The Influence of Sex and Fly Species on the Development of Trypanosomes in Tsetse Flies

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Abstract

Unlike other dipteran disease vectors, tsetse flies of both sexes feed on blood and transmit pathogenic African trypanosomes. During transmission, Trypanosoma brucei undergoes a complex cycle of proliferation and development inside the tsetse vector, culminating in production of infective forms in the saliva. The insect manifests robust immune defences throughout the alimentary tract, which eliminate many trypanosome infections. Previous work has shown that fly sex influences susceptibility to trypanosome infection as males show higher rates of salivary gland (SG) infection with T. brucei than females. To investigate sex-linked differences in the progression of infection, we compared midgut (MG), proventriculus, foregut and SG infections in male and female Glossina morsitans morsitans. Initially, infections developed in the same way in both sexes: no difference was observed in numbers of MG or proventriculus infections, or in the number and type of developmental forms produced. Female flies tended to produce foregut migratory forms later than males, but this had no detectable impact on the number of SG infections. The sex difference was not apparent until the final stage of SG invasion and colonisation, showing that the SG environment differs between male and female flies. Comparison of G. m. morsitans with G. pallidipes showed a similar, though less pronounced, sex difference in susceptibility, but additionally revealed very different levels of trypanosome resistance in the MG and SG. While G. pallidipes was more refractory to MG infection, a very high proportion of MG infections led to SG infection in both sexes. It appears that the two fly species use different strategies to block trypanosome infection: G. pallidipes heavily defends against initial establishment in the MG, while G. m. morsitans has additional measures to prevent trypanosomes colonising the SG, particularly in female flies. We conclude that the tsetse-trypanosome interface works differently in G. m. morsitans and G. pallidipes.

Introduction

During a transmission of a pathogen, selection in the invertebrate vector may be of profound importance in dictating which pathogen genotypes are most prevalent in mammalian hosts. This evolutionary pressure can select for particular combinations of pathogen and vector species, and weed out less fit pathogen phenotypes regardless of any competitive advantage in the mammalian host, such as virulence or drug resistance.

Tsetse flies (Diptera: Glossinidae) serve as vectors of several pathogenic trypanosome species in subsaharan Africa, but typically manifest high levels of resistance to infection [1,2]. Resistance mechanisms operate at a number of levels and time points during the trypanosome’s complex developmental cycle within the fly. For Trypanosoma brucei, trypanosomes first establish infection in the tsetse midgut (MG), initially in the gut lumen with subsequent invasion of the ectoperitrophic space via the peritrophic matrix (PM) enclosing the bloodmeal. The antimicrobial defences operating in the MG, such as antimicrobial peptides, lectins and reactive oxygen intermediates [3,4,5,6,7], ensure that a high proportion of infections are cleared at this early stage. In the laboratory, these defences can be counteracted by, for example, feeding the flies on lectin-binding sugars or anti-oxidants [8,9,10] or knocking down expression of specific antimicrobial peptides or proteins using RNA interference [4,11].

Of the MG infections that persist, few subsequently result in a salivary gland (SG) infection and it is evident that the trypanosomes experience a severe population bottleneck, as the SG are invaded and colonised by very small numbers of trypanosomes [12,13]. The barriers to SG infection are unknown, but there are several points along this complex pathway where progression may potentially be blocked. From the MG, trypanosomes move anteriorly to invade the proventriculus and penetrate through the PM before migrating to the SG via the foregut. The proventriculus is known to be a highly immunogenic tissue [5] and this could influence the success of trypanosome invasion of the foregut or the differentiation from MG procyclics to migratory forms. Escape of trypanosomes from the proventriculus into the foregut would also be blocked if trypanosomes were unable to penetrate the PM. Little is known of SG immune responses, but these are also likely to be vigorous judging by the frequent failure of migratory trypanosomes to colonise the SG and establish infection [2,12,13]. A recent survey of genes expressed in tsetse SG revealed a large variety of potential immunity-related molecules, some of which are also expressed by MG and fat body tissues [14].

Unlike other dipteran vectors such as mosquitoes, sand flies and black flies, both male and female tsetse feed on blood and hence...
In tropical Africa human and livestock diseases caused by parasitic trypanosomes are transmitted by bloodsucking tsetse flies. In the fly, trypanosomes undergo a complex cycle of proliferation and development during their remarkable journey from the midgut to the salivary glands. At every step of the way, the flies mount robust immune defences against trypanosome infection and consequently most flies fail to develop a transmissible infection. Previous work has shown a sex difference in the numbers of salivary gland infections with Trypanosoma brucei: male flies are more susceptible to salivary gland infection than females. Here we explored possible reasons for this. Infections developed in the same way in both male and female flies until the final stage of salivary gland invasion and colonisation. We conclude that the salivary gland environment in the female fly is much more inhospitable for trypanosomes, perhaps because of a greater immune response. Comparison of two different tsetse species showed very different levels of trypanosome resistance in the midgut and salivary glands.

Authors Summary

In tropical Africa human and livestock diseases caused by parasitic trypanosomes are transmitted by bloodsucking tsetse flies. In the fly, trypanosomes undergo a complex cycle of proliferation and development during their remarkable journey from the midgut to the salivary glands. At every step of the way, the flies mount robust immune defences against trypanosome infection and consequently most flies fail to develop a transmissible infection. Previous work has shown a sex difference in the numbers of salivary gland infections with Trypanosoma brucei: male flies are more susceptible to salivary gland infection than females. Here we explored possible reasons for this. Infections developed in the same way in both male and female flies until the final stage of salivary gland invasion and colonisation. We conclude that the salivary gland environment in the female fly is much more inhospitable for trypanosomes, perhaps because of a greater immune response. Comparison of two different tsetse species showed very different levels of trypanosome resistance in the midgut and salivary glands.

**Results**

**Glossina morsitans morsitans**

Comparison of male and female G. m. morsitans infected with *T. b. brucei* J10 confirmed previous findings that male tsetse flies establish greater numbers of SG infections of *T. brucei* than females [15,16,17]. While there was no significant difference in MG infection rates, a significantly higher proportion of MG infections progressed to SG infection in male than in female flies. The transmission index (TI = infected salivary glands/infected MGs) for male flies was over twice that for female flies (P=0.045; Fig. 1A).

The progression of infection in these flies was monitored at points of transition in the developmental cycle to investigate the nature of the barriers to SG infection and influence of fly sex. In established MG infections, the first event we recorded was invasion of the proventriculus by trypanosomes migrating anteriorly within the ectoperitrophic space. In flies dissected 10–14 days after infection, only about three quarters had an infected proventriculus and there was no difference in infection rates between males and females (Table 1). In the proventriculus, trypanosomes arrest in G2 before undergoing an asymmetric division that yields one short and one long epimastigote; these are migratory stages and the short epimastigote is believed to invade the SG [18,19,20]. Asymmetric dividers were found in about 75% of infected proventriculi, with no significant difference between male and female flies (Table 1).

The next event is that the migratory trypanosomes invade the foregut and can be found in the salivary exudate or spit, a mixture of regurgitated foregut contents and saliva from the SG produced by flies when they probe a surface with the proboscis [18,21]. To examine the foregut contents, we used individually-caged flies, which were allowed to probe onto warm microscope slides 7–28
days after infection; dissection results for these flies at day 28 are shown in Fig. 1A. Trypanosome-positive spit samples were only obtained from those flies subsequently found to have MG infection, but some flies (about 20%) with MG infection did not produce a positive spit sample during the whole observation period (Fig. 1B). This reflects the failure of about 40% (27 of 64) MG infections to infect the proventriculus and produce asymmetric diverters (Table 1). Only a small proportion of spit-positive flies finally developed SG infection, just over 20% combining males and females (Fig. 1B), which means that in the majority of infections the migratory trypanosomes either failed to reach the SG or to colonise them. Attrition was greater in female than male flies (Fig. 1B), although the sex difference was not statistically significant.

The relative proportions of trypanosome developmental stages in individual stained spit samples from male and female flies were similar (Table 2); at this early stage of infection, few metacyclics were present. Additionally, the trypanosome composition of the spit sample had no bearing on whether a fly subsequently developed SG infection, as there was no significant difference in numbers of developmental stages in spit samples from flies with or without SG infection when dissected at 28 days (Table 2). However, there was a significant effect of gender on the rate at which flies became spit-positive, the females lagging behind the males (Fig. 2: median time to positivity 10 or 14 days for males or females, respectively, P<0.05). It was noticed that often very few trypanosomes (typically <5) were present in spit samples from flies that became positive on or after 12 days, the majority of which were female. It is possible that colonisation is adversely affected by the later arrival and smaller numbers of migratory trypanosomes in female compared to male flies. However, this hypothesis was not borne out by statistical analysis of the combined data from male and female flies: of 48 flies that gave their first positive spit sample early (7–11 days after infection), 9 had positive SG at dissection (19%), whereas of 29 flies that produced their first positive sample late (12–21 days after infection), 6 were SG positive at dissection (21%) (P=1.00). So time of migration to the SG does not affect the success of SG colonisation.

Glossina pallidipes

We compared infection rates of G. m. morsitans with those of G. pallidipes using the same strain of T. b. brucei J10. Without immunosuppressive supplements, MG infection rates were very low in G. pallidipes compared to G. m. morsitans (Table 3). The addition of N-acetyl-glucosamine (NAG) or L-glutathione (GSH) to the infected feed has been shown to enhance MG infection rates [8,9] in G. m. morsitans by blocking antimicrobial lectins or reactive oxygen species respectively, and this is also evident from the data collected here for G. m. morsitans (Table 3); there is no effect of these supplements on SG infection rates except as a result of increased numbers of MG infections [8,22]. However, in contrast to G. m. morsitans, NAG appeared to be totally ineffective in boosting numbers of MG infections in G. pallidipes: no infected MG were found with NAG in G. pallidipes compared with 54.4% infected MG in G. m. morsitans (Table 3). The addition of GSH resulted in a large increase in numbers of infected MG for G. pallidipes (50.0%
with GSH versus 1.3% without GSH), similar to the effect seen in *G. m. morsitans* (81.0% with GSH, 11.3% without GSH), but significantly lower comparing the two fly species (*P* = 0.018) (Table 3).

The high MG infection rates obtained with GSH enabled us to examine SG infection rates in *G. pallidipes* (Fig. 3). Comparison with *G. m. morsitans* showed that transmission was far more efficient in *G. pallidipes*, despite lower MG infection rates; the SG infection rates and TI for both sexes of *G. m. morsitans* were significantly higher than for *G. m. morsitans* (*P* < 0.0001; Fig. 5). In fact, all 17 male *G. pallidipes* with MG infection also had infected SG (TI = 100%) compared to only three of 38 male *G. m. morsitans*, and 15 of 17 female *G. pallidipes* with MG infection also had infected SG whereas none of 48 female *G. m. morsitans* with infected MG had infected SG. As for *G. m. morsitans*, there was a sex difference in TI and *G. pallidipes* males had a higher TI than females, though this was not significant. Although male *G. pallidipes* had higher MG and SG infection rates than males, the differences were not significant either (Fig. 3).

Salivary gland hypertrophy

The *G. pallidipes* colony from which our experimental flies were derived suffers from infection with a virus that causes the SG to hypertrophy (SGH) [23,24]. Although the prevalence of SGH is relatively low in the colony (3.8%), PCR diagnosis indicates that almost all flies are infected with SGH virus [23]. Viral load is significantly higher in symptomatic flies [24], suggesting that while most flies control viral infection and are asymptomatic, a minority succumb and develop SGH.

As it is not known how SGH affects trypanosome infection, we analysed whether there was an association between trypanosome infection and SGH in *G. pallidipes* infected with *T. b. brucei*. Of the 43 flies with SGH, 30 had infected SG (88%), a significantly greater level of infection than flies with normal SG (24% infected of 359 flies, 69%; *P* = 0.008). Although SGH is positively correlated with trypanosome infection, the large number of SG positive flies without SGH (69%) shows that SGH is by no means essential for SG colonisation by trypanosomes in *G. pallidipes*.

### Discussion

The tsetse fly is unusual among dipteran vectors of disease because both sexes feed on blood and hence transmit pathogenic trypanosomes. However, the sexes are not equally efficient vectors and males have been found to be more susceptible to infection with *T. brucei* than females [15,16,17]. To explore the underlying basis of this sex difference, we compared infections in male and female *G. m. morsitans* at a number of points in the trypanosome's developmental cycle within the alimentary tract and SG of the fly. Levels of attrition were similar in both male and female flies, until the final stage of SG invasion and colonisation. The only difference detected was among the trypanosomes that migrate from the MG to the SG via the foregut: in female flies these appeared later than in males. However, there was no detectable difference in the success of early or late migrating populations in invading and colonising the SG, so differential attrition at the trypanosome-SG interface remains the only underlying explanation for the observed sex difference.

It appears that migratory trypanosomes encounter a very hostile environment in the SG. In *G. m. morsitans* SG colonisation was frequently unsuccessful and only about 20% of flies positive for migratory trypanosomes in spit samples were subsequently found to have SG infection. Compared to the MG, little is known about the functional immune response of the SG to trypanosomes, but...
Development of Trypanosomes in Tsetse

Materials and Methods

Tsetse flies and trypanosomes

Tsetse flies were kept at 25°C and 70% relative humidity and fed on sterile defibrinated horse blood via a silicone membrane. Flies were given the infected bloodmeal for their first feed 24–48 hours post-eclosion, which consisted of cryopreserved blood-stream form trypanosomes of T. b. brucei J10 (MCRO/ZM/74/J10 [clone 1]) in defibrinated horse blood (approximately 10⁶ cells/ml). Infective bloodmeals were supplemented if necessary with final concentrations of 60 mM N-acetyl-glucosamine (NAG) [8] or 10 mM L-glutathione (GSH) [9] to increase infection rates. Results were usually combined from two replicate experiments to increase sample size, except for those shown in Tables 1 and 2 which were each derived from a single batch of flies.

Spit samples

Spit samples were obtained from flies as described [13]: male and female flies were sampled on days 8–18 and 7–28 in two replicate experiments. Slides were fixed with 2% paraformaldehyde (PFA), washed three times with phosphate buffered saline (PBS) and then incubated with 1:100× Hoechst 33258 DNA stain for 15 minutes. The slides were mounted using FluorSave reagent and viewed by fluorescence imaging to record the life cycle stage of the parasites using a DMRB microscope (Leica) equipped with a Colour Coolview camera (Photonic Science) and ImagePro Plus software (Media Cybernetics). Digital images of life cycle stages were quantified using Image J (http://rsb.info.nih.gov/ij/). Morphology and relative positions of the nucleus and kinetoplast were used to identify developmental stages [18,19,20]. Cells were assigned to the following developmental stages: long procyclic trypomastigote, asymmetrically dividing cell, short or long epimastigote, metacyclic.

Dissection

Flies were killed by removing the head. Salivary glands were placed into a drop of PBS. Salivary gland hypertrophy (SGH) was recorded if the glands were grossly swollen; such glands also appear white rather than transparent. Whole salivary alimentary tracts, from the proventriculus to the rectum, were placed into a
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7. Numbers of trypanosomes were square-root transformed prior to analysis to normalise variances. The rate at which flies became positive for trypanosomes in spit samples was analysed by Kaplan Meier survival followed by Breslow (generalized Wilcoxon) testing. ANOVA and Kaplan-Meier data were processed using the statistical package SPSS version 18.0.

Acknowledgments

We are immensely grateful to IAEA, Vienna, for supply of tsetse pupae.

Author Contributions

Conceived and designed the experiments: LP MB WG. Performed the experiments: LP VF WG. Analyzed the data: LP MB WG. Wrote the paper: LP MB WG.

www.plosntds.org 6 February 2012 | Volume 6 | Issue 2 | e1515