Flying trypanosomes in tsetse flies

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Survival in and colonization of the tsetse fly midgut are essential steps in the transmission of many species of African trypanosomes. In the fly, bloodstream trypanosomes transform into the procyclic stage within the gut lumen and later migrate to the ectoperitrophic space, where they multiply, establishing an infection. Progression of the parasite infection in the fly depends on factors inherent to the biology of trypanosomes, tsetse, and the bloodstream flies. Flies usually eradicate infection early on with both pre-existing and inducible factors. Parasites, in contrast, respond to these stimuli by undergoing developmental changes, allowing a few to both survive and migrate within the tsetse. Here we discuss parasite and fly factors determining trypanosome colonization of the tsetse, focusing mainly on the midgut.

Tsetse–trypanosome interactions: a matter of life and death

African trypanosomes alternate their life cycles between a vertebrate host and tsetse, Glossina spp. (see Glossary). These parasites cause human African trypanosomiasis (HAT, or sleeping sickness) and animal African trypanosomiasis (AAT). In addition to the considerable human morbidity caused by HAT, AAT has an estimated annual cost of $4.75 billion in lost agricultural gross domestic product [1]. The best-studied species, Trypanosoma brucei and Trypanosoma congolense, first have to establish an infection in the midgut and then migrate to the salivary glands (SGs) or the mouthparts of the fly, respectively, in order to mature into the mammalian infectious form (see Box 1 for a summary of the stages of infection in the tsetse fly). Despite the high incidence of trypanosomiasis in mammals in sub-Saharan Africa, it is puzzling that relatively few tsetse captured in the wild show midgut trypanosome infections, with estimates ranging from 2% to 20% in different tsetse species and sample sites [2,3]. Even within HAT foci the prevalence of Trypanosoma brucei rhodesiense in tsetse can be astonishingly low: for example, 0.0064% of individuals of the tsetse species Glossina swynnertoni in the Serengeti, Tanzania, were estimated to be infected [3]. In laboratory infection experiments, multiple colonization failures and bottlenecks in parasite survival imply that tsetse infection by trypanosomes is likely to be a strongly contested process. There is a balance between the ability of the tsetse to eliminate the infection and the capacity of the parasite to evade the hostile environment of the fly, physical defenses, and immune response in order to survive and develop. In this review, we discuss the recently discovered factors and the molecular events thought to control the success of T. brucei and T. congolense infections within the tsetse midgut and their respective maturation to the SGs or mouthparts. Other reviews cover specific aspects of these processes [4–6].

Glossary

Animal African trypanosomiasis (AAT): disease of mammalian livestock caused by infection with African trypanosomes including T. b. brucei and T. congolense, among others.

Antimicrobial peptides (AMPs): short peptides produced by the host in response to pathogens and parasites, which act directly on parasites to kill them.

Chitin: long chain polymers of N-1,4-acetylglucosamine residues found in many species, including insects.

Eclosion: emergence of a teneral fly from a pupal case.

Ectoperitrophic space (ES): the space between the peritrophic matrix and gut epithelium in the insect midgut.

Epimastigote: form of trypanosome in which the kinetoplast is anterior to the nucleus, found in both the PV and SGs of infected tsetse. Examples mentioned in the text include short epimastigotes (SES), long epimastigotes (LES), and attached epimastigotes.

Extrachromosomal susceptibility factor: a heritable factor determining the susceptibility of tsetse to trypanosome infection that is not present on the chromosomal DNA of the tsetse; instead showing cytoplasmic inheritance. Classical examples of extrachromosomal heritable factors include organelles such as mitochondria and maternally inherited bacteria.

Fat body: fat storing cells in the fly that store and secrete metabolites into the hemolymph.

Glutamic acid/alanine-rich protein (GARP): abundant glycoprotein expressed on the surface of epimastigote T. congolense.

Glycosaminoglycans (GAGs): large branched polysaccharides made of repeating disaccharide units [N-acetylhexosamine and uronic acid].

Glycosylphosphatidylinositol (GPI): conserved glycolipid particularly abundant on the plasma membrane of trypanosomatid parasites. In trypanosomes, it can occur as free or anchoring surface proteins (e.g., variant surface glycoproteins, VSGs).

Human African trypanosomiasis (HAT): human disease caused by infection with T. b. rhodesiense or Trypanosoma brucei gambiense.

Kinetoplast: a DNA network of kinetoplastids (including trypanosomes) containing the mitochondrial genome. The kinetoplast, also known as kDNA, is found adjacent to the basal body of the flagellum.

Long slender (LS): replicative T. brucei bloodstream trypanastigote form in which the kinetoplast is posterior to the nucleus and undergoes antigenic variation in the mammal. LS cannot transform to the PF in the tsetse midgut.

Maturation index (MI): the proportion of midgut infections, which go on to generate infected SGs (T. brucei) or mouthparts (T. congolense).

Metacyclogenesis: process by which trypanosomes transform into mammalian infective, metacyclic trypanastigotes.

Midgut: the middle portion of the tsetse gut, located between the foregut and hindgut, and lined with the PM. Digestion and absorption of the bloodmeal occur in the midgut, and it is in the midgut that both T. brucei and T. congolense first establish infection in the fly.
Monomorphic trypanosomes: bloodstream form (BSF) trypanosomes which do not appear to show morphological differences correlated with their ability to transform to the PF, that is, lacking separate ST and LS forms.

N-Acetyl glucosamine (GlcNAC) and glycosamine (GlcN): amino sugars differing by the presence of an acetyl group on the nitrogen-containing side chain.

Peptidoglycan recognition protein (PGRP): protein used in the innate immune response of insects to recognize surface peptidoglycans of pathogens and parasites.

Peritrophic matrix (PM): a sheath of acellular matrix separating the midgut lumen of some insects, including tsetse, from the gut epithelium. There are two types of insect PMs: type I, in which the PM is secreted directly from gut epithelial cells, sometimes in response to feeding, and type II (e.g., Glossina spp.), which continually secretes the PM from specialized cells in the PV, anterior to the midgut.

Peritrophins: proteins of the PM characterized by containing cysteine-rich CBDs, and usually modified with both O-linked and N-linked glycans.

Procyclic form (PF): trypanosome procyclomastigote form in which the kinetoplast is posterior to the nucleus and which colonizes both the midgut and PV (as late procyclic) of infected flies.

Procytoplasmic acidic glycoproteins expressed abundantly on the surface of procyclic trypanosomes of T. brucei and other insect stages of T. congolense.

Proventriculus (PV): portion of the gut at the foregut midgut boundary and connected to the crop, resembling a mushroom-shaped bulge. In tsetse, the PV may also act as a reservoir and contains specialized enlarged epithelial cells that secrete the PM. It may also be referred to as the cardio.

Sialic acids (SAs): negatively charged sugar residues widely distributed in nature. In trypanosomes, SAs can be found attached to surface GPIs and are essential for survival in the fly.

Short stumpy (ST): cell-cycle arrested T. brucei bloodstream procyclomastigote form, which is preadapted for transformation to the PF in the tsetse midgut.

Teneral: young, immature adult fly that has not yet received a bloodmeal.

Trans-sialidase (TS): enzyme expressed by all African trypanosomes and Trypanosoma cruzi, and used to transfer host SAs onto trypanosome surface molecules.

Tricarboxylic acid (TCA) cycle: aerobic metabolic cycle used by procyclic but not bloodstream trypanosomes to release energy from amino acids.

Trypomastigote: form of trypanosome, including bloodstream, metacyclic, and procyclic stages, in which the kinetoplast is posterior to the nucleus.

Tsetse: obligate blood feeding viviparous flies of the genus Glossina in the superfamily Hippoboscoidea. Found only in sub-Saharan Africa, tsetse are the only known cyclical vectors of the African trypanosomes T. b. brucei and T. congolense. On the basis of morphology, habitat, and phylogeny, Glossina has been divided into three sub-genera, here referred to as the morsitans, palpalis, and fusca groups. The best-studied laboratory species, G. morsitans, is in the morsitans group.

Variant surface glycoprotein (VSG): abundant GPI-anchored proteins, tightly packed on the surface of bloodstream and metacyclic trypanosomes, and responsible for antigenic variation in LS forms.

Trypanosome infection and variation between fly sexes and species

Susceptibility to Trypanosoma brucei brucei midgut infections and the maturation index (MI) vary between tsetse species. For example Glossina morsitans morsitans has a relatively weak barrier to infection with T. brucei strain J10, with 11.3% of flies becoming infected at age 24–48 h, but with a MI below 20%. By contrast, Glossina pallidipes has a huge barrier to midgut establishment, with only 1.3% of flies developing midgut infections under the same conditions, but a MI as high as 88% [7].

T. congolense susceptible and refractory lines selected in G. m. morsitans [8] and Glossina morsitans centralis [9] demonstrate that refractoriness is not an all-or-nothing phenotype; rather it affects the proportion of flies infected. Midgut susceptibility to T. congolense was maternally inherited, suggesting an extrachromosomal susceptibility factor. The refractory lines were more refractory to other trypanosome strains and species, including Trypanosoma vivax, which develops outside the midgut, suggesting a general effect of the factor on fly immunity [9,10].

The extrachromosomal susceptibility factor might be the genotype or density of rickettsia-like organisms (RLOs), later identified as Sodalis glossinidius [5]. In regions where Sodalis is variably present in the field, trypanosome-infected flies are more likely to harbor Sodalis than uninfected flies [11]. Consistently, selective elimination of Sodalis using streptozotocin (which did not kill the obligate tsetse symbiont Wigglesworthia glossinidius) led to almost 40% reduction in susceptibility to midgut infection in the progeny of treated flies [12]. Therefore, Sodalis appears not to be essential for trypanosome midgut infection, but increases the proportion of susceptible flies. How Sodalis influences vector competence remains to be elucidated.

The possible involvement of heritable factors has also been considered for the MI. The higher T. brucei MI in males led to the postulation that maturation is under the control of one or more non-dosage-compensated X-linked loci [7]. However, it is uncertain if tsetse have non-dosage-compensated X-linked genes [13], a question that should soon be answered using the genome and transcriptome data becoming available. Unlike T. brucei, T. congolense metacyclogenesis occurs in the proboscis of G. morsitans independently of fly sex [14], suggesting that independent mechanisms may determine T. congolense and T. brucei maturation. For T. brucei, the MI is higher for faster maturing strains [14], suggesting a limited time window for successful maturation after midgut infection. The mechanism governing this is still poorly understood: despite the increasing information available on trypanosome gene expression during different life stages in the fly [15], to date the only known T. brucei gene required specifically for maturation of infections (but not for midgut colonization) is PSSA-2, which encodes for a transmembrane glycoprotein of unknown function [16].

Variation between trypanosome species and strains

Tsetse transmissibility varies not only between trypanosome species but also between strains. Human pathogenic T. b. rhodesiense strains show lower MI in G. m. morsitans than closely related T. b. brucei strains [17]. T. congolense strains with a high virulence in mice showed higher tsetse midgut infection rates than strains with moderate or low virulence [18]. Isogenic clones of T. congolense differing only in the mutations underlying resistance to isometamidium chloride differed in the midgut infection rate in G. m. morsitans, with the highly resistant strain giving a higher infection rate [19]. T. congolense is believed to be monomorphic [lacking separate long slender and short stumpy (ST) forms], thus no variation in infectivity within a strain would be expected. However, significantly higher rates of tsetse midgut infection establishment have been observed in the acute than in the chronic phase of infection in mice, independent of parasitemia level [20]. Differences in the MI have also been observed between flies fed on infected mouse blood from days 4 to 10 post-mouse infection, also independent of parasitemia level [21]. These results suggest that either changes may occur in T. congolense itself during the mammalian infection or that the T. congolense infection causes changes in the blood that then impact on T. congolense midgut colonization.
Box 1. Life cycle of *T. brucei* and *T. congolense* in tsetse

*T. brucei* long and proliferative slender (LS) and ST blood forms are ingested with blood. In the tsetse midgut, ST forms transform into the proliferative PF, whereas LS forms die. Three to six days post-infection (dpi) PFs cross the PM (Box 3), and proliferate in the ES [52]. Both survival and establishment of a parasite infection in the midgut are major bottlenecks (Figure I). Even in successful midgut colonization, survival estimates range from 1% [74] down to 0.013–0.027% by 3 dpi [75]. Trypanosomes migrate anteriorly to the PV at 6 days [75], transforming to long trypomastigotes, which divide asymmetrically, producing long and short epimastigotes (SEs) [76]. In *T. brucei*, parasite differentiation in the fly appears to be controlled by a series of RNA-binding proteins [72,77]. How PF trypanosomes sense to migrate to either the ES or the PV is unknown, but it may have to do with the capacity to communicate and cooperate in response to an external signal (also known as social motility) [78]. After differentiation in the PV, *T. brucei* is then thought to re-enter the gut lumen and migrate through the foregut to the SGs [75]. The time at which parasites reach the foregut is strain-dependent, ranging from 6 [75] to 28 dpi [7]. Strains also vary in the time taken to reach the SG; the fastest produces mammalian infectious, metacyclic trypomastigotes from day 12 [75]. Most strains mature considerably slower. SG colonization is a narrow bottleneck: a recent estimate using genetically tagged trypanosomes suggests it takes as few as five trypanosomes to colonize the SG [74]. There is clear evidence that meioiotic stages of *T. brucei* (not depicted) develop in the infected SGs, which are suggested to be responsible for genetic exchange [79]. However, there is no evidence that meiosis is necessary for metacyclogenesis. Establishment of SG infection is a major barrier to *T. b. brucei* transmission; most midgut infections fail to mature. However, if successful, in a yet-to-be defined mechanism, parasite development in the SG induces a downregulation in expression of tsetse salivary proteins, which modifies feeding behavior and may enhance transmission [80].

The initial barriers and bottlenecks to *T. congolense* are also midgut survival and colonization of the ES. The transition between the proliferative BSF into PF occurs entirely in the tsetse midgut. Unlike *T. brucei*, *T. congolense* matures to infectious metacyclics in the mouthparts (proboscis). The majority of midgut infected *G. morsitans* develop proboscis infections [81]. A maturation barrier has, however, been observed in fusca group tsees [82]. In the PV, the parasites differentiate into long trypomastigotes [81], then migrate through the foregut to the proboscis. Differentiation to long epimastigote forms occurs in the proboscis [81]. SEs are generated by an unknown process [81]. SEs colonize the proboscis and attach via the flagellum to the proboscis and cibarium, where metacyclogenesis occurs. Epimastigote attachment is required for *T. congolense* metacyclogenesis [81].

**Factors determining tsetse–trypanosome transmission**

**Bloodmeal species**

When laboratory-reared *G. m. morsitans* and *G. m. centralis* are fed infected goat or pig blood, there is a higher rate of infected midguts compared with flies fed with meals made with blood from other mammals [4], although no blood factors were identified. One bloodmeal component that may control trypanosome infection in the tsetse is serum complement. Procyclic form (PF) trypanosomes are highly susceptible to killing by vertebrate complement in vitro [22]. This information is puzzling considering that tsetse take a bloodmeal every 2 days, implying that PF in the fly gut are in contact with a fresh source of complement after feeding. This suggests that trypanosomes could have evolved ways to either neutralize or evade the action of serum complement in the midgut and proventriculus (PV). Trypanosomes may evade complement by escaping to the ectoperitrophic space (ES, see below), although it is not known whether complement can be activated in this region. Another possibility, which does not exclude the first one, is that trypanosomes take advantage of an existing tsetse anti-complement system in order to survive. Tsetse
flies express a family of midgut serine protease inhibitors (serpins) that appear to regulate complement activity in the fly midgut (C.P. Ooi, PhD thesis, University of Liverpool, 2011). Trypanosomes may also evade complement by acquiring sialic acids (SAs) from host blood glycoproteins. SAs are negatively charged sugar residues with many biological functions depending on the cell type and organism, including prevention of complement killing of some pathogenic bacteria [23]. In African trypanosomes, SAs are only acquired by means of a surface trans-sialidase (TS). TS is only expressed in the procyclic stage, and the transfer of SAs presumably occurs while the parasite is in contact with the bloodmeal as it is thought that most insects are unable to make SAs [24]. Trypanosome mutants lacking TS activity are completely unable to colonize the tsetse midgut, suggesting that the transfer of SAs is essential to establish a midgut infection [25], although it remains to be determined if this is due to an increased sensitivity to vertebrate complement. Mammalian sera differ in the content of SAs transferable to procyclic trypanosomes (M.L.S. Güther et al., unpublished), and thus when tsetse take bloodmeals with different availabilities of SAs this may impact on trypanosome survival and establishment in the fly midgut.

Gut pH, proteases, and lectins
Midgut proteases and bloodmeal digestion of Glossina have been reviewed [4]. Digestion is compartmentalized spatially within the tsetse midgut: (i) the anterior midgut is used for storage of blood, (ii) secretion of digestive proteases occurs in the middle portion, and (iii) absorption of nutrients and digestion is thought to occur in the posterior region. Recent measurements revealed alkaline conditions in both teneral and fed flies (pH 10.6 at the PV to 7.9 in the hindgut lumen 48 h after feeding) [26]. Thus, trypanosomes remaining in the anterior midgut will be exposed to a different environment and fewer proteases than those in the posterior midgut. By contrast, PF T. brucei are cultured in vitro in pH 7.1–7.7 media; outside this range the pH is unfavorable for growth [27].

In addition to protease activity, Glossina chymotrypsin may also function as a lectin with red blood cell (RBC) and trypanosome agglutination and protease activities inhibitable by glucosamine (GlcN) and N-Acetyl glucosamine (GlcNAc) (Box 2) [28]. Recombinant Glossina fuscipes proteolytic lectin has GlcN and GlcNAc sensitive agglutinating activity [28]. Glossina palpalis palpalis and G. pallidipes harbor an additional trypanocidal lectin, inhabitable by galactose [29].

Clearing of T. rhodesiense is faster in more refractory tsetse species, and faster in a refractory than a susceptible G. morsitans line, suggesting that early trypanosome death may be an important susceptibility determinant [30]. However, inhibition of midgut trypsin activity does not increase infection rates [30], and trypsin activity is the same in conspecific infected and refractory flies, suggesting that trypsin activity is not the primary factor responsible for midgut refractoriness [30].

Far from being a helpless protease victim, T. brucei inhibits midgut trypsin in gut extracts [31] and may use a protease/lectolytic lectin signal for stumpy to procyclic transformation [28,32]. N-terminal domains of T. brucei surface procyclins are cleaved in the midgut, leaving the protease resistant C-terminal domain intact [33], which might protect the parasites from further proteolysis. Consistent with this protection hypothesis, mutant T. brucei lacking all procyclins, although fly transmissible, have a reduced midgut colonization rate [34]. In T. congolense, glutamic acid/alanine-rich protein (GARP) and protease resistant surface molecules initially replace variable surface glycoprotein (VSG) [35]. The structure of GARP suggests tight intramolecular packing, implying that in analogy to the T. brucei procyclins [35], it may also have a protective role in the tsetse midgut.

Possible competition for resources
Tsetse are exclusive blood feeders and obtain most of their energy by metabolizing protein. Proline is the main energy source for tsetse flight [36]. Trypanosomes undergo a metabolic switch in the tsetse gut, shifting from using glucose to proline metabolism for energy. During transformation to PF, trypanosome mitochondria gain cytochromes and an active tricarboxylic acid (TCA) cycle [37]. With both PF and the fly reliant on proline, they may compete for this limited resource. The effect of trypanosome infection on proline levels in the midgut and the hemolymph, and the impact of this possible competition on the establishment or maturation of infections, is unknown. Starvation, which probably lowers the availability of proline to both trypanosomes and tsetse, did not lead to a change in parasite numbers 4–7 days after infection compared with fed controls [38].

Once T. b. brucei infection is established in G. morsitans, the parasite number in the midgut stays relatively constant [38]. Trypanosomes may use programmed cell death

Box 2. Increased trypanosome infection by GlcN and GlcNAc: sweet fiction or fact?
It is well reported that addition of either GlcN [17,83] or GlcNAc [29,84] to trypanosome-infected bloodmeals increases midgut infection prevalence in tsetse flies. However, the physiological significance of this observation is still debated. It has been suggested that release of GlcNAc may occur if Glossina or Sodalis chitinases degrade the tsetse PM chitin [84], as trypanosomes lack chitinases. GlcN, in contrast, may originate by the action of a soluble GlcNAc-de-N-acetylase, although such activity has not been reported in midgut extracts. The activity of tsetse midgut lectin, as estimated by midgut extract hemagglutination activity, is very low until 3 days after eclosion, and then increases with fly age [52]. These observations have led to the ‘lectin hypothesis’, which states that Sodalis abundance impacts on the refractoriness of teneral (young unfed) flies via release of proteolytic lectin that affect GlcNAc activity, which might also explain the greater susceptibility of teneral flies. However, GlcNAc also stimulates trypanosome growth, causing a shift from glucose to proline as the main energy source [85]. Data must be interpreted with caution: using GlcN or other means of perturbing the activity of the proteolytic lectin affects bloodmeal digestion, fly mortality [86], and trypanosome metabolism, as well as affecting the lectin trypanosome interactions. The generality of this hypothesis is also in question, as the midgut infection rate of another species, G. pallidipes, is not affected by addition of GlcNAc to bloodmeals [7]. Both amino sugars can also influence maturation; in G. morsitans, GlcN inhibits maturation of both T. congolense and T. brucei infections [17], whereas GlcNAc has no effect on T. b. brucei maturation in this tsetse species [86].
to avoid compromising fly fitness by removing too many resources (such as proline) from the fly [38]. The tsetse immune response may also limit parasite number in established midgut infections, although an immunogenic T. b. rhodesiense strain reached a similar density in the fly gut as a less immunogenic T. b. rhodesiense strain [39], suggesting this may not be the case.

**Tsetse immune response**

The two main humoral immune response pathways of insects, including the tsetse fly [40], are the Toll and immune deficiency (Imd) pathways [41]. Trypanosomes stimulate the Imd pathway [40]. The Imd pathway acts in both systemic and epithelial immunity, in which effector molecules are released into the hemolymph or secreted by an epithelium, respectively [41]. Epithelial immunity in tsetse is likely to be the more significant barrier to trypanosome establishment as procyclic trypanosomes exist in close proximity to tsetse epithelia, and hemolymph is not thought to be a major invasion route. For many tsetse genes induced by trypanosome infection, it is still unclear if they form part of the immune response. An example of this type of molecule is TsetseEP. TsetseEP is a glutamic acid-proline (EP) repeat containing protein found in the tsetse midgut, hemolymph, and SGs, with no known orthologs in other insects, although Drosophila has hypothetical proteins with similar cysteine structure and carboxy-terminal acid rich repeats [42,43]. Expression of TsetseEP in the midgut is strongly upregulated by injection of dead Escherichia coli to the hemolymph [43]. TsetseEP protein may be involved in immune modulation as RNAi knockdown of TsetseEP increases susceptibility of the fly midgut to trypanosome infection [44]. Starvation lowers the amount of TsetseEP after 3 days, concurrent with increasing susceptibility to midgut trypanosome infection [44]. The mechanisms by which TsetseEP interacts with trypanosomes are unknown, although it may have some agglutination/lectin activity [42]. Interestingly, EP repeats are also found in the C-termini of the T. brucei EP-procyclins [42], although there is no evidence of horizontal gene transfer between these organisms [42,43].

**Antimicrobial peptides (AMPs)**

Imd pathway effectors include the AMP attacin. Recombinant attacin kills PF *in vitro*, and supplementing attacin into bloodmeals subsequent to the initial infected one decreases midgut infection prevalence [45]. AMP expression can be constitutive or trypanosome inducible, depending on the fly species. When G. m. morsitans ingest T. b. brucei, the AMPs attacin, cecropin, and defensin are detectable in the hemolymph by 6 days after the infectious bloodmeal [45]. As fat body expression of transcripts for attacin and defensin is not observed until late in the midgut colonization process, it is unclear whether this induced expression plays any role in eliminating midgut infections [45].

Knockdown of attacin or the Imd pathway transcriptional activator Relish, prior to a trypanosome-infected bloodmeal, significantly increased midgut infection prevalence in G. morsitans [45]. RNAi prevented the accumulation of attacin transcripts in the PV, midgut, and fat body after trypanosome feeding, thus it is not clear which of these attacin sources prevents midgut trypanosome establishment. The level of attacin present before trypanosomes enter the midgut is a determinant of infection outcome. Before ingestion of the infected bloodmeal, constitutive systemic (fat body) attacin expression has been observed in *G. pallidipes* and *G. p. palpalis*, species refractory to establishing midgut trypanosome infections, but not the susceptible species *G. m. morsitans* [46]. Refractory tsetse species had higher baseline attacin transcript levels in the PV and midgut and upregulated attacin in the fat body 6 h after blood feeding [46].

In addition to AMPs, it is likely that tsetse also use reactive oxygen species and reactive nitrogen species to fight establishment and maturation of trypanosome infections. These are discussed elsewhere [17,47].

**Peptidoglycan receptors**

The Glossina homolog of the Drosophila scavenger peptidoglycan receptor PGRP-LB, which both detects and breaks down bacterial peptidoglycans, plays a role in the detection and elimination of midgut trypanosomes [48]. PGRP-LB knockdown leads to immune system hyperstimulation and high attacin expression. However, increased PGRP-LB levels correlate with refractoriness to trypanosome infection, and knockdown of either PGRP-LB, or the Imd pathway by knockdown of the receptor PGRP-LC, increases midgut infection prevalence. This suggests that the effect of PGRP-LB is either direct or via a different effector than attacin. Simultaneous PGRP-LB and -LC knockdown has an even stronger effect on midgut trypanosome establishment than either knockdown separately [48].

The symbiotic bacteria Wigglesworthia also plays a role in the development of the tsetse immune system [49,50] and the refractoriness of tsetse to trypanosomes [48,51] partially mediated through modulation of PGRP-LB expression. The role of the tsetse microbiota on tsetse vector competence is reviewed elsewhere [5].

**Crossing of the tsetse peritrophic matrix (PM)**

Trypanosomes cross the PM at least twice in their life cycle, a process that is essential to development within the tsetse (Box 1 and Box 3). Parasites cross near the middle or anterior end of the PM, possibly because of the unfavorable conditions in the posterior midgut. Only very low numbers of parasites can be found in the tsetse hindgut, and none have been observed crossing the PM in this posterior region [52–54]. What signals attract trypanosomes to migrate to the ES remains to be investigated. Trypanosomes appear to exit the PM towards the ES flagellum first [54]. The presence of trypanosomes in holes between layers of the tsetse PM is suggestive of PM damage occurring during penetration [53]. PM crossing could take as long as 2 days for *T. brucei* [54]. Whether trypanosomes use physical disruption or either their own or tsetse or symbiont enzymes to degrade and invade the PM is still unknown. Other parasites do use enzymes to invade the PM in their insect vectors. Plasmodium ookinetes secrete a chitinase and an aspartic protease to invade the mosquito PM [55,56], and Leishmania may use both sandfly and their
Box 3. The tsetse PM: a barrier to trypanosome infection?

The PM of insects acts as a physical and biochemical barrier to abrasive food particles, digestive enzymes, ingested toxins, and pathogens [87]. Perfusion studies demonstrated that the effective pore size of the intact tsetse PM is ~9 nm, which is too small for a procyclic trypanosome (~2.5 μm) to pass through [4]. The tsetse PM, therefore, creates a hurdle for trypanosomes, which must be overcome by the parasite in order to ensure their survival and subsequent transmission (Figure II). Glossina spp. secrete a type II PM into the midgut as a continuous sleeve or concentric sleeves [4]. Although type II PMs are typically composed of chitin (poly[1,4]-GlcNAc), glycosaminoglycans (GAGs), peritrophins, and other glycoproteins [87], the exact molecular composition and architecture remains to be elucidated. This, the G. m. morsitans PM has been characterized at the ultrastructural level and was shown to consist of three layers of differing thicknesses [4]. Lectin staining and immunohistochemistry suggested that each layer contains GAGs, glycoproteins, and chitin in differing amounts [4], but there is a potential for cross-specificity of staining by each lectin. To fully understand the glycan composition of the tsetse PM, a mass spectrometrical approach will be necessary. Moreover, the chitin content of the tsetse PM is still unknown, but is probably low, like other type II PMs such as that of Lucilia cuprina [87]. At least one tsetse peritrophin gene, GmPro2, has been partially characterized [22]. Other PV-specific cDNAs and proteins with multiple chitin-binding domains (CBDs) in Glossina sequence data suggest that more PV proteins exist [22]. Proteomic approaches similar to those used to characterize the PM of the mosquito Anopheles gambiae [55] would yield further information on the protein composition of the tsetse PM. The majority of lectins, important in immunity of the tsetse fly to trypanosome midgut establishment, are bound to the PM [4]. GlcNAc is present in all three layers of the PM but is most abundant in the inner and outermost layers [4]. This may provide a binding site for the GlcNAc sensitive proteolytic lectin discussed elsewhere, potentially determining tsetse susceptibility to trypanosomes.

Figure II. Schematic diagram of the development of Trypansomona brucei within the tsetse midgut. Once inside the tsetse gut, whereas the LS trypanosome form is unable to further differentiate and dies within a few hours, the ST form (small green) transforms into the PF (yellow) within the lumen of the midgut. PFs multiply, cross the PM (yellow arrow), and then invade the PV (invasion not depicted for simplicity), where they further differentiate into a series of developmental forms (not depicted; Box I), and re-enter the gut lumen, again crossing the PM. In a yet-to-be determined mechanism, it is suggested that the short epimastigote (blue) then migrates (via the esophagus) to the salivary glands where metacyclogenesis occurs. In the case of the monomorphic Trypanosoma congoensis, there is also a cycle of colonization in both the midgut and the PV, which involves the same PM crossing events. The left inset depicts an epimastigote form crossing through the three-layered tsetse PM. The right inset shows a transmission electron micrograph of a procyclic T. brucei crossing the PM of Glossina morsitans (N. Dyer et al., unpublished). Parasite crossing involves detachment of the luminal electrondense layer of the tsetse PM (asterisks). The mechanisms used by either species of trypanosomes to cross the tsetse PM remain to be elucidated. Abbreviations: ES, ectoperitrophic space; L, midgut lumen or endoperitrophic space; PF, procyclic form trypanosome; PV, proventriculus; PM, peritrophic matrix; LS, long slender; ST, short stumpy.

own endogenous chitinases to invade the sandfly PM and colonize the midgut [57,58]. Interestingly, both genomic and biochemical analyses of African trypanosomes have shown that these parasites do not express chitinases [59]. However, PF do express several types of surface-bound and released protease activities, which, in the absence of chitinases, may be enough to degrade the tsetse PM and facilitate the escape of parasite into the ES [60,61]. We also do not know how and whether the trypanosomes sense and/or bind to the tsetse PM. Trypanosomes may bind a PM lectin, akin to the mechanism used by Plasmodium to bind the mosquito midgut epithelium using a glycosylated aminopeptidase N [62]. The PM barrier function to parasites or pathogens may vary between refractory and susceptible individuals. For example, the PM of lepidopteran larvae (Anticarsia gemmatalis) resistant to gemmatalis multicapsid nucleopolyhedrovirus has higher chitin content and is more robust than that of susceptible larvae [63]. It is not known if the ultrastructure or composition of the tsetse PM varies between species refractory and susceptible to midgut trypanosome establishment, although increasing length of the PM in teneral flies does correlate with increasing refractoriness [22]. Also, the male tsetse PM is significantly more permeable than that of the female
[4], suggesting sex differences in composition or structure, although there is no significant difference between male and female refractoriness to establishment of a midgut infection in G. morsitans [7].

Teneral phenomenon

During the first 48 h after emergence, tsetse susceptibility to midgut trypanosome infection declines [22]. This change in susceptibility is temporally correlated with multiple factors including increasing PM maturity, the disappearance of milk gland protein from the gut, and declining symbiont numbers. Consuming uninfected bloodmeals also decreases susceptibility, so that at second and subsequent bloodmeals the susceptibility is lowered, with fewer than 20% of G. morsitans establishing either T. brucei or T. congolense midgut infections [64]. The molecular basis of the teneral phenomenon remains elusive.

Fly starvation

Starvation of teneral G. morsitans prior to a T. congolense or T. b. brucei infected meal increases midgut infection prevalence. Starving 20-day-old flies increases the colonization rate of the midgut with T. congolense [64], and the MI of T. b. brucei, when the midgut infection was established prior [65] or subsequent [64] to starvation. Starvation alters the tsetse immune system, lowering basal AMP mRNA expression levels in teneral flies, and reducing induction of AMP expression in response to T. b. brucei in older flies [66].

Trypanosome ‘cold shock’ in the fly

Bloodstream form (BSF) trypanosomes quickly transform into PFs to survive in the fly. Cold shock alone causes reversible EP-procyclin expression, but other signals are needed to commit to PF fate [67]. In vitro studies on signals used by T. brucei to trigger the ST to PF transition have shown that cold shock from 37 °C to 20 °C sensitizes the cells to the presence of the differentiation signals citrate or cis-aconitate (TCA cycle intermediates) [67]. Given the alkaline environment of the tsetse gut [26], it seems likely that TCA cycle intermediates, cold shock, and/or proteases are likely to provide the differentiation signal in vivo. cis-aconitate enters the cell via PAD (proteins associated with differentiation) surface transporters [32]. Cold shock increases PAD2 expression, which presumably allows more cis-aconitate into the cell [32]. Uptake of cis-aconitate induces the phosphorylation of PIP39 phosphatase, which then enters into glycosomes, where it triggers a differentiation response [68].

Despite these elegant in vitro experiments, it is unknown if cold shock influences trypanosome differentiation and midgut colonization in vivo. Design of in vitro experiments has sometimes been based on the premise that flies feed at dawn and dusk and thus are most likely to be at around 20 °C for a few hours after feeding. In contrast, colonized flies are usually kept at a warmer temperature such as 26 °C. In the field, a large proportion of G. pallidipes feed or at least were observed on oxen when the air temperature was in excess of 20 °C [69]. The temperature which flies are at during and after feeding is likely to vary with the climate over the space of a year.

Concluding remarks

Many factors regulate the establishment of midgut trypanosome infections and their later maturation in the tsetse. Particularly when performing in vivo experiments in the fly, technical challenges have precluded the elucidation of molecular mechanisms underlying the observed results. There are several exciting outstanding questions regarding the tsetse—trypanosome interactions (Box 4), but improvement of high-throughput sequencing methods, the relative ease of generating transgenic trypanosomes (including tetracycline-inducible systems in the fly) [70], RNAi knockdown of tsetse genes [71], and the sequencing of the genome of several African trypanosome species are already boosting the field. The generation of RNAi libraries in a fly transmissible strain of T. brucei could help to determine the trypanosome genes essential for progression through the fly, although the assays of infection in the fly

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**Box 4. Outstanding questions**

**Species and strain differences**
- Why do tsetse species differ in susceptibility to trypanosome infection?
- Are there heritable or genetic components to tsetse refractoriness other than Sodalis?
- Why are some strains of T. brucei faster at colonizing the SGs than others?

**Bloodmeal factors**
- Which components of blood underlie the effect of host species on trypanosome infection rate?
- How do PF escape killing by blood complement in the midgut?

**Midgut environment**
- Does cold shock impact on trypanosome transformation and survival in the tsetse fly?
- How do trypanosomes manage to survive and replicate in the high pH environment of the gut?
- How much do conditions in the ES differ from those in the gut lumen?

**Tsetse response to trypanosomes**
- What are the relative contributions of the pre-existing defenses and the immune gene expression that is induced upon feeding on trypanosomes?
- Does the systemic immune response impact on trypanosome establishment or maturation, or is this only dependent on the epithelial immune response?
- How do the different parasite surface proteins help them to survive in the different environments they encounter in different compartments of the fly?

**PM crossing**
- How do trypanosomes sense or bind to the tsetse PM?
- What strategy do trypanosomes use to cross tsetse PM?

**Parasite migration and maturation within the fly**
- Does social motility take place during PM crossing or migration to the PV?
- What physical or chemical cues guide the different migration patterns of T. brucei and T. congolense in the fly?
- What are the receptors involved in the attachment of T. brucei and T. congolense epimastigotes to the tsetse SG epithelial cells and proboscis, respectively?
- What are the differentiation signals used by trypanosomes in the different compartments of the tsetse?
would with current methods require a phenomenal amount of work. Moreover, the recent discovery that overexpression of a trypanosomal RNA-binding protein leads to the complete progression through all tsetse life-cycle stages of *T. brucei in vitro* will probably revolutionize our understanding of the molecular basis of parasite differentiation in the fly [72]. Equally, *T. congolense* can complete its entire life cycle in *vitro*, but only recently have genetic tools to perform functional genetics become available [73]. Complementing all the exciting developments in the trypanosome field, the anticipated completion of the *G. m. morsitans* genome will also be a big step in our understanding of the fascinating molecular interactions between trypanosomes and the tsetse.

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