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MINI REVIEW

Adult blood-feeding tsetse flies, trypanosomes, microbiota and the fluctuating environment in sub-Saharan Africa

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The tsetse fly vector transmits the protozoan *Trypanosoma brucei*, responsible for Human African Trypanosomiasis, one of the most neglected tropical diseases. Despite a recent decline in new cases, it is still crucial to develop alternative strategies to combat this disease. Here, we review the literature on the factors that influence trypanosome transmission from the fly vector to its vertebrate host (particularly humans). These factors include climate change effects to pathogen and vector development (in particular climate warming), as well as the distribution of host reservoirs. Finally, we present reports on the relationships between insect vector nutrition, immune function, microbiota and infection, to demonstrate how continuing research on the evolving ecology of these complex systems will help improve control strategies. In the future, such studies will be of increasing importance to understand how vector-borne diseases are spread in a changing world.
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Introduction

African unicellular protozoa belonging to the genus *Trypanosoma* are the causative agents of sleeping sickness in humans, where it is known as Human African Trypanosomiasis (HAT), as well as in animals, where it is known as Animal African Trypanosomiasis (also known as Nagana). They infect successively two hosts during their life cycle (thus they are called 'digenetic' parasites): an insect, the tsetse fly (*Glossina* spp.) which is required for their transmission to the second host, most often a mammal (and for the transmission from one mammal host to another). After the strictly hematophagous tsetse fly bites a trypanosome-infected mammal and takes an infected blood meal, the ingested trypanosomes reach the fly midgut, where they differentiate from the bloodstream form (short stumpy trypomastigotes—present in the mammal blood) into the early procyclic form (Sbicego *et al.*, 1999). The parasites then differentiate into several forms during their migration from the gut to the salivary glands. This sequential process includes differentiation from the early procyclic form into the late procyclic form (which is established in the gut), followed by the mesocyclic form (a maturation step in the anterior midgut), followed by the proliferating epimastigote form (in the salivary glands or proboscis, depending on the trypanosome species), and finally differentiation into the non-proliferating metacyclic form (in the salivary glands or proboscis, depending on the trypanosome species) (Van den Abbeele *et al.*, 1999). This last form is the only one that is infective for mammals, and is transmitted from the fly's saliva into a subsequent mammal host's bloodstream during ingestion of a new blood meal. If susceptible, this host will possibly become infected and develop sleeping sickness. Thus, in addition to its role as a trypanosome 'transporter', the tsetse fly is crucial in providing a milieu where the parasite can differentiate, multiply and become infective to mammals. The ability of the fly to acquire the parasite, favor its maturation, and transmit it to a mammalian host is called 'vector competence', and depends on both the *Glossina* and trypanosome species, among other factors.

Interestingly, when flies are fed on trypanosome-infected blood under optimal laboratory conditions, less than 50% become infected. This demonstrates that resistance (usually designated as 'refractoriness') to trypanosome infection is the normal status of the fly. Midgut infection rates rarely exceed 10%

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in natural fly populations. In addition, many midgut-infected flies do not produce mature parasites, indicating that they will never become infective (Moloo *et al.*, 1986; Dukes *et al.*, 1989; Maudlin and Welburn, 1994).

Four species groups, *morsitans*, *palpalis*, *austeni* and *fusca*, within tsetse flies are known to transmit different species or subspecies of trypanosomes. The '*morsitans* group' is the major vector for trypanosomes of the subgenera *Trypanozoon* and *Nannomonas*, which respectively include *Trypanosoma brucei brucei* (Tbb) and *Trypanosoma congolense* (Tc; the savanah type being the most prevalent in cattle); these are the main nagana-causing parasites in sub-Saharan Africa (Nyeko *et al.*, 1990; Reifenberg *et al.*, 1997; Solano *et al.*, 1999). This disease is responsible for dramatic losses in livestock production, estimated at US$ 4.5 billion/year (Reinhardt, 2002). Furthermore, the *morsitans* group is the vector of *Trypanosoma brucei rhodesiense* (Tbr), the causative agent of the acute form of HAT that is endemic in 13 east African countries (Welburn *et al.*, 2009). On the other hand, the *palpalis* group, which poorly transmits Tbb and Tc (Kazadi, 2000), is the vector of *Trypanosoma brucei gambiense*, which is responsible for the chronic form of HAT in 24 countries of western and central Africa (Hoare, 1972; Kennedy, 2008; Welburn *et al.*, 2009).

HAT develops in two phases. During the first stage (which is hematolymphatic), the parasite proliferates in the blood and the lymph. This may progress into the second stage (which is meningoencephalitic) if trypanosomes cross the blood–brain barrier and subsequently invade the central nervous system. The second stage is usually characterized by severe neurological disorders and is frequently fatal if not treated. The signs and symptoms are generally similar for the acute and chronic form of HAT. They differ however in frequency, severity and kinetic appearance. Acute form usually progresses to death within 6 months. Chronic form has a more progressive course with an average duration of almost 3 years (reviewed in Dumas and Bouteille, 1996 and reviewed in Franco *et al.*, 2014).

HAT is one of the most neglected tropical diseases in the world (Brun *et al.*, 2010), even though in terms of mortality it ranks ninth out of 25 human infectious and parasitic diseases in Africa (Welburn *et al.*, 2009). To this day, sleeping sickness is responsible for major disruptions to social, agricultural and economic development in Africa (Simarro *et al.*, 2011). For instance, the disease was recently estimated to cause the loss of 1.5 million disability-adjusted life years per year (Hotez *et al.*, 2009). As sleeping sickness mostly affects marginalized populations living in isolated rural areas, the disease is a severe burden to rural poor populations that often do not have access to health facilities (Odiit *et al.*, 2004).

The serious nature of this disease has led to its targeting for elimination by the WHO and PATTEC (Pan-African Tsetse and Trypanosomiasis Eradication Campaign), and subsequently by the London Declaration on Neglected Tropical Diseases. The number of new cases has begun to decrease in recent years, mirroring a situation that was observed in the 1960s before the last heavy outbreak in the 1990s. So, in spite of this decrease, the severity of the situation demands a deeper understanding of HAT to improve current treatment approaches, as well as to help design novel strategies to control the disease. These goals are in line with PATTEC and the WHO-fixed objective to eliminate HAT. As sleeping sickness is a vector-borne disease, control strategies can be focused on the patient (by developing preventive and/or curative approaches) and/or on the tsetse fly vector (to eradicate or impede its vector competence). Unfortunately, these approaches are hindered by a lack of vaccines and a limited drug toolbox that produces harmful side effects (Simarro *et al.*, 2008). To complicate matters, current drug treatments have led to the emergence of resistant trypanosome strains (Baker *et al.*, 2013).

Domestic (that is, pigs) and wild animals (that is, diverse rodents, carnivores and primates) found within HAT zones are a valuable nutritional asset for people living in these areas. At the same time, these animals present a risk to humans, since they may harbor different trypanosome species, including those specific to HAT. This unfortunately creates a situation where these animals act as trypanosome reservoirs (Njiokou *et al.*, 2006; Simo *et al.*, 2006; Farikou *et al.*, 2010a), from which their parasites are spread by tsetse flies that feed indifferently on humans or other domestic or wild mammals.

The cyclical transmission of trypanosomes is highly dependent on the biochemical and physiological interactions that occur between the parasite and its insect host. These in turn depend on a variety of biotic and abiotic factors including: climate change; geographical distribution and environmental conditions of HAT foci; tsetse fly population flow between foci; disease epidemiology; type and distribution of trypanosome reservoirs; changes in tsetse fly nutritional behavior; and finally the nature and diversity of fly intestinal microbiota, including symbiotic (*Wigglesworthia glossinidia*, *Sodalis glossinidius* and *Wolbacchia* spp.) and diverse non-symbiotic bacteria (Dale and Maudlin, 1999; Wang *et al.*, 2013a). Investigations of these factors have already begun in the past several years.

By revisiting the existing literature, the present review on microbial ecology aspects of HAT aims to advance research on how changes in environmental conditions can affect trypanosome–tsetse fly–gut microbiota interactions, and consequently, the dynamics of the disease. Successful pursuits in these areas will enable the design of novel strategies for disease control.

Global changes and sleeping sickness transmission

Global climate changes are of particular importance to arthropod-borne diseases (Rogers and Randolph, 2006; Moore *et al.*, 2012). The spread of sleeping sickness is tripartite, involving the trypanosome, the tsetse fly vector (and its symbionts) and the vertebrate hosts. The perpetuation of the parasite itself relies on two connected populations, the adult tsetse flies as well as the mammals from which they take their blood meal. Both the fly vectors and vertebrate hosts require specific climatic conditions (for example, temperature and humidity) for their survival, reproduction and propagation (Dean *et al.*, 1969). The ability of trypanosomes to establish in the midgut, and then to migrate to and mature within the salivary glands, depends on several biotic and abiotic factors. Modification of these factors may affect vector competence, which may then impact trypanosome transmission to host vertebrates and thus the spread of the disease (Figure 1). There is clearly a need for interdisciplinary investigations to determine how global changes (that is, changing temperature, rainfall patterns, increasing urbanization, deforestation, grassland degradation and overgrazing) could affect a variety of factors that include: the geographical distribution of trypanosome vertebrate-host reservoirs; the nutritional behavior of tsetse flies, the development of trypanosome and tsetse flies; and interactions between the tsetse fly vector, the vertebrate hosts and the trypanosome. Detailed studies of these factors would improve our understanding of how the disease is spread in environments affected by socioeconomic, environmental and climatic changes. So, the increasing world population that will, soon, reach seven billion people (Tollefson, 2011) requires an ever-increasing number of livestock and new farmlands to satisfy our nutritional needs. Increasing the number of livestock in farmlands drastically alters the sub-Saharan African environment by modifying not only the livestock distribution, but also the distribution of tsetse flies that feed on it (and possibly their nutritional behavior). Indeed, the importance of environmental factors to transmission intensity and trypanosome distribution is increasingly being recognized (Van den Bossche *et al.*, 2010; Bouyer *et al.*, 2013). Accordingly, a recent study of climate change effects on the evolution of African trypanosomiasis predicts that 46–77 million additional people will be at risk of sleeping sickness by 2090 (Moore *et al.*, 2012).

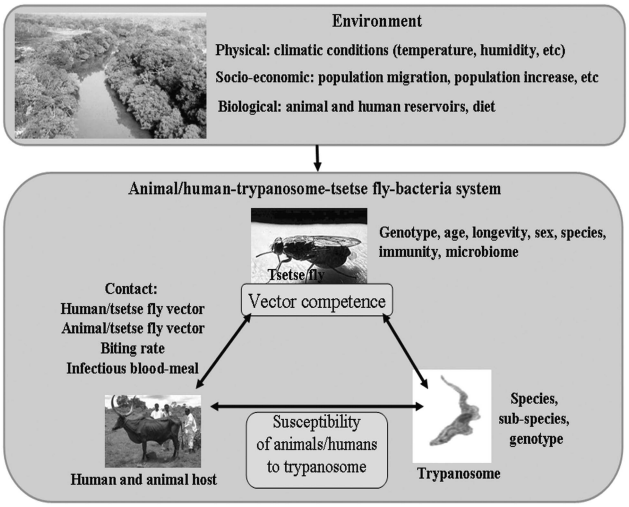

Figure 1 Factors that may influence tsetse fly susceptibility for trypanosome infection. For trypanosome transmission to occur, the parasite must first be established in the tsetse fly midgut following an infective blood meal taken from a mammal source acting as a host reservoir of trypanosomes; the trypanosome must then mature in the salivary glands or the mouthparts, depending on the trypanosome species. Many abiotic and biotic factors may affect the success or failure of trypanosome development. The role of these factors in vector competence depends on how they affect trypanosome development in the tsetse fly, and the fly's susceptibility or resistance to trypanosome infection.

Impact of global changes on the developmental rates of trypanosomes and tsetse flies

In vector-borne diseases, temperatures above 34 °C frequently have a negative impact on the survival of both the insect vectors and the parasites (Rueda *et al.*, 1990). Thus, such high temperature may also impact unfavorably tsetse fly and trypanosome populations. Tsetse fly pupation and survival requires favorable environmental conditions, including moderate temperature (23–25 °C), high relative humidity (75–90%) with weak saturation deficit (to avoid high evaporation power) and shade (Ndegwa *et al.*, 1992; Courtin *et al.*, 2010; Pagabeleguem *et al.*, 2012). Nevertheless, higher temperatures induce a more rapid blood meal digestion by the tsetse fly; consequently, the fly may feed more frequently, which can increase both the rate of trypanosome ingestion (when the fly feeds on an infected host) and transmission (when the fly have become trypanosome infected and feeds on a non trypanosome-infected host) (Terblanche *et al.*, 2008). Thus, higher temperatures could have both positive and negative effects on HAT transmission. Finally, there may exist an optimal temperature that would favor an optimal balance between vector and parasite populations development rates and the parasite transmission rate.

Moreover, seasonal alternation, local environmental changes and differences between geographic areas may modify the balance between the different species; this is particularly relevant among wild vertebrates that are fed upon by tsetse flies (Staak *et al.*, 1986; Mukabana *et al.*, 2002; Farikou *et al.*, 2010a). Any modification of tsetse fly nutritional behavior may impact trypanosome transmission, as

well as the spread of HAT and Animal African Trypanosomiasis. A fly's nutritional behavior can be determined by identifying the origin of its ingested blood meal and which residues are still present in the gut. Such an investigation was performed in the Bipindi and Campo HAT foci of south Cameroon in 2008, which established that the collected flies had taken their blood meals from humans (46%), pigs (37%) and wild mammals (17%). Notable differences between the two foci were nevertheless recorded: in Bipindi, 23% and 67% of flies took their blood meal from humans and pigs, respectively, whereas these figures were respectively 63% and 23% for flies in Campo. There were also substantial differences observed between years: in 2004, 45% and 7% of flies respectively from Bipindi and Campo took their blood meal from pigs, versus 67% and 23%, respectively, in 2008 (Simo *et al.*, 2008, Farikou *et al.*, 2010a). These results illustrate how the nutritional behavior of tsetse flies depends on the geographical area and how quickly it can change over a relatively short period.

Developmental and immune responses in the trypanosome–tsetse fly association

Trypanosomes undergo several rounds of differentiation and proliferation during their life cycle. Although the development cycle differs somewhat between trypanosome species, the two main stages consist of establishment and maturation steps. Both *T. congolense* (subgenus *Nannomonas*) and *T. brucei* (subgenus *Trypanozoon*) establish within the fly midgut (midgut colonization step) but mature in the tsetse proboscis and salivary glands, respectively; the development cycle then culminates with the metacyclic form that is infective for mammals (humans, wild or domestic animals) (Van den Abbeele *et al.*, 1999).

As stated in the introduction, the rate of trypanosome midgut colonization in the tsetse fly is generally low (Moloo *et al.*, 1986; Maudlin and Welburn, 1994). During this midgut colonization step, tsetse flies employ mechanisms for eliminating the trypanosomes, whereas the parasites attempt to evade the tsetse fly immune system for their own survival (Aksoy *et al.*, 2003). Several molecules can be released during the time course of these interactions (Table 1), either by tsetse flies or by trypanosomes. This release can be modified according to fly-intrinsic (for example, sex of flies) and fly-extrinsic factors (for example, trypanosome species; starvation). Therefore, successful establishment (that is, midgut colonization) of trypanosomes in tsetse depends on their ability to adapt, transform, survive and grow rapidly after their quick transition from the vertebrate host blood to the different environment of the tsetse gut (Simo *et al.*, 2010).

Trypanosome invasion activates innate immune responses within tsetse flies (Hao *et al.*, 2001; Hu and Aksoy, 2006) by inducing the tsetse production of several molecules including: antimicrobial peptide; glutamine/proline-rich (EP) protein; reactive oxygen species; and several other molecules involved in the immunodeficiency (Imd) pathway (Hao *et al.*, 2001; Lehane *et al.*, 2003; Hu and Aksoy, 2006; Nayduch and Aksoy, 2007; MacLeod *et al.*, 2007a; Haines *et al.*, 2010). In fact, the balance between the released molecules has an important role in the success or failure of trypanosome establishment within the tsetse fly midgut, as it is crucial for creating a suitable environment for their survival and development. For example, the prevalence of trypanosomes increased in flies when the expression of either the Imd pathway or the downstream-expressed antimicrobial peptide effector was downregulated by RNAi (Hao *et al.*, 2001; Hu and Aksoy, 2006). MacLeod *et al.* (2007a) have also shown that antioxidants promote the establishment of trypanosome infections in tsetse flies.

Table 1 Effects of various intrinsic and extrinsic factors on trypanosome development within the tsetse fly vector

Factors	*Produced by*	*Effect*	*References*
Attacin	Tsetse fly	Trypanocidal activity	Hao *et al.*, 2001; Hu and Aksoy, 2006
Diptericin	Tsetse fly	Trypanocidal activity	Hao *et al.*, 2001; Hu and Aksoy, 2006
Glutamin/proline-rich (EP) protein	Tsetse fly	Inhibits trypanosome establishment	Haines *et al.*, 2010
Reactive oxygen species	Tsetse fly	Inhibits trypanosome establishment	Hao *et al.*, 2001; Lehane *et al.*, 2003; Hu and Aksoy, 2006; Nayduch and Aksoy, 2007; MacLeod *et al.*, 2007a
Nitrogen monoxide (NO)	Tsetse fly	Promotes trypanosome migration to salivary glands and maturation	MacLeod *et al.*, 2007b
L-Cysteine	Tsetse fly	Promotes trypanosome migration to salivary glands and maturation	MacLeod *et al.*, 2007b
Purines	Tsetse fly	Promotes trypanosome survival	Henriques *et al.*, 2003
Heat-shock protein 70 Heat-shock protein 83	Trypanosome	Reaction against stress in fly midgut	Simo *et al.*, 2010
Starvation	Tsetse fly	Increases establishment and/or maturation of trypanosome in tsetse fly and offspring	Kubi *et al.*, 2006

Subsequently, trypanosomes require signals such as L-cysteine and/or nitric oxide, as well as environmental stimuli, for their migration to the salivary glands, where they mature (MacLeod *et al.*, 2007b).

In the presence of trypanosomes, tsetse flies will modify the expression of several of their genes. In response to this differential gene regulation, trypanosomes regulate the expression of their own genes for their survival (Savage *et al.*, 2012). For example, *T. b. gambiense* and *T. b. rhodesiense* express genes associated with reactions against stress (Simo *et al.*, 2010), indicating that trypanosomes are exposed to environmental stress within the tsetse fly midgut. Owing to the delicate equilibrium governing these molecular interactions, any internal or external perturbation may impact the fly–trypanosome relationship. For instance, injecting tsetse flies with *E. coli* was shown to stimulate their immune system, resulting in a severe blocking of trypanosome establishment subsequent to any infected blood meal (Hao *et al.*, 2001). Therefore, the upregulation of several immune responsive genes early in infection can act to block parasite transmission. These results have previously been discussed in the context of potentially using transgenic approaches to modulate tsetse fly vector competence (Hao *et al.*, 2001). A deficit in mammal blood meal availability, and other environmental factors, can also cause nutritional stress in a tsetse population; in addition to making tsetse flies significantly more susceptible to midgut infection, these factors boost the maturation of midgut infections (Akoda *et al.*, 2009a). These examples illustrate how external factors, which do not depend on the trypanosome or tsetse fly, can modify their association. In this context, environmental changes can impact the biochemistry, physiology and even survival of tsetse flies and trypanosomes. Their interactions will also be affected as a consequence, since both organisms must first adapt their physiology to the modified environmental conditions. Very recent developments in the genomics of the tsetse fly (International Glossina Genome Initiative, 2014) now provide novel ways to further investigate these tsetse–trypanosome interactions through comparative and functional genomics.

Impacts of the tsetse fly microbiome and nutrition on fly physiology and *Trypanosoma* transmission

Tsetse flies harbor three bacterial symbionts, including the obligate primary (essential) symbiont *Wigglesworthia glossinidia* (Wang *et al.*, 2013a) and the secondary (non-essential) symbiont *Sodalis glossinidius* (Dale and Maudlin, 1999). Both symbionts, which belong to the Enterobacteriaceae family, colonize the tsetse fly gut (Aksoy *et al.*, 2013) and are vertically transmitted to the intrauterine-developing larvae *via* milk gland secretions (Wang *et al.*, 2013a). *Wigglesworthia* encodes vitamins that may promote host reproduction as well as fly nutrition throughout its development (Nogge, 1982; Rio *et al.*, 2012). In addition to the midgut, *Sodalis* develops in several other tsetse fly (*Glossina* spp.) organs (Wang *et al.*, 2013a). The specific elimination of *Sodalis* has been reported to result in reduced tsetse fly longevity (Dale and Welburn, 2001; Wang *et al.*, 2013b).

Tsetse flies can also harbor a third symbiont, the α-proteobacterium *Wolbachia* (O'Neill *et al.*, 1993), which is a non-essential bacterium that infects many different invertebrates (Werren *et al.*, 2008). The presence of this bacterium is restricted to the reproductive organs of the tsetse fly and is transmitted transovarially (Wang *et al.*, 2013a). Although it is highly prevalent within laboratory-reared tsetse fly colonies (Cheng *et al.*, 2000), its prevalence in natural tsetse fly populations is variable (Doudoumis *et al.*, 2012). *Wolbachia* has also been shown to induce strong cytoplasmic incompatibility in tsetse, as by the second gonotrophic cycle, none of the females in an incompatible cross yield any progeny (Alam *et al.*, 2011). This phenomenon occurs when a *Wolbachia*-infected male mates with an uninfected female resulting in degeneration of the future embryo. In contrast, when a *Wolbachia*-infected female mates with either an uninfected male or a male infected with the same strain as the female, the female will produce viable *Wolbachia*-infected offspring. Furthermore, these offspring will be more numerous than those produced by a non *Wolbachia*-infected female after mating with a non *Wolbachia*-infected male (Alam *et al.*, 2011). This reproductive advantage for infected females has two implications. First, it results in the spread of *Wolbachia* infections along with the other traits (*Sodalis*) that the infected insects might display (Hoffman *et al.*, 1998; Dobson *et al.*, 2002). Second, it produces a progressive replacement of the initial fly population by a population of *Wolbachia*-infected flies (Alam *et al.*, 2011; Medlock *et al.*, 2013).

Recent investigations of the midgut microbiota composition in natural tsetse fly populations collected from HAT foci in three African countries (Angola, Cameroon and Kenya) have revealed the presence of an unexpectedly diverse bacterial community (more than 10 bacteria species in *Glossina fuscipes fuscipes* from Kenya (identified using culture-depending and non culture-depending methods), more than 5 bacteria species in *G. p. palpalis* from Cameroon (using culture-depending methods)) (Geiger *et al.*, 2009, 2011; Lindh and Lehane, 2011). Their diversity was shown to depend on the tsetse species or subspecies, as well as on the geographic origin, although differences in environmental conditions and food supply may also influence the diversity of the harbored bacterial communities. Recently, Aksoy *et al.* (2014) investigated the different levels and patterns of gut

microbial diversity among individuals from tsetse fly populations in Uganda (*Glossina morsitans morsitans*, *G. f. fuscipes* and *Glossina pallidipes*), using multiple approaches such as deep sequencing of the V4 hypervariable region of the 16S rRNA gene, 16S rRNA gene clone libraries, and bacterium-specific quantitative PCR. In contrast to the former, this study revealed an extremely limited microbiota diversity in the investigated flies. The obligate endosymbiont *Wigglesworthia* was dominant in all samples (>99%), and a wide prevalence of low-density *Sodalis* infections (<0.05%) was also observed. However, 22% of the samples displayed high *Sodalis* density colonization; they also carried co-infections with *Serratia*. The wild fly microbiomes display more bacterial species than insectary-reared flies, where, by now, only one species, a novel one named *Serratia glossinae*, has been previously identified using a culture-dependent method (Geiger *et al.*, 2010). Finally, bacteria diversity characterized in wild flies was very variable depending on fly species, geographical origin as well as on the different microbiome analysis techniques used. Thus, the need to pursue and extend such investigations.

Colonization of the gut by microbial communities may or may not increase tsetse fly resistance against trypanosome invasion. Underlying mechanisms include competition for nutrients, niche occupation and stimulation of immune responses (Stecher and Hardt, 2011; Brestoff and Artis, 2013; Engel and Moran, 2013; Furusawa *et al.*, 2014). Recent data suggest that *Sodalis* and *Wigglesworthia* can modulate trypanosome development (Table 2). *Wigglesworthia* must be present during the immature larval stages for the adult tsetse fly immune system to develop and function properly (Weiss *et al.*, 2011). The artificial elimination of *Wigglesworthia* from flies does not only render them sterile, but will also compromise their immune system development. This in turn increases fly susceptibility to gut trypanosome infection; in contrast, flies carrying *Wigglesworthia* are highly resistant (Wang *et al.*, 2009). Furthermore, comparisons between *Wigglesworthia* spp. from *G. morsitans morsitans* and from *G. brevipalpis* have revealed metabolic variations. These differences involve the chorismate, phenylalanine and folate biosynthetic pathways, which are only present in *Wigglesworthia* from *G. morsitans morsitans*. African trypanosomes are auxotrophic for these molecules and salvage them exogenously. This could explain the differences observed in trypanosome susceptibility between these two tsetse species (Rio *et al.*, 2012).

Colonization with *S. glossinidius* has been shown to increase susceptibility for trypanosomes in tsetse flies (Welburn *et al.*, 1993) through a mechanism involving the production of *N*-acetyl glucosamine (Maudlin and Ellis, 1985; Welburn and Maudlin, 1999). This sugar results from the hydrolysis of pupae chitin, by an endochitinase from *S. glossinidius*. Furthermore, this sugar is reported to inhibit a midgut lectin from the tsetse fly, which is lethal to trypanosome procyclic forms (Welburn and Maudlin, 1999; Dale and Welburn, 2001). More recently, it was reported that the ability of two different trypanosome subspecies to establish in the tsetse fly midgut is significantly linked to the presence of *S. glossinidius*-specific genotypes (Geiger *et al.*, 2007). This suggests that different *Sodalis* genotypes might be associated with differing capacities for trypanosome-establishment facilitation. In addition, susceptibility may increase in response to a greater density of the symbiont in the fly gut (Cheng and Aksoy, 1999). The favorable effect of *Sodalis* on fly infection by trypanosomes has recently been assessed in large tsetse fly sampling campaigns conducted in two sleeping sickness foci in southern Cameroon (Farikou *et al.*, 2010b). Finally, additional diversity analyses have shown that the geographical isolation of the two foci may have induced the independent evolution of *Sodalis* and tsetse fly populations, suggesting a probable

Table 2 Effects of various tsetse fly symbionts on tsetse fly susceptibility to trypanosome infection

Factors	*Symbiont*	*Effect*	*References*
Unknown	*Wigglesworthia glossinidia*	Development of tsetse immune system and resistance to trypanosome infection	Wang *et al.*, 2009; Weiss *et al.*, 2011
Chorismate, phenylalanine, folate	*Wigglesworthia glossinidia* strain	Increases susceptibility of *Glossina morsitans morsitans* species to trypanosomes	Rio *et al.*, 2012
Chitinase	*Sodalis glossinidius*	Increases susceptibility of tsetse to trypanosomes	Welburn *et al.*, 1993
Density	*Sodalis glossinidius*	Increases susceptibility of tsetse to trypanosomes	Cheng and Aksoy, 1999
Genotypes	*Sodalis glossinidius*	Associated with tsetse fly infection by different trypanosome species	Geiger *et al.*, 2007
Unknown	*Sodalis glossinidius*	Effect on fly infection	Farikou *et al.*, 2010b
Haplotypes	*Sodalis glossinidius*	Associated with prevalence of tsetse fly infection	Farikou *et al.*, 2011
Phage	*Sodalis glossinidius*	Associated with tsetse fly resistance to trypanosome infection	Hamidou Soumana *et al.*, 2014
Unknown	*Wolbachia* sp.	Inhibits development of trypanosome in tsetse fly	Aksoy *et al.*, 2013

coevolution between *Sodalis* and tsetse flies (Farikou *et al.*, 2011). Taken together, these data could help explain reported epidemiological differences in HAT cases between HAT foci. Recently, the transcriptional signature of *Sodalis* hosted by trypanosome-infected flies was compared with that of *Sodalis* hosted by refractory flies (that is, flies that were not infected despite having taken a trypanosome-infected blood meal). Many of the modulated transcripts in the symbiont population within flies refractory to trypanosome infection cluster within networks involving lysozyme activity, bacteriolytic enzymes, bacterial cytolysis and cell wall macromolecule catabolic processes. These observations suggest the possible involvement of a *Sodalis*-hosted prophage in tsetse *Trypanosoma* resistance (Hamidou Soumana *et al.*, 2014).

Several other mechanisms may be involved in the modulation of trypanosome infection by midgut microbiota including the production of anti-parasitic molecules by the bacteria inhabiting the tsetse fly vector gut (reviewed in Azambuja *et al.*, 2005). For example, pigment-producing bacteria have already been identified in the tsetse fly midgut (Geiger *et al.*, 2009, 2010, 2011; Lindh and Lehane, 2011) and the prodigiosin pigment is reported to be toxic to *Plasmodium falciparum* (Lazaro *et al.*, 2002) and *Trypanosoma cruzi* (Azambuja *et al.*, 2004).

Nutrition also affects the susceptibility of tsetse flies to trypanosomal infections, since extreme starvation periods in teneral (young flies that have never taken a blood meal) and non-teneral tsetse flies can increase the proportion of adult flies, and their offspring, that will develop mature trypanosome infections that can be transmitted to humans (Kubi *et al.* 2006; Akoda *et al.*, 2009b). Previous studies have suggested that immune function is affected by the nutritional state of the fly, as well (Attardo *et al.*, 2006). Bacterial populations may also vary in persistence, abundance and species composition within the tsetse fly host; the host environment and nutrition are major determinants in this case (Chandler *et al.*, 2011). This underscores the importance of defining the relationships between diet and the composition and function of gut microbiota (Ponton *et al.*, 2011, 2013). For example, blood meals have been associated with massive proliferation of bacteria residing in the digestive tract (Kumar *et al.*, 2010; Oliveira *et al.*, 2011; Wang *et al.*, 2011) through the effects of reactive oxygen species levels (Oliveira *et al.*, 2011). This process has been demonstrated in the mosquito midgut, where a blood meal immediately decreases the level of reactive oxygen species through a mechanism involving heme-mediated protein kinase C activation, creating a favorable environment for bacterial proliferation (Oliveira *et al.*, 2011).

As a food source, blood contains a number of components that can interfere with insect physiology (Luckhart and Riehle, 2007; Kang *et al.*, 2008; Pakpour *et al.*, 2013), such that the quality of ingested blood can be just as important as the quantity (Broderick *et al.*, 2004; Chandler *et al.*, 2011). Preference for tsetse fly mammalian hosts (human, wild or domestic animals) can differ greatly according to *Glossina* species, wild life and geographical locations (Omolo *et al.*, 2009; Farikou *et al.*, 2010a; Muturi *et al.*, 2011). Due to the difference in blood composition between different mammalian hosts (human, wild or domestic animals), it can be expected that blood meals taken from different host types will differentially influence gut microbiota composition in tsetse flies, which might explain some of the geographical variation previously observed (Geiger *et al.*, 2009, 2011). In addition, flies may ingest bacteria within the environment, particularly from the skin surface of hosts during blood meals (Poinar *et al.*, 1979; Simo *et al.*, 2008, Farikou *et al.*, 2010a). Blood composition and sources may therefore be important factors that modulate vector competence, through complex interactions between nutrients, immunity and bacterial communities.

Implications for large-scale tsetse fly control

Current strategies for insect pest control management include the application of chemical insecticides, the dissemination of sterile male insects and the introduction of natural predators (including lady beetles) or parasites (including parasitic wasps) (Engel and Moran, 2013). Environmental factors influence microbial interactions and the resilience of a community, which is in turn influenced by microbial diversity (Masurekar, 2008). These factors will then influence vector competence. Previous insight on the interactions between tsetse flies, trypanosomes, microbiota and the environment can also be used to improve the control of tsetse flies, by providing clues on how to manipulate tsetse fly gut microorganisms. This approach may also be of practical value for generating novel modes of pest biocontrol. A number of bacteria-based approaches have also been suggested, some of which have been successfully implemented (Aksoy *et al.*, 2008; Engel and Moran, 2013).

One recent review (Engel and Moran, 2013) has reported that the composition of the gut microbiota in invertebrate hosts could influence vector competence via different approaches including the modulation of immune responses, niche competition or production of inhibitory molecules (Azambuja *et al.*, 2005; Dong *et al.*, 2009; Hoffmann *et al.*, 2011; Cirimotich *et al.*, 2011a, b). Therefore, it is likely that induced modifications of the gut microbiota composition could impact the vector competence of flies. As mentioned above, natural tsetse flies are colonized by a taxonomically diverse array of microbiota (Geiger *et al.*, 2009, 2011; Aksoy *et al.*, 2014).

One pest control method in particular, paratransgenesis (Aksoy *et al.*, 2008), uses modified symbionts to express molecules that could increase tsetse fly resistance to trypanosomes, by stopping the development of parasites. This method is suggested instead of fly transgenesis, since tsetse flies are viviparous (Attardo *et al.*, 2006): their embryos and larvae develop *in utero*, rendering microinjection of transgenes into the embryo very difficult. The symbiont *Sodalis* may be used for paratransgenesis (Medlock *et al.*, 2013), as it is cultivable and thus suitable for genetic manipulation *in vitro* (Aksoy *et al.*, 2008). *Wigglesworthia*, by contrast, cannot be used as it is uncultivable. Importantly, the *Sodalis* symbiont inhabits the tsetse gut in immediate proximity with trypanosomes, thereby directly exposing them to *Sodalis* effector proteins (De Vooght *et al.*, 2012). *Sodalis* is vertically transmitted to tsetse offspring and can thus transmit the manipulated character from one generation to the next. Finally, the *Sodalis* genome is rich in pseudogenes, making it susceptible to large-scale gene erosion (Toh *et al.*, 2006). Due to its reduced functional genome, *Sodalis* is metabolically dependent on tsetse flies for survival; it has never been found associated with other insects. This makes *Sodalis* a potentially safe candidate for a paratransgenesis approach. In practice, tsetse flies harboring a recombinant *Sodalis* strain encoding trypanosome resistance genes must be disseminated into natural fly populations, so as to replace the current susceptible population (Alam *et al.*, 2011). *Wolbachia*-induced cytoplasmic incompatibility can be exploited to drive this rapid dissemination within natural populations of tsetse fly vectors and progressively replace it, thereby allowing disease control (Alam *et al.*, 2011).

In addition to its role in the *Sodalis*/*Wolbachia* couple (dissemination of flies harboring the recombinant *Sodalis*), *Wolbachia* may be used alone. The embryonic death caused by *Wolbachia*-induced cytoplasmic incompatibility can be applied to suppress tsetse fly populations (Alam *et al.*, 2011; Aksoy *et al.*, 2013). Another advantage of the *Wolbachia* symbiont is its capability to inhibit the development of trypanosomes in tsetse (Table 2) (Aksoy *et al.*, 2013).

One alternative to paratransgenesis could be to increase the prevalence of gut bacteria that are naturally present in tsetse flies and that are capable of reducing the trypanosome load in tsetse fly natural populations.

Finally, several examples of the effect of insect gut bacteria on the fitness or sexual competitiveness of insect vectors have been reported. One promising approach has been observed by feeding the tephritid fly *Ceratitis capitata* with a specific bacterial diet that can improve the fitness and sexual competitiveness of γ-irradiated sterile male insects (Gavriel *et al.*, 2011; Engel and Moran, 2013). Similar experiments could be conducted on γ-irradiated tsetse flies, an approach used to eradicate isolated tsetse populations (Vreysen *et al.*, 2000), since irradiation may modify their intestinal bacterial community content and so decrease their fitness and sexual competitiveness.

Concluding remarks and future perspectives

In spite of the recent decline in cases, the possibility of an expansion of HAT and other diseases must not be underestimated, as reflected by the recent outbreak of Ebola virus disease in western Africa. Today, a broad range of data are available in disease fields concerned with sleeping sickness. However, these data are dispersed and one major difficulty is

Box 1 Further issues to develop.

Some proposals we provide have immediate practical applications, whereas others will first require fundamental investigations.

1. Realize an exhaustive cartography of the HAT foci, especially in isolated bush areas.
2. Evaluate the asymptomatic HAT prevalence, and the expansion of this disease.
3. Develop dispensaries in isolated areas.
4. Generate a 'sentinel' network.
5. Develop multidisciplinary investigations to improve our understanding of the environmental and human factors that favor the maintaining of the endemic stage of the disease, or epidemic outbreaks.
6. On the basis of (5), develop predictive approaches to identify areas potentially at risk of sleeping disease following environmental modifications.
7. More extensive characterization of the composition of tsetse fly microbiota.
8. Develop integrated investigations of the interactions between the fly, its microbiome and the trypanosome, to identify genes involved in susceptibility/refractoriness of the fly to trypanosome infection.
9. Investigate the vertical and horizontal transmission of the bacteria species hosted by the female fly.
10. Characterize the genetic diversity within populations of the different fly species, as well as their indigenous symbiont *Sodalis glossinidius* populations, to detect a possible relationship between tsetse fly and *Sodalis* genotypes.
11. Evaluate the feasibility of using genetically engineered bacteria that would express trypanocidal molecules in the fly's gut or molecules capable of blocking the trypanosome development cycle, to block the fly's vector competence.

to assemble them into an integrated and dynamic overview that depicts the events during disease development, as well as guidelines to detect the factors that control them. Globally, the disease develops in two distinct phases: one within the insect host, the tsetse fly, another in the mammal host, including humans. These two phases are closely interconnected by the exchange of the trypanosome between tsetse flies and the mammal hosts. The interruption of this active and necessary 'exchange' step could lead to parasite elimination, and consequently the disappearance of the disease from the mammal hosts. Tsetse flies clearly have a central role in parasite exchange, due to the intimate link between their feeding behavior and disease transmission. Thus, the primary focus of interrupting parasite exchange should be placed on anti-vector strategies. These could include eradicating flies or blocking their vector capabilities, as well as: research on the ecological/climatic/nutritional conditions favoring fly population development and spread; trypanosome survival; and fly infection processes. Some of these approaches could be used in the future to determine possible geographic areas, and the corresponding human populations at risk of sleeping sickness. Exceptional recent progress in genomics, transcriptomics, proteomics and biotechnology provides hope for characterizing the factors governing the tripartite interaction between the fly, its microbiota and the parasite, which must then be confirmed by functional analyses. At the same time, progress in diverse biotechnologies may open novel uses for practical application based on former findings. The collection of environmental data and analyses of its effects on disease development and propagation will similarly benefit from further studies. We have provided several points for consideration in Box 1, which may yield success with these approaches.

Conflict of Interest

The authors declare no conflict of interest.

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