six trapping days for each trap revealed no deviation of the monthly catches. Therefore, even if the real tsetse population cannot be explored by simple trapping, the results qualified for statistical evaluation on a comparative basis.

Biconical traps were used in this study since they are considered the gold standard device for trapping riverine tsetse (Challier and Laveissière, 1973) although other traps may be more efficient for *savannah* group tsetse (Malele et al., 2011). Less bulkier monoscreen traps catch more tsetse per used metre of fabric (Abila et al., 2007) but the actual trap did not attract significantly more tsetse than the biconical one, making monoscreen traps an option for control rather than for catching tsetse. However, since most tsetse tend to circle traps without actually landing and only 11% of tsetse that land on a trap eventually enter (Rayaisse et al., 2011), optimizing tsetse catching devices is warranted.
This is closely related to the question why flies approach a trap and what makes them enter, whereby reasons are greatly unknown. While blue and black surfaces both attract tsetse equally, royal blue traps with low UV-reflectivity continuously catch more flies than black ones (Green and Flint 1986). It was assumed that riverine tsetse associate blue and black coloured traps with shady resting sites (Kappmeier and Nevill, 1999), which would indicate an over-abundance of engorged flies wanting to rest and males seeking resting females. But in our study catches of hungry flies outnumbered those of engorged ones while there was no significant difference between males and females, making the resting-site hypothesis appear rather unlikely.

The next step of data collection was the laboratory analysis of trapped tsetse, whereby it has already been mentioned that native dissection under the stereo-microscope provides excellent information on the age of females (Saunders, 1960). But for trypanosome diagnostics it is lacking in sensitivity because only alive parasites are visible (Ouma et al., 2000). At the same time, detecting regularly occurring multiple trypanosome species in one fly (Malele et al., 2011) is hardly possible. Nevertheless, this field-applicable method was able

to reproduce similar trypanosome infection rates in tsetse as found recently in other studies of *T. congolense*-infested areas (Kubi et al., 2007; Mekata et al., 2008; Malele et al., 2011). Further, a high tsetse trypanosome infection rate in flies of a trapping location could be linked to high trypanosomosis prevalence of cattle herds that were watered at the corresponding site, which indicates the value of tsetse dissection for the purpose of this work.

Tsetse bloodmeals were investigated in two steps: through the use of a universal *cyt b* primer pair that amplified vertebrate DNA (Kocher et al., 1989) prior to applying species-specific *cyt b* primers for cattle (Bartsch et al., 2009) and humans (Matsuda et al., 2005) subsequently. This procedure seems laborious compared to multiplex PCR approaches that could potentially reveal these results in a single working step (Tobe and Linacre, 2008). However, recent studies on tsetse bloodmeals that applied multiplex PCR did not detect multiple bloodmeals, which might be due to interference of various primers with DNA from various hosts in differing concentrations (Kent, 2009). So the subsequent application of species-specific primers in this project was justified especially with the limitation of two investigated species.
Initially, the emphasis was put on tsetse challenge, so bloodmeals deriving from cattle were detected first. Therefore cattle-specific primers were applied to every host DNA-containing sample. The cattle primers that were used in this study showed no cross-reactivity between wild and domestic bovines when applied for identifying hosts of biting midges in Brandenburg (Bartsch et al., 2009). Within domestic bovines, Zebu DNA and that of taurine cattle did not vary in binding intensity with the bovine-specific primer although their *cyt b* sequence does exhibit minor differences of single base pairs.

Eventually, more vertebrate DNA-positive samples than expected turned out to be cattle-DNA negative. Given that the host preference of tsetse let alone the abundance of wild animals were not known for the Sikasso region, some of those samples were commercially sequenced in order to obtain an overview of the local host spectrum. When the results were matched with NCBI's sequence database BLAST (Altschul et al., 1990), humans turned out to be the main hosts apart from cattle. The question of how far tsetse host preference would shift towards humans under limited host abundance came up. So human-specific primers (Matsuda et al., 2005) were used on every DNA-containing sample, including those containing bovine DNA, which uncovered the presence of multiple host feeding tsetse. When working with human-specific primers contamination always threatens the outcome of bloodmeal analysis. In order to prevent non-sample human DNA in the outcome, every gel contained a negative and positive control. However, confirmation in this matter could be reached through cloning and sequencing (Kent, 2009).

It was concluded that the proportion of cattle-DNA containing bloodmeals influenced the tsetse challenge in the study villages. The tsetse challenge is a simplified formula that neglects biological factors like vegetation, climatic conditions and other hosts besides cattle as do general transmission models (Milligan and Baker, 1988; Rogers, 1988). For small-scale vector control projects funding usually does not support laborious data acquisition, rendering tsetse challenge valuable at the community level. However, it would be interesting to analyse AAT transmission cycles in Sikasso more in detail since ecological pressure might have led to unique mechanisms. This needs to be investigated by large-scale tsetse sampling assisted by geo-referenced data on cattle herding routes, supported by AAT monitoring of these herds.

Tsetse challenge could be computed from trap locations directly at the river only, because no tsetse were caught along transects that were placed every 100m near cattle routes from the villages to the respective watering sites. According to unpublished data of the study group, herds of farmers who watered their animals at wells during the dry season presented a lower trypanosomosis prevalence compared to the traditionally watered herds. It follows that tsetse remain at the river, in suitable habitat during the dry season. This makes the strategy of

avoiding the water sites applicable for this particular season. For the case that wells are unavailable, shortening of the exposure time at the watering sites when the AAT risk is highest would also help.
Another option would be vector control of designated watering sites through rationally applied insecticide treated targets (Rayaisse et al., 2011; Lindh et al., 2012; Mungube et al., 2012b; Rayaisse et al., 2012), organised at the community level. This would be practicable for those villages that share cattle routes and thus watering sites, but the sustainability of this approach is not assured which has been proven in other works (Sindato et al., 2008). However, the willingness to participate in vector control programs comes with knowledge on AAT (Joja and Okoli, 2001; Grace et al., 2008), which is best to be mediated for broad audiences for instance in plays (Okoth et al., 1998).

During the wet season when animals are watered at scattered puddles and in the case of failed community-based protection of watering sites, it is best to treat cattle with insecticides. This should be done preferably through spraying only adult stock at the lower body parts for protecting the entire herd (Torr et al., 2001; Torr et al., 2005; Bouyer, 2007; Torr et al., 2007). Farmers who are isolated by distance due to widely scattered herd locations as found in one of the study villages become less eligible for community-based vector control. There, in some cases it might be necessary to additionally apply trypanocides on a curative basis although in sub-urban areas trypanocidal use proves less efficient in disease containment than insecticides (Hargrove et al., 2012). Unfortunately, farmers often do not have the means to invest in disease prevention (Bouyer et al., 2011), so drug applications are rather limited to the treatment of obviously sick animals, neglecting occult carriers. If trypanocidal treatment seems necessary, every aenemic animal of the herd should be treated for instance with the help of the FAMACHA eye colour chart (Grace et al., 2007). Generally, the Sikasso region has been found to be endemic for AAT (Mungube et al., 2012), thus the adoption of trypanotolerant N'Dama cattle, rather than the preferred Zebu breeds, is highly recommendable (Murray et al., 1990; d'Ieteren et al., 1998; Maichomo et al., 2009).

The second project of this work dealed with establishing a MALDI TOF MS-based in-house database for 5 laboratory-reared tsetse species with the aim of setting a basis for a rapid high-throughput analysis. Recent results of the identification of biting midges (Kaufmann et al., 2011a; Kaufmann et al., 2011b) and drosophila (Campbell, 2005; Feltens et al., 2010) confirmed that intact protein profile (IPP) -based diagnostics of insects may become tool for routine diagnostics as it has for the identification of microbes (Welker, 2011). Three savannah group tsetse *G. morsitans morsitans, G. pallidipes* and *G. austeni,* the riverine species *G. palpalis gambiensis* and the forest group fly *G. brevipalpis* were chosen for analysis due to their significance in trypanosomosis transmission (Leak, 1998; Nayduch and Aksoy, 2007; Motloang, 2012). The insects were obtained as teneral flies since bloodmeals are known to impair protein and peptide peaks (Kaufmann et al., 2011b).

In order to extract the protein, the tough chitin shell had to be broken first. In other studies this has been done through manual grinding (Kaufmann et al., 2011a) or by the use of glass beads (Feltens et al., 2010). In this study, the standard acetonitrile/formic acid procedure with an additional sonication step was performed which simplified the process and led to comparable protein yields.
Only minimal differences were observed between the protein yields of insects that were freshly stored in 70% ethanol and several months old samples. Kauffman et al. (2011b) who stored biting midges at room temperature noticed decreasing peak intensities in older samples. Storing samples at 7°C as done in this work, or even at -20°C as done by Feltens et al. (2010), preserve the isolates much better without significantly altering the results. Storage of the tsetse in 70% ethanol though may change the protein pattern of incorporated blood meals (Zellner et al., 2005), an issue that needs to be addressed when extending the database to peak patterns of potential hosts.

A quality check of the obtained spectra was then performed by creating the composite correlation index (CCI) (Arnold and Reilly, 1998) comparing all 60 spectra sets with one another. The resulting colour-coded heat map demonstrated the uniqueness of each sample of the respective body parts and species. This was not the case in analysed biting midges where head and thorax demonstrated resembling peak patterns (Kaufmann et al., 2011b). While most isolates matched exclusively with themselves at rates of 0.98, others presented some minor deviations probably due to differences in peak intensities. On the other hand, some samples resulted in close matches (0.8-0.9) with completely different species or organs, whereby in all cases the correct match out-ruled this close match. If some peaks are missing in an isolate, this could lead to mismatches. Then, 60 main spectra (MSP) were created by the Biotyper software Flex control 3.0 with a set range of 2-20 kDa which has proven as the most convenient m/z range due to mechanisms of the ionisation process and the detectors (Hortin, 2006).

The observed high protein yield of the thorax and entire flies is considered to originate from muscle tissue (Feltens et al., 2010). However, legs, that were expected to contain sufficient muscle tissue, often reproduced low peak intensities. This might be due to an unfavourable chitin-muscle ratio and the tube form of the leg, which perhaps inhibits the protein extraction by ultrasound. Wings on the other hand have proven to demonstrate high protein yields, probably because of their haemolymph and thus protein content (Nogge, 1979).
When integrating the 70 most reproducible peaks as reference spectra in the Bruker in-house database they were checked for viability with freshly extracted tsetse samples by the Biotyper software add-on Real Time Classification (RTC). This resulted in 100% correct labelling, where scores of 2.0-3.0 represented a match at the species level and a score of 1.7-1.9 seemed to stand for matches at the genus level. The next step would be testing the created library with wild caught tsetse, which was successfully demonstrated in the biting midges proteomic library from Switzerland (Kaufmann et al., 2011a).

The CCI index and the RTC results of the analysed tsetse isolates indicate that sex differentiation is not always reliable which corroborates findings in biting midges (Kaufmann et al., 2011a). This outcome is rather unsatisfying because other than male non-biting midges tsetse males are haematophagous and the sex ratio of a population has an impact on the trypanosomosis risk of a region (Rogers, 1988). The only species showing consistently different peak patterns between males and females in the CCI and 100% correct RTC identification according to sex were *Gpg.* Distinctive peaks can be discerned in the pattern of males and females that are absent in the opposite sex. Possible reasons for this phenomenon may be found when considering the general polymorphism in tsetse chromosomes (Krafsur, 2009).
The created dendrogram corroborated findings of genomic studies that placed *G. brevipalpis* widely apart from the other tsetse species, indicating their sister status (Petersen et al., 2007). *G. austeni* either clustered with riverine tsetse or savannah group flies, depending on what body part was compared. This mirrored the uncertainty about their group status in contemporary tsetse literature based on PCR findings (Gooding and Krafsur, 2005). Even though the contribution of MALDI TOF MS to taxonomy matters is viewed critical (Murray, 2010), proteomic findings in insects and microbes have so far supported results of studies that applied genomic methods (Stackebrandt et al., 2005; Feltens et al., 2010; Kaufmann et al., 2011b; Ng et al., 2012).
Thus, the established tsetse species database based on MALDI TOF MS was able to rapidly classify subjected lab-reared tsetse automatically and precisely according to their species and body part. However, further research at the population-level is warranted because risk assessment data needs to be drawn from fly populations at a given location rather than from rigid species parameters (Eldridge, 2003). As demonstrated in chapter 2 of this work, differences in fly behaviour, for instance host preference, do occur not only between species but also between flies of different populations. Recent work in the field of genomics applies work-intensive microsatellite techniques in order to classify populations or subspecies (Dyer

et al., 2009). Less time-consuming IPP of tsetse at the population-level could add to a more effective risk assessment.
Bloodmeal analysis may be possible by MALDI TOF MS since host proteins could be traced in tick intestines (Sonenshine et al., 2005). Another field of application could become trypanosomosis diagnostics by proteomics since first results indicate its feasibility (Ndao, 2012). In microbial identification researchers were even able to differentiate methicillin-sensitive and –resistant *Staphylococcus* strains (Edwards-Jones et al., 2000). If resistances could be equally traced in trypanosomes by MALDI TOF, it would revolutionise current diagnostics. All of the above requires protein profiling for which IPP is not the method of choice. But if peptides of macerated tsetse would be subjected to a MALDI-MS analysis as done in shotgun mass mapping (Vogel and Marcotte, 2012), diagnostics of tsetse at the population-level, trypanosome infection status and incorporated bloodmeals may become possible.

In conclusion, the above-described results of the studies that were presented in this work may contribute to rational vector control. A simplified AAT risk estimation through the tsetse challenge formula proved to be valid at the community-level and data acquisition could be supported through the use of species-specific PCR for determining the bloodmeal. Both will facilitate the planning of future vector control interventions. The study also revealed the over-proportional existence of multiple host feeding *G. tachinoides* compared to *G. palpalis gambiensis.* This has not been described so far prompting questions about epidemiological consequences. The successful establishment of a tsetse MALDI database forms the basis for elaborated studies on rapid and accurate tsetse analysis.

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Summary

Tsetse flies inhabit 10 million km^2 of subsaharan Africa, transmitting Human African Trypanosomoses (HAT) and African Animal Trypanosomoses (AAT). Public health services of most African countries are not able to reach the affected rural communities. Besides, trypanocides often are inefficient and vaccinations are unavailable. Thus, various means of vector control remain for disease management. In order to avoid unreasonable interventions against tsetse, decision support tools help defining the most efficient control strategies: trypanosomosis risk assessment and profound knowledge on local tsetse populations and their behaviour. Large-scale risk surveys and tedious serological laboratory analyses are too expensive at the community-level. That is why the objective of this work was rationalising trypanosomosis risk assessment and improving current tsetse analysis methods.

Chapter 1 provides a literature review on trypanosomosis epidemiology, tsetse biology, physiology, control means and methods for risk assessment and bloodmeal analysis.

Chapter 2 deals with the application of a tsetse challenge formula that simplified relative AAT risk estimation in 2 villages of the Sikasso region in southeast Mali. During 6 months tsetse were trapped at animal watering sites, followed by microscopic examination of the flies for trypanosome infection rates and by PCR analysis of tsetse bloodmeals. Bloodmeals were identified by species-specific *cytochrome b* primers that amplified vertebrate mitochondrial DNA and by sequencing unidentifiable samples. The outcome of the field study revealed that *Glossina morsitans submorsitans* had vanished, while *Glossina palpalis gambiensis (Gpg)* and *Glossina tachinoides (Gt)* were still present in this area with 369 and 105 caught tsetse, respectively. Further, it became obvious that the tsetse were unevenly distributed with catches of 2-152 flies per trap with the majority in direct proximity of watering places while being absent from distances of 20 metres and onwards from a river. Trypanosome infection rates of the flies varied between 0% and 33.3% depending on the trapping location. The analysis of 120 bloodmeals revealed cattle and humans as main hosts while 2 samples showed crocodile DNA. The tsetse challenge of the 2 villages differed significantly with 6 days *vs.* 77 days that had to be spent by cattle at the watering site in order to contract AAT. The obtained value could in both cases be linked to the trypanosome prevalence of nearby cattle herds.

Further analysis of tsetse deriving from 20 traps in 4 villages revealed unexpected differences between the 279 analysed *Gt* and *Gpg. Gt* demonstrated no host preference whatsoever because their feeding pattern comprised in equal shares humans, cattle and surprisingly mixed meals of both. Multiple host feeding, yet rarely been described in tsetse research, did occur significantly less often in *Gpg* ($p<0.05$). *Gpg* showed a preference for humans over cattle (66.5% and 10.3%, respectively). The infection rate also differed with *Gt* being 3-fold more likely to be infected with trypanosomes (18.5%) than *Gpg* (5.5%). Therefore, **chapter 3** contains a logistic regression analysis of the factor *mixed bloodmeal* towards the factors *species*, *infection, hunger stage* and *sex.* The statistics demonstrated that multiple host feeding was not linked to high infection rates or age but that it positively correlated with female sex in *Gt* and fully engorged *Gpg.* It is then discussed how multiple feeding possibly impacts trypanosomosis transmission mechanisms, assuming a higher vectorial competence of *Gt* compared to *Gpg.*

Although PCR has proven more sensitive than serological methods, the development of MALDI TOF MS (matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry) has become a more rapid tool for routine microbial diagnostics. Insects have rarely been specified by proteomic means, so **chapter 4** consists of a proteomic database construction for the tsetse species *G. morsitans morsitans, G. pallidipes, G. austeni, G. palpalis gambiensis and G. brevipalpis* based by MALDI TOF MS. Lab-reared flies were analysed as entire insects and dissected, obtaining their head, wings, legs, thorax and abdomen. After a simple protein extraction, 60 mass spectrum peak patterns were created as reference spectra. The following principle component and cluster analysis confirmed that each body

part was suitable for exact speciation. Evaluation of the database by crosschecking with newly extracted isolates resulted in a composite correlation index that demonstrated reliable tsetse speciation. Dendrograms drawing on peak similarity showed that *G. brevipalpis* stood consistently apart from the other species, confirming genomic findings that suggested their sister group status. As expected, tsetse of the *morsitans* group tended to cluster, with the exception of *G. austeni* that did not show consistent affinities to any of the 3 groups reflecting uncertainties about their group status in recent tsetse taxonomy literature. So, the constructed database apparently displayed genomic findings at the protein level and it proved to be a rapid and accurate tool for tsetse species determination.
The results are discussed in **chapter 5**. It could be demonstrated that a simplified risk assessment formula is able to provide AAT risk trends. This will be useful for planning future vector interventions more rationally, making it available for community-based projects. Thereby, species-specific PCR proved more efficient for bloodmeal analysis than serological methods. Still, obtaining the host preference remains the most laborious tsetse parameter, making it the limiting factor to a more time-efficient risk evaluation. Since rapid MALDI-based diagnostics at the species-level could be established, extending the database is warranted for high-throughput proteomic tsetse identification at the population-level, trypanosome diagnostics and bloodmeal analysis.

Zusammenfassung
Blutmahlzeitanalyse von Tsetsefliegen (*Glossina* spp.) mittels PCR und Spezies-differenzierung mit MALDI TOF MS als Beiträge zu rationaler Vektorbekämpfung

Tsetsefliegen sind in über 10 Millionen km^2 Land in Afrika südlich der Sahara verbreitet und übertragen die durch Trypanosomen verursachte menschliche Schlafkrankheit (HAT) und die Viehseuche Nagana (AAT). Den Gesundheitsbehörden ist es oft unmöglich, die betroffenen Kommunen zu erreichen. Außerdem sind viele Trypanozide unwirksam und Impfungen nicht verfügbar, weswegen die verschiedenen Methoden der Vektorbekämpfung oft effektiver sind. Um die verfügbaren Mittel sinnvoll einzusetzen und Fehlentscheidungen zu vermeiden, werden komplizierte Transmissions-Risiko-Modelle eingesetzt. Dazu ist fundiertes Wissen über regionale Tsetsepopulationen und deren Verhalten nötig. Da groß angelegte Studien und aufwändige Laboranalysen für Projekte auf kommunaler Ebene unbezahlbar sind, hatte diese Arbeit das Ziel, die Risikoanalyse zu vereinfachen und konventionelle Labormethoden zu verbessern.

Kapitel 1 beinhaltet eine Literaturübersicht der HAT- und AAT-Epidemiologie, Tsetsebiologie, ihrer Physiologie sowie Bekämpfungsmethoden, Transmissionsmodelle und zu Methoden der Blutmahlzeitanalyse. **Kapitel 2** beschreibt die Anwendung der „*tsetse challenge*"-Formel, um das relative AAT-Risiko für Rinderherden in zwei Dörfern Südostmalis einzuschätzen. Während sechs Monaten wurden Tsetse an Wasserstellen gefangen, mikroskopisch auf Trypanosomen untersucht und anschließend Blutmahlzeiten (BM) mit spezies-spezifischen *Cytochrom-b*-Primern und Sequenzierung auf deren Herkunft untersucht. Es stellte sich heraus, dass *Glossina morsitans submorsitans* nicht mehr in der Region vorkommt, dafür wurden 369 *Glossina palpalis gambiensis (Gpg)* und 105 *Glossina tachinoides (Gt)* gefangen. Dabei wurde deutlich, dass die scheinbare Abundanz mit Fängen von zwei bis 152 Fliegen pro Falle und Tag stark schwankte und dass sie nur in direkter Nähe zu den Flussläufen vorkamen. Die Infektionsraten der Fliegen variierten zwischen 0% und 33.3% und die Analyse von 120 BM ergab Hausrinder und Menschen als Hauptwirte, während nur zwei BM Krokodil-DNS enthielten. Das relative AAT-Risiko (*tsetse challenge*) der beiden Dörfer unterschied sich signifikant mit sechs und 77 Tagen, die ein Rind an einer Wasserstelle verbringen müsste, um mit AAT infiziert zu werden. Das Ergebnis spiegelte sich in beiden Fällen in der AAT-Prävalenz umliegender Rinderherden wider.

Als die Studie auf vier Dörfer ausgeweitet wurde, stellten sich signifikante Unterschiede zwischen den 279 analysierten *Gt* und *Gpg* heraus. *Gt*-BM bestanden in gleichen Anteilen aus Rindern, Menschen und aus gemischten Anteilen beider Wirte. Frakturierte (F) BM in Tsetse sind bisher kaum beschrieben worden und sie kamen signifikant häufiger in *Gt* als in *Gpg* vor ($p<0.05$). *Gpg* zeigten dabei eine deutliche Präferenz für Menschen (66.5%). Weil die Infektionsrate von *Gt* (18.5%) deutlich höher war als die von *Gpg* (5.5%), wurde eine logistische Regressionsanalyse durchgeführt: **Kapitel 3** stellt den Einfluss des Faktors FBM auf Spezies, Alter, Infektionsrate, Hungerzustand und Geschlecht dar. Dabei wurde demonstriert, dass FBM unabhängig vom Infektionsstatus waren, aber sie korrelierten positiv mit dem Merkmal „weiblich" bei *Gt* und „voll gesogen" bei *Gpg.* Es wird diskutiert, inwiefern FBM Infektionsmechanismen beeinflussen, wobei von einer höheren Vektorkapazität von *Gt* gegenüber *Gpg* ausgegangen wird.

Die angewandte PCR ist zwar sensitiver als etablierte serologische Methoden, aber MALDI TOF MS (*matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry*) bietet schnellere Ergebnisse und ist in der mikrobiellen Diagnostik bereits Routine. **Kapitel 4** beschäftigt sich deswegen mit dem Erstellen einer proteomischen Datenbank für die Tsetsespezies *G. morsitans morsitans, G. pallidipes, G. austeni, G. palpalis gambiensis und G. brevipalpis* mittels MALDI TOF MS. Laborgezüchtete Fliegen wurden als ganze Individuen und seziert in Kopf, Flügel, Beine, Thorax und Abdomen analysiert. Nach einer einfachen Proteinextraktion, wurden 60 MSP's (*main spectra*) als Referenzspektren geschaffen und

eine Komponenten- und Cluster-Analyse durchgeführt, wobei sich jedes Körperteil als nutzbar für eine exakte Spezifizierung erwies.
Die Zuverlässigkeit der Datenbank wurde erfolgreich mit neu extrahierten Tsetse-Isolaten getestet, dargestellt in dem farblich abgestuften CCI (*composite correlation index*). Dendrogramme, die Ähnlichkeiten zwischen den 70 meistreproduzierten Peaks darstellen, zeigten eine große Distanz von *G. brevipalpis* zu den anderen Spezies. Dies bestätigte Ergebnisse einer Studie des Genoms, in der ein Schwesterstatus von *G. brevipalpis* zu anderen Tsetse postuliert wird. Auch *G. austeni* spiegelte Kontroversen aus Taxonomiestudien über deren Gruppenzugehörigkeit wider, da sie entweder mit der Savannen- oder der Flussgruppe Cluster bildete, abhängig vom analysierten Körperteil. Insgesamt bot die MALDI-Datenbank eine schnelle und exakte Speziesbestimmung von Tsetse und lieferte nebenbei nützliche taxonomische Informationen.
Die Ergebnisse werden in **Kapitel 5** diskutiert. Auch eine vereinfachte Formel der Risiko-Einschätzung bietet wertvolle Informationen über AAT, was eine rationale Planung von Vektorbekämpfungsprojekten auf kommunaler Ebene möglich macht. Dabei erwies sich spezies-spezifische PCR der BM als effizient, auch wenn das Ermitteln der Wirtspräferenz aufwändig bleibt. Seitdem sich eine MALDI-basierte Tsetse-Spezifizierung als möglich erwiesen hat, könnte eine Ausweitung der proteomischen Analyse von Tsetsefliegen auf BM, Infektionsstatus und Populationszugehörigkeit zu einer Routine-Methode in der Tsetsediagnostik werden.

Résumé

Analyse de repas sanguins des mouches tsé-tsé (Diptera: *Glossinidae*) par PCR et de la différenciation d'espèces par le MALDI TOF MS en vue d'une contribution à la lutte rationnelle contre les vecteurs

Les mouches tsé-tsé sont présentes sur une aire de 10 million km² en Afrique subsaharienne et transmettent les THA (Trypanosomoses Humains Africains) et les TAA (Trypanosomoses Animaux Africains). Les services de santé publique de la plupart des pays africains ont des difficultés d'atteindre les communautés. De plus, plusieurs tripanocides sont inefficaces et les vaccins sont rarement disponibles. Toutefois, différentes possibilités de lutte contre les vecteurs restent efficaces pour lutter contre les THA et les TAA. Pour employer de façon appropriée les moyens disponibles et éviter des interventions inappropriées contre les mouches tsé-tsé, on définit la stratégie de lutte la plus adaptée en appliquant des modèles complexes de risque de transmission. Pour cela, il faut avoir des connaissances approfondies sur les populations locales de glossines et leur comportements. Étant donné que les études de grande envergure et les analyses de laboratoire élaborées sont beaucoup trop chères pour les projets au niveau des communautés rurales, ce travail avait pour but de simplifier l'analyse de risque et d'améliorer les méthodes habituelles de laboratoire.

Le chapitre 1 comprend un survol de la littérature sur l'épidémiologie des THA et de la TAA, la biologie de la glossine, sa physiologie, les méthodes de lutte contre celle-ci, les modèles de transmission et les méthodes d'analyse de repas sanguins. **Le Chapitre 2** décrit l'emploi d'une formule de «tsetse challenge» pour évaluer le risque relatif de la TAA pour les troupeaux de bovins dans deux villages du sud-est du Mali. Durant 6 mois, des mouches tsé-tsé ont été piégées sur différents points d'eau, examinées au microscope pour dépister les trypanosomes et ensuite trouver l'origine des repas sanguins (RS) par l'utilisation de primer *cytochrome b* spécifique à l'espèce et du séquençage. Il s'est avéré que les *Glossina morsitans submorsitans* n'étaient plus présentes dans la région. Par contre, 369 *Glossina palpalis gambiensis* (*Gpg*) et 105 *Glossina tachinoides* (*Gt*) ont été piégées. Il est aussi apparu clairement qu'avec 2 à 152 mouches par piège, l'abondance apparente variait fortement et que la majorité des glossines n'existaient qu'à proximité des cours d'eau. Le taux d'infection des mouches variait entre 0 % et 33,3 % et l'analyse de 120 repas sanguins a démontré que les bovins et les humains étaient les hôtes principaux, tandis que seulement deux repas sanguins contenaient de l'ADN de crocodile. Le «tsetse challenge» des deux villages se différenciait de façon significative avec, respectivement, une durée de 6 ou 77 jours qu'un bovin devait passer sur le bord d'un cours d'eau avant d'être infecté avec la TAA. Ce qui se reflétait dans la prévalence de la TAA des troupeaux de bovins avoisinants dans les deux cas.

Lorsque l'étude fut étendue à quatre villages, des différences signifiantes se sont concrétisées entre les 279 *Gt* et *Gpg*. Les repas sanguins des *Gt* se composaient en parties égales de bovins, d'humains et de façon étonnante de mélanges des deux hôtes. Des RS fracturés (F) chez les mouches tsé-tsé ont été jusqu'à maintenant très peu décrits dans la littérature et ils apparaissaient d'une façon significative plus souvent chez la *Gt* que chez la *Gpg* ($p<0.05$). Par contre, la *Gpg* montrait une claire préférence pour les humains (66.5%). Lorsque le taux d'infection de la *Gt* (18.5%) était nettement plus haut que celui de la *Gpg* (5.5 %), une analyse par régression logistique a été faite: **Le chapitre 3** montre l'influence des RSF sur l'espèce, l'âge, le taux d'infection, l'état de faim et du sexe. Il fut démontré que les RSF étaient indépendants des degrés d'infection, correspondaient positivement aux mouches femelles chez la *Gt* et chez les *Gpg* gorgées. Il fut aussi discuté jusqu'à quel point les RSF influencent le mécanisme d'infection en supposant une plus haute capacité vectorielle des *Gt* par rapport aux *Gpg*.

La PCR utilisée est en fait plus sensible que les méthodes sérologiques, mais la méthode MALDI TOF MS (matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry) offre des résultats plus rapides, ce qui est déjà de la routine pour les diagnostics microbiens. **Le chapitre 4** est donc dédié à la mise au point d'une banque de donnés protéomique pour

les mouches tsé-tsé au moyen la MALDI TOF MS. Des mouches élevées en laboratoire ont été analysées en entier ou disséquées en tête, ailes, pattes, thorax et abdomen. A la suite d'une simple extraction de protéines, il fut créé 60 MSP (main spectra) comme référence et une analyse en composante principale une recherche de cluster ont été conduites. Ces analyses confirmèrent que parties du corps suffisaient pour une spécification exacte. La fiabilité de la banque de donnés a été testé avec succès en utilisant des mouches tsé-tsé nouvellement extraites, ce qui fut représenté en dégradé de couleur par le CCI (composite correlation index). Une grande distance des *G. brevipalpis* par rapport aux autres espèces a été démontrée par des dendrogrammes basés sur la ressemblance des 70 pics les plus souvent reproduits. Ce résultat a confirmé les résultats d'une étude génomique qui supposait un groupe frère des *G. brevipalpis* avec les autres mouches tsé-tsé. Les *G. austeni* suscitaient aussi des controverses dans des études de taxonomie sur leur appartenance à certains groupes, lorsqu'elles formaient des «cluster» soit avec les groupes des savanes soit avec ceux des cours d'eau, dépendant de la partie du corps analysée. Finalement, la banque constituée offrait une identification rapide et exacte de l'espèce de glossine ainsi que des informations qui pourraient être utilisées pour des études taxonomiques.
Les résultats sont discutés dans **le chapitre 5**: une formule simplifiée de l'estimation de risque offre aussi des informations de grandes valeurs sur la TAA, ce qui pourrait contribuer à une planification rationnelle de projets de lutte contre les vecteurs au niveau des communes rurales. Les PCR de repas sanguins spécifiques aux espèces se sont démontré très efficaces, même s'il savèree difficile de déterminer la préférence de l'hôte. La spécification des mouches tsé-tsé basée sur le MALDI TOF, pourrait permettre une extension de l'analyse protéomique de la glossine concernant les repas sanguins, les infections trypanosomiennes et à l'appartenance à une population, ce qui pourrait constituer une méthode de haut débit pour les diagnostiques futures.

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Hoppenheit, A., Steuber, S., Bauer, B., Ouma, E.M., Diall, O., Zessin, K.H., Clausen, P.-H., 2010. **Host preference of tsetse: an important tool to appraise the Nagana risk of cattle in the cotton zone of Mali**. Wien Klin Wochenschr 122 Suppl 3, 81-86.

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Awards

Junior Award (First Price) 2009:
Scientific conception and presentation
Host preference of tsetse: an important tool to appraise the Nagana risk of cattle in the cotton zone of Mali.
Annual Meeting of the Austrian Society for Tropical Medicine and Parasitology, Nov 19-21, Vienna, Austria.

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Disclosure of the Own Share in the Body of Work

The share of the authors who were involved in the publications of this work is listed under the following criteria:

1. Idea

2. Design of experiments

3. Test execution

4. Analysis of test data

5. Compilation of the manuscript

6. Acquirement of third-party funds

Publication I
Hoppenheit A, Steuber S, Bauer B, Ouma EM, Diall O, Zessin K-H, Clausen P-H
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6. Clausen, Hoppenheit

Publication II

Hoppenheit A, Bauer B, Steuber S, Terhalle W, Diall O, Zessin KH, Clausen PH
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Publication III

Hoppenheit A, Murugaiyan J, Bauer B, Steuber S, Clausen PH, Rösler U
Rapid speciation of Tsetse (*Glossina* spp.) using MALDI TOF MS.
To be submitted to PLoS Negl Trop Dis

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3. Hoppenheit, Murugaiyan
4. Hoppenheit, Murugaiyan
5. Hoppenheit, Murugaiyan
6. Rösler, Clausen

Acknowledgements

Throughout the four years of working on this thesis I could always count on the help of a number of people whom I would like to thank here.

My sincerest gratitude is dedicated to my professors and mentors: Peter-Henning Clausen, Burkhard Bauer, Stephan Steuber and Karl-Hans Zessin for helping me through every step of this work, providing scientific and moral support at all times.

I would also like to thank the German Academic Exchange Service (DAAD) for funding my stay in Mali and especially for the thoroughly nice support of Mrs. Miriam Condé of the ALUMNI network.

This work would not have been possible without my job at the International Animal Health and the many times I received help from Max Baumann and Norbert Miethe when dealing with tedious MS Office issues. And last but not least, thanks to Madame Louise LeBel, the multi-tasking talent, who is best in coping with officials, interpretations or generally in being a good friend.

Furthermore, I am grateful for the guidance I received from my graduate school, the Dahlem Research School, especially from Angela Daberkow and Achim Gruber.

Then, many thanks go to those who have enabled my research in Mali: Oumar Diall for his advice and the warm welcome in his family, Bila Cene who taught me how to dissect tsetse in Burkina, Erick Mungube for being in the same boat and hanging in there, „les techniciens“ Goita, Bengaly and Maiga who helped me hunting down the flies, Dri for the countless times he saved one of the „motos“, further Yao, Kim, Yang, Ali, Chahal, the Sensei, Demba and Oumou and of course the volunteers of the FU Berlin Sebastian Fritz, Mareen Kohn, Szilvia Vincze and Janine Vögely. I would also like to express my thanks to Janette and Abou Camara and everyone else at Miriya Walé for all the encouragement and inspiration.

My sincere thanks also go to Jay Murugaiyan and Uwe Rösler for providing the MALDI and their advice.

At the end, I particularly like to thank: Nicolas Kellner, Friederike Bredy, my mother, Nuri Encinas, Anja Blasse, Juliane Fischer, MBF, Taent, Barbara Hinney, Wendy, all the “paradocs” from the “(mental) institution“ and finally yt, Jon LaJoie and the Lab God.

Statement of Authorship

Except where reference is made in the text, this dissertation contains no material published elsewhere or extracted in whole or in part from a dissertation presented by me for another degree or diploma. No other person's work has been used without due acknowledgement in the main text of the dissertation. This dissertation has not been submitted for the award of any other degree or diploma in any other tertiary institution.

Berlin, December 17 2012 Antje Hoppenheit