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Trypanosome Transmission Dynamics in Tsetse

Serap Aksoy, **Brian L. Weiss**, and **Geoff M. Attardo**

Abstract

Tsetse flies (Diptera:Glossinidae) are vectors of African trypanosomes. Tsetse undergo viviparous reproductive biology, and depend on their obligate endosymbiont (genus *Wigglesworthia*) for the maintenance of fecundity and immune system development. Trypanosomes establish infections in the midgut and salivary glands of the fly. Tsetse's resistance to trypanosome infection increases as a function of age. Among the factors that mediate resistance to parasites are antimicrobial peptides (AMPs) produced by the Immune deficiency (Imd) signaling pathway, peptidoglycan recognition protein (PGRP) LB, tsetse-EP protein and the integrity of the midgut peritrophic matrix (PM) barrier. The presence of obligate *Wigglesworthia* during larval development is essential for adult immune system maturation and PM development. Thus, *Wigglesworthia* prominently influences the vector competency of it's tsetse host.

1. Tsetse Disease Vectors

1.1 Tsetse (Diptera: Glossinidae)

The genus *Glossina* contains 22 species within 3 subgenera; the *fusca, palpalis*, and *morsitans* species groups [1]. While all tsetse species are disease vectors, their ability to transmit pathogenic African trypanosomes varies, with the *palpalis* group including the most prolific human disease vector species. The different species complexes occupy varying ecological niches. *Morsitans* group taxa are adapted to relatively dry savannah habitats. Conversely, *palpalis* group flies tend to inhabit riverine and lacustrine habitats while the majority of *fusca* group flies can be found mainly in moist West African forests [2]. The host-specificity of the different species groups also vary, with the *palpalis* group flies displaying strong anthrophilic tendencies, while the others are more zoophilic in preference.

1.2 Trypanosomiasis

Two trypanosome species, *Trypanosoma brucei rhodesiense* and *T. b. gambiense*, are the causative agents of Human African trypanosomiasis (HAT). *Trypanosoma b. rhodesiense* occurs east of the Rift valley and causes acute human disease that is rapidly fatal if not properly treated. *Trypanosoma b. gambiense* occurs in west/central Africa, and infection with this parasite causes a chronic disease that results in eventual death if left untreated [3]. In addition to HAT, a non-human animal form of the disease called Animal African

Trypanosomiasis (AAT; commonly referred to as nagana) is caused by *T. b. brucei* and the related trypanosomatids, *T. congolense* and *T. vivax*. AAT severely reduces the availability of meat and milk products in large regions of Africa by excluding cattle rearing from ten million square kilometers of grazable land [4]. This widely impacts land use practices by reducing the availability of animal labor for plowing and placing constraints on the use of mixed agriculture [5]. The Programme on African Animal Trypanosomiasis (PAAT) estimates that AAT causes approximately 3 million cattle deaths per year and requires farmers to administer approximately 35 million doses of costly trypanocidal drugs [6]. Economic losses in cattle production are estimated at US$ 1–1.2 billion and total agricultural losses caused by AAT are estimated at US$ 4.75 billion per year [7].

Trypanosomes were determined to cause HAT over a century ago, and since this time, several epidemics have plagued the African continent [8]. After the devastating epidemics that occurred between 1920–1940 subsided, HAT control programs in endemic countries were gradually eliminated during the post-independent 1960s. Regretfully, a steep rise in disease incidence occurred during the following 40 years. Estimating the true burden of HAT is difficult, as the disease afflicts the poorest and most neglected populations that live in remote and rural settings located beyond the reach of health care systems [9]. In 2008, mortality associated with HAT ranked ninth out of 25 among human infectious and parasitic diseases in Africa [10]. After intense international interventions, HAT cases in Africa have recently dropped below 10,000 for the first time in 50 years, signaling a possible end to the latest epidemic cycle as a major public health crisis [11].

1.3 Trypanosomiasis Control

HAT and AAT are both fatal if left untreated. Chemotherapy is expensive [12], and current treatments for late stage disease are complicated [13,14]. Mammalian vaccines are not available due to the antigenic variation capacity of trypanosomes. Active surveillance and treatment of patients are essential for effective disease control, but such programs can be too expensive to operate at times of low endemicity. Traps and targets can reduce local tsetse populations and thus disease transmission. However, they are not widely explored for HAT control due to problems in implementation and lack of effective attractants to improve their efficacy, particularly for human disease-transmitting tsetse species [15]. New genetic approaches that aim to reduce tsetse's vector competence by blocking parasite transmission through the tsetse fly vector are of interest [16].

2. Unique Aspects of Tsetse Biology

Multiple aspects of tsetse's physiology differentiate them from other insects. These distinctions include a diet consisting exclusively of vertebrate blood, the utilization of proline rather than sugars as an energy source, the nourishment and birthing of live offspring (viviparous reproduction) and their essential relationship with an obligate symbiont (*Wigglesworthia*) to maintain fecundity and for development of the immune system.

2.1 Viviparous Reproduction

Tsetse's mode of reproduction is one of the fly's most dramatic biological adaptations [17]. Rather than laying eggs, tsetse develop a single offspring per reproductive cycle, which is carried and nourished by the mother throughout embryonic and larval development. Female flies are limited to a maximum of 8–10 progeny due to the time and nutrient intensive nature of this process. Females birth fully developed larvae that burrow into the ground, pupate, spend ~30 days undergoing metamorphosis and eclose as adults. Nutritional requirements for larval and pupal development are supplied by the mother [18]. Each gonotrophic cycle a single egg is fertilized and undergoes embryonic development within the mother's uterus. Embryos hatch into larva that undergo three developmental instars also within the uterus [19]. Nutrients are provided to the developing larva via a specialized accessory gland termed the milk gland that empties its secretions into the uterus (Figure 1). The milk constituents fulfill a variety of functions, including provision of amino acids, lipid emulsification, iron transport, assisting larval digestion and immunity [20–23]. Tsetse's obligate symbiont *Wigglesworthia* is also transmitted to the feeding offspring via maternal milk [24,25] and colonizes the larval milk gland and gut bacteriome organs [26]. Interestingly, during each lactation cycle, transcription of the major milk proteins is tightly regulated by a transcription factor, LadyBird Late [27,28]. Each reproductive cycle generates significant oxidative stress for the mother, yet tsetse females can produce offspring for almost their entire lifespan. This is accomplished in part by mitigating oxidative stress with antioxidant enzymes during and after pregnancy to prevent oxidative damage [29].

2.2 Tsetse Symbiosis for Nutrient Supplementation

The diet of both the male and female tsetse is restricted to a single food resource: vertebrate blood. To supplement their diet, tsetse harbor the enteric endosymbiont, *Wigglesworthia*, which is a member of γ-proteobacteria. In tsetse's gut, *Wigglesworthia* lives intracellularly in bacteriocytes that collectively form the bacteriome organ in the fly's anterior midgut (Figure 1; [30]). The tsetse-*Wigglesworthia* symbiosis is ancient, as reflected in the concordant evolutionary history shared between organisms [31]. Although *Wigglesworthia's* genome is dramatically reduced in size (about 700 kb; [32,33]), the bacterium has retained the ability to synthesize a plethora of B vitamins. Studies that utilize different antibiotic supplementation regimens to remove specific symbionts result in different fecundity outcomes. Fertile adults that received ampicillin-supplemented diets, which clears extracellular bacteria only, were not impaired in fecundity but gave birth to progeny that were free of *Wigglesworthia* [34]. In contrast, adults that received tetracycline-supplemented blood meals, which clears both intracellular and extracellular bacteria, became reproductively sterile likely due to the elimination of bacteriome-localized *Wigglesworthia* [34,35]. This sterility was partially recovered by supplementing the blood diet of these flies with micronutrients, particularly B vitamins [36] and yeast extract [37]. *Wigglesworthia* produced vitamin metabolites play a crucial role in proline homeostasis, and this amino acid is tsetse's single energy source [38]. The *Wigglesworthia* produced co-factor pyridoxal phosphate (the active form of vitamin B6) is an essential co-factor for alanine-glyoxylate aminotransferase (AGAT), which catalyzes the conversion of alanine to proline in tsetse's fat body. In the absence of AGAT (or *Wigglesworthia)*, females are unable to maintain

proline homeostasis and their fecundity decreases [38]. Interestingly, trypanosomes also utilize proline as their sole source of energy during their development in the tsetse host. Thus, *Wigglesworthia* produced vitamins are also indirectly essential for trypanosome fitness in tsetse [38] and may also play a yet unknown role in fly immunity.

2.3 Tsetse Symbiosis and Immune System Maturation

Wigglesworthia also exhibits an essential role in host immune functionality. Tsetse that undergo intrauterine larval development in the absence of obligate *Wigglesworthia* (Gmm^{Wgm-}) present a severely compromised cellular immune system during adulthood. Specifically, Gmm^{Wgm-} flies present a highly depleted population of hemocytes and loss of phagocytosis and melanization functions [39]. The immuno-compromised phenotype exhibited by Gmm^{Wgm-} can be partially reversed when their moms are fed a diet supplemented with *Wigglesworthia* cell extracts [40]. As such, *Wigglesworthia*-derived molecule(s) may play a role to induce the maturation of cellular immune system during larvagenesis. Alternatively, the presence of *Wigglesworthia* (or its products) in pregnant females may induce a signaling cascade that stimulates immune system development in intrauterine larvae.

3. Tsetse-Trypanosome Biology

3.1 Trypanosome Transmission Dynamics in Tsetse

To survive in tsetse's midgut, mammalian bloodstream form (BSF) adapted to survival in the midgut radically transform their metabolism [41] so that within several hours viable procyclic form trypanosomes (PF) that express a new surface coat (procyclin) become visible in the midgut and divide exponentially [42]. At around three days post infection, in a high proportion of the flies, the parasites are eliminated likely through the actions of host immunity proteins including antimicrobial peptides (AMPs) produced by the Immune deficiency (Imd) signaling pathway [43–45], Peptidoglycan Recognition Protein (PGRP)-LB [46] and tsetse-EP protein [47]. In susceptible flies, PF parasites continue to replicate, cross the chitinous gut peritrophic matrix (PM) that separates the gut lumen (the "endoperitropic space") and its contents from immuno-reactive epithelial cells, and establish infections in the ectoperitrophic space of the midgut (Figure 1). Subsequently, PF parasites differentiate into epimastigote forms (EP) and accumulate around the proventriculus organ (Figure 1). In a proportion of flies, the EP parasites (*T. brucei* complex) depart the proventriculus, enter the foregut and invade the salivary glands (SG) of the fly through the mouthparts. In the salivary gland lumen, the EP parasites mature into mammalian infective metacyclic forms for transmission to the next mammalian host in fly saliva. A recent study that compared normal and parasitized SG transcriptomes revealed that transcripts for the most abundant putative secreted SG proteins with anti-hemostatic functions present in saliva were significantly reduced upon infection [48]. In contrast, expression of putative host proteins associated with immunity, stress, cell division and tissue remodeling were enriched in infected SG suggesting that parasite infections induce host immune and stress response(s) that likely results in tissue renewal [48]. The same study also identified novel parasite surface proteins that are expressed uniquely in the metacyclic stage of the parasite. Future characterization of

the metacyclic proteins can reveal new candidate molecules that can be targeted for parasite control.

The host gut immune environment [43,45] as well as its nutritional status at the time of parasite acquisition [49] play important roles in determining the efficiency of parasite infection establishment. Tsetse's symbiotic microfauna also contribute to the fly's parasite resistance phenotype [50].

3.2 *Wigglesworthia* Mediates Trypanosome Infection Outcomes in Tsetse

Replication of *Wigglesworthia* cells, and/or *Wigglesworthia* cell death, results in the release of peptidoglycan (PGN) into tsetse's bacteriome environment. PGN is a potent inducer of the fly's immune deficiency (IMD) signaling pathway, the end products of which are bacteria-damaging antimicrobial peptides (AMPs) [51]. To evade destructive tsetse immune responses, *Wigglesworthia* induces expression of the secreted host protein, PGRP-LB in the fly's bacteriome organ [51]. Through its amidase activity, PGRP-LB degrades *Wigglesworthia* released PGN to prevent the expression of host immune effectors and thereby protects the tsetse-*Wigglesworthia* symbiosis [51]. Interestingly, PGRP-LB also exhibits anti-trypanosomal activity and can act as the first line of defense against trypanosome infection establishment [46]. Female tsetse that harbor trypanosome infections in their gut display heightened immunity, which in turn decreases host fecundity [52]. This phenotype is characterized by lengthened gonotrophic cycles and thus decreased cumulative reproductive output. Thus, in addition to stimulating host immune system development and providing vitamins absent from vertebrate blood, *Wigglesworthia* also protects its host from fecundity-reducing parasite infections.

3.3 Role of PM for Trypanosome Establishment

Tsetse's gut is lined by a chitinous, sleeve-like PM structure that acts as a biophysical barrier that regulates pathogen infection outcomes, prevents pore forming microbial toxins from damaging midgut epithelial cells and alters the temporal kinetics of host immune responses [53–55].

Adult tsetse (8 days post-eclosion) that have fed multiple times are highly refractory to infection with trypanosomes. Conversely, newly eclosed adults (referred to as 'teneral') are highly susceptible to infection when their first blood meal contains infectious parasites [56]. Similarly, adults that are starved are also highly susceptible to infection [49]. The structural integrity of tsetse's PM increases as a function of adult age post-pupal eclosion, and the higher parasite susceptibility presented by young adults has been largely attributed to the absence of a robust PM at this stage of host development [57,58]. In comparison to their wild-type counterparts, adult *Wigglesworthia*-free (Gmm^{Wgm-}) flies (progeny of females receiving ampicillin-supplemented blood diet) are highly susceptible to trypanosome infection [34,51]. This phenotype may result from the fact that Gmm^{Wgm-} individuals house a structurally compromised PM [59]. The absence of a robust PM significantly alters the dynamics of trypanosome infection establishment. Specifically, in the wild-type environment, tsetse's immunoreactive midgut epithelium does not detect parasites until after they have completed differentiation to PF forms (~ 12 hours post-ingestion) and

circumvented the PM midgut barrier (~ 2–3 days post-ingestion). However, in *Gmm*$^{Wgm-}$ gut, mammalian blood stream form (BSF) trypanosomes are detected by midgut cells immediately following ingestion. Early detection of BSF parasites by tsetse's midgut epithelium results in a dysfunctional immune response that is most conspicuously characterized by the absence of induced *attacin* expression [60]. This gene, which is normally up-regulated following trypanosome challenge [59], encodes a potent trypanocidal AMP [43,44,60]. Experimental elimination of tsetse's PM through a gene silencing strategy renders normally resistant adults susceptible to parasitism [60]. These findings imply that tsetse's PM is not a physical impediment to infection establishment, but instead serves as a barrier that regulates the fly's ability to immunologically detect and respond to the presence of these microbes. Additionally, tsetse's immune system may differentially recognize distinct parasite forms and react less antagonistic against PF trypanosomes that have established successful midgut infections.

4. Conclusion

Tsetse vector African trypanosomes, which are the causative agents of deadly HAT and AAT. Tsetse give birth to live young and depend on obligate endosymbionts for the maintenance of fecundity and immune system development. Particularly the indirect contributions of obligate symbionts for gut PM development mediates parasite establishment. Recent technological advances in high-throughout sequencing methodologies and functional genomics have allowed us to obtain the whole genome sequence of the tsetse host [61] and better understand the molecular mechanisms that underlie tsetse's unusual physiological characteristics. This information can now be exploited to develop novel control strategies aimed at reducing tsetse's reproductive output and/or the fly's competence as a vector of pathogenic trypanosomes. Towards this end a paratransgenic gene expression system has been developed using tsetse's commensal symbiont *Sodalis* [16]. Expression of trypanocidal products in the gut in the symbiotic bacteria can modify the gut environment to reduce parasite transmission.

Acknowledgements

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60. Weiss BL, Savage AF, Griffith BC, Wu Y, Aksoy S. The Peritrophic Matrix Mediates Differential Infection Outcomes in the Tsetse Fly Gut following Challenge with Commensal, Pathogenic, and Parasitic Microbes. J Immunol. 2014; 193:773–782. [PubMed: 24913976] ** The structurally robust PM was eliminated through RNA interference-approach. In such flies, bacteria *Enterobacter* and *Serratia* proliferation was impeded because these flies expressed the antimicrobial peptide gene, *attacin*, earlier in the infection process than did wild type flies. After challenge with trypanosomes, *attacin* expression was latent in tsetse that lacked an intact PM, and these flies were highly susceptible to parasite infection. Results suggest that Imd signaling pathway effectors, as opposed to reactive oxygen intermediates, serve as the first line of defense in tsetse's gut after the ingestion of exogenous microorganisms. Furthermore, tsetse's PM is not a physical impediment to infection establishment, but instead serves as a barrier that regulates the fly's ability to immunologically detect and respond to the presence of these microbes.

61. Initiative IGG. Genome sequence of the tsetse fly (Glossina morsitans): vector of African trypanosomiasis. Science. 2014; 344:380–386. [PubMed: 24763584] * The whole genome sequence data from the tsetse species *Glossina morsitans* is presented. Particularly a reduction in the host pattern recognition proteins (PGRPs) are noted in relation to closely related other Diptera. This reduction may reflect tsetse's evolutionary association with obligate microbes and restricted exposure to a wide array of microbes as a strict blood feeder.

Highlights

1. We describe tripartite interactions between tsetse, trypanosome and symbionts

2. Tsetse undergo intrauterine larvagenesis and lactate (viviparous reproduction)

3. Fecundity relies on obligate symbiont for diet supplementation and proline synthesis

4. Symbiont presence during larval growth influences adult immune and gut development

5. Gut peritrophic matrix barrier integrity affects trypanosome transmission dynamics
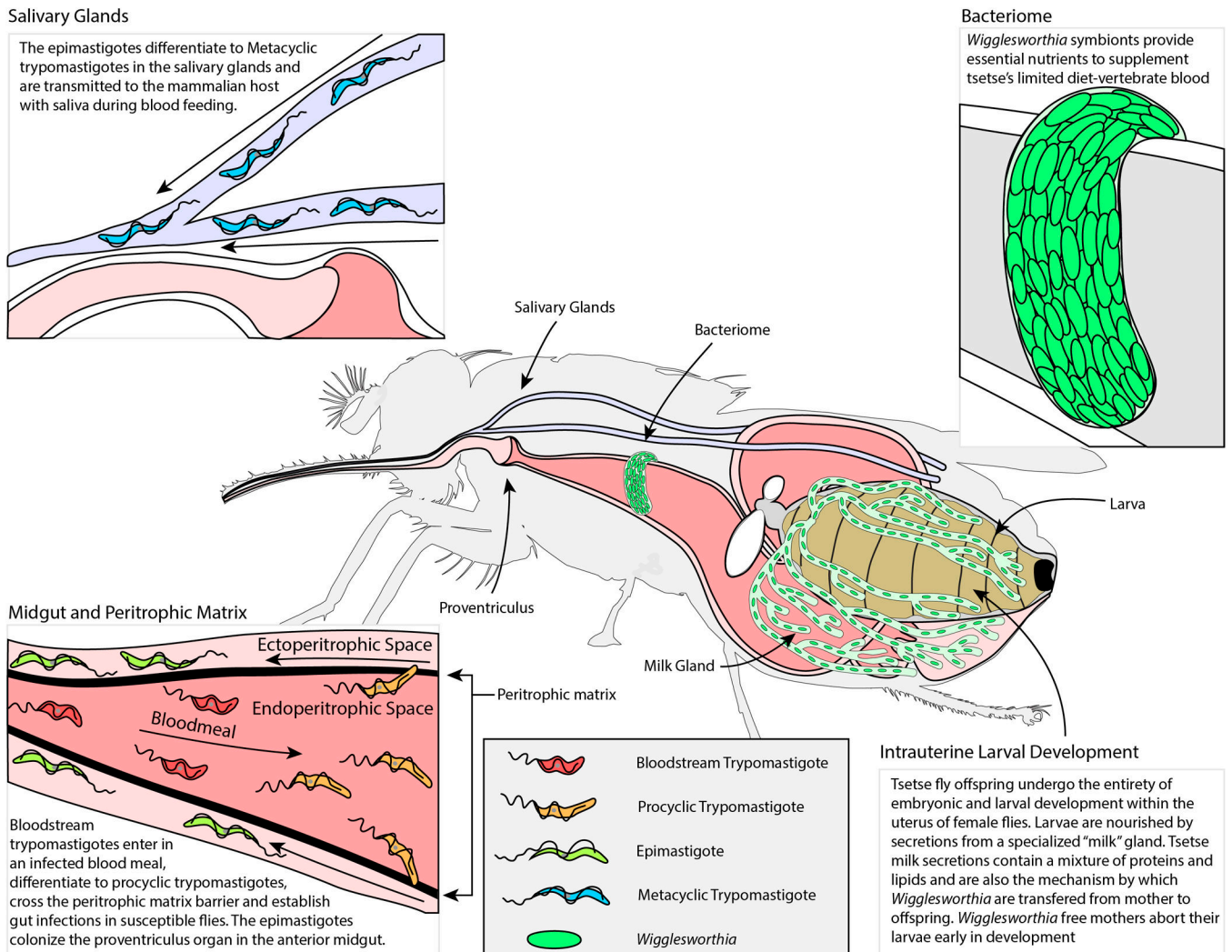
Figure 1.
Diagrammatic Representation of the Interactions between Tsetse, Trypanosomes and the Obligate Symbiont *Wigglesworthia*. This figure represents the life cycle and metamorphosis of trypanosomes within the tsetse beginning with their introduction via an infective blood meal, followed by their escape from the peritrophic matrix into the endoperitrophic space and subsequent invasion of the salivary glands for transfer to a vertebrate host. The diagram also illustrates the localization of the obligate endosymbiont *Wigglesworthia* in tsetse's bacteriome in the anterior midgut as well as its transmission into tsetse's intrauterine larva in milk secretions. The viviparous reproductive physiology is depicted with an intrauterine larva and the large network of milk gland tubules that provide nutrients.