Maturation of a *Trypanosoma Brucei* Infection to the Infectious Metacyclic Stage Is Enhanced in Nutritionally Stressed Tsetse Flies

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ABSTRACT We report on the effect of tsetse fly starvation on the maturation of an established *Trypanosoma brucei brucei* midgut infection, i.e., the development of procyclic infection into the infectious metacyclic parasites in the tsetse fly salivary glands. *Glossina morsitans morsitans* flies were nutritionally stressed 10 d after the uptake of a *T. b. brucei*-infected bloodmeal by depriving these flies from feeding for seven consecutive days, whereas the control fly group (nonstarved group) continued to be fed three times a week. After this period, both fly groups were again fed three times per week on uninfected rabbit. Thirty days after the infected bloodmeal, all surviving flies were dissected and examined for the presence of an immature midgut and a mature salivary gland trypanosome infections. Results showed a significantly increased proportion of flies with salivary gland infection in the nutritionally stressed fly group suggesting an enhanced maturation of the trypanosome infection. These data suggest that environmental factors that cause nutritional stress in a tsetse population do not only make tsetse flies significantly more susceptible to establish a midgut infection as was shown previously but also boost the maturation of these midgut infections.

KEY WORDS tsetse fly, *Glossina*, *Trypanosoma brucei brucei*, nutritional stress, trypanosome maturation

The developmental cycle of African trypanosomes in tsetse flies (Diptera: Glossinidae) starts when the blood-feeding insect feeds on a trypanosome-infected mammalian host. Then, the ingested parasites undergo a series of developmental stages including the establishment of a procyclic infection in the fly's midgut as well as the upward migration to the mouthparts (for *Trypanosoma congolense*) or salivary glands (for *Trypanosoma brucei* spp.) where a complex differentiation takes place to the final mature metacyclic stage. This end stage is again infectious for a mammalian host and will be transmitted at every blood-feeding event of the infected tsetse fly. The existence of developmental barriers is evidenced by the low proportion of trypanosome infections in the midgut of experimentally infected flies and the fact that only a limited proportion of these midgut infections will finally give rise to a mature infection, especially in the case of *T. brucei* spp. (Roditi and Lehane 2008). The proportions of infected flies in a tsetse population as well as the age-specific susceptibility are important factors that affect the epidemiology of tsetse-transmitted trypanosomiasis. Tsetse flies are considered to be most susceptible for *T. congolense* and *T. brucei* spp. infection in their teneral state, i.e., before their first bloodmeal (Welburn and Maudlin 1992). The majority of infected tsetse flies are considered to have acquired the infection during their first bloodmeal, whereas those that do not become infected at an early stage are not believed to contribute to the trypanosome infection rate of the tsetse population. However, under specific physiological conditions, both teneral and older flies can become more susceptible to develop a trypanosome infection. Indeed, Kubi et al. (2006) showed that nutritional deprivation of tsetse flies lowers the developmental barrier to establish a trypanosome infection, particularly at the tsetse midgut level. However, the effect of this nutritional stress on the maturation of an established procyclic infection to the infective metacyclic stage has not been examined thoroughly. Therefore, the experimental work presented in this study focused on the effect of starvation of tsetse flies on the maturation of a *T. brucei* procyclic midgut infections into a metacyclic salivary gland infection.

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Materials and Methods

Tsetse Flies and Trypanosome Strain. Freshly emerged male *Glossina morsitans morsitans* Westwood flies (<32 h old) from the colony maintained at the

Table 1. Proportion (+95% confidence interval) of *G. m. morsitans* male flies that developed a trypanosome infection with *T. brucei brucei* AnTAR 1 in the midgut (procyclic stage) and salivary glands (mature, metacyclic stage), 30 d after the infected bloodmeal

Group[a]	Total no. flies dissected	Proportion of infected flies		
		Midgut	Salivary gland	Maturation[a]
1	332	0.50 (0.44–0.55)	0.32[b] (0.26–0.36)	0.63[b] (0.55–0.69)
2	331	0.45 (0.39–0.50)	0.21 (0.16–0.25)	0.47 (0.38–0.54)

In group 1, flies were starved for seven consecutives days after the trypanosome midgut establishment period (10 d after the infected bloodmeal), whereas group 2 flies continued to be fed three times a week.

[a] Maturation is the proportion of midgut infected flies that developed a mature, metacyclic infection in the salivary glands.

[b] $P = 0.002$.

Institute of Tropical Medicine (Antwerp, Belgium) were used in the experiment. The origin of this tsetse colony and the rearing technique are described by Elsen et al. (1993). *Trypanosoma brucei brucei* AntAR1 strain derived from the stock EATRO 1125 (Le Ray et al. 1977) was used in the experiments.

Experimental Design. Twenty six cages of 40 newly emerged male flies each were given a single bloodmeal on anesthetized trypanosome-infected mice (NMRI) (one cage per mouse) showing a parasitemia of $10^{8.1}$–$10^{8.4}$ trypanosomes/ml of which >50% were short-stumpy bloodstream forms. Mice were anesthetized by an intraperitoneal injection of a xylazine/ketamine mixture. After the infected bloodmeal, unfed flies were removed. During a period of 10 d corresponding to the trypanosome establishment period in the tsetse fly's midgut (Van Den Abbeele et al. 1999), all these flies were fed three times per week on uninfected rabbits. After this 10-d period, flies were divided into two experimental groups, each containing 13 replicates (i.e., cages of flies fed on a different infected mouse). Group 1 consisted of starved flies that were deprived of blood feeding for seven consecutive days, whereas group 2 consisted of nonstarved flies that continued a normal feeding regimen of three times per week. At the end of the 7-d starvation period for group 1, this normal feeding regimen was resumed until 2 d before dissection. To avoid reinfection of the flies during the maintenance feeding, the rabbits were replaced at weekly intervals. Thirty days after the infective meal, surviving flies were dissected and midgut and salivary glands were examined by phase-contrast microscopy (400×) to determine the presence of trypanosomes. The proportion of midgut procyclic infections (immature infection) was calculated as the proportion of dissected flies that developed a trypanosome infection in the midgut. The proportion of metacyclic infection (mature infection) was calculated as the proportion of dissected flies that developed an infection in the salivary glands. The maturation rate was calculated as the proportion of immature infections that developed into mature infections.

Statistical analyses were carried out in STATA (Stata Corporation 2006) by using a logistic regression. The proportions of dissected flies showing infection in the midgut and in the salivary glands were tested separately. Starvation was taken as explanatory variable. Clusters resulting from flies infected on the same mouse and maintained in the same cage were considered as primary sampling units. The maturation was analyzed in a similar way but on a data set restricted to midgut-infected flies.

Animal ethics approval for the experimental infections was obtained from the Ethics Commission of the Institute of Tropical Medicine, Antwerp, Belgium (Refs. PAR003-MC-K-Try and PAR004-MC-M-Try).

Results and Discussion

Of a total of 1,040 newly emerged male flies, 755 (72.5%) ingested a trypanosome-infected bloodmeal and were retained for the experiment. Throughout the experiment, the mortality rates were similar in both experimental groups, 8.8 and 5.5%, respectively, in group 1 (starved) and group 2 (nonstarved) flies. At day 30, 663 flies in total were dissected to determine the infection status in the midgut and salivary glands (Table 1). In both groups, the percentage of midgut-infected flies was high and was not significantly different (50 and 45%, respectively; $P = 0.2$). However, in group 1 flies a significantly higher number of these midgut-infected flies developed a mature metacyclic infection in the salivary glands ($P = 0.002$). These results clearly demonstrate that an established *T. brucei* midgut infection remains persistent even during a period in which the tsetse fly is exposed to a high nutritional stress. In addition, our data suggest that the maturation of this persistent procyclic midgut infection is significantly enhanced by the fly starvation resulting in a higher proportion of flies that finally carry mature, infectious metacyclic trypanosomes. Previous studies have already demonstrated that a period of starvation of newly emerged and older tsetse flies before the infective bloodmeal increases the susceptibility of these flies to establish a *T. congolense* or *T. b. brucei* midgut infection (Kubi et al. 2006). However this is, to our knowledge, the first time to demonstrate unambiguously that starvation of tsetse flies also significantly affects the further development of established *T. b. brucei* procyclic trypanosomes to metacyclic forms. The underlying mechanism for trypanosome maturation enhancement is not clear. We could hypothesize that, as a result of the nutritional stress of the tsetse fly, the presence of factors that prevent trypanosomes to mature is reduced or that the level of factors that promote maturation is increased. However, the nature of these factors affecting the

maturation of a midgut *T. brucei* infection remains unknown. In *G. morsitans* flies, maturation of *T. brucei* spp. is greatly affected by fly sex with male tsetse flies producing significantly more mature trypanosome infections than female flies (Welburn and Maudlin 1999). In a recent study, Macleod et al. (2007) suggested that oxidative stress (i.e., presence of reactive oxygen species such as nitric oxide) might be involved in the triggering of the maturation process of a *T. b. brucei* midgut infections in tsetse. In the same study, the authors demonstrated that pregnancy in female tsetse flies has a detrimental effect on the parasite maturation that they attributed to the altered physiology/biochemistry in the pregnant females and to a reduction of free nutrients available for the trypanosomes. According to our study, however, the trypanosome maturation process was enhanced when male midgut-infected tsetse flies were deprived of nutrients as a result of starvation. However, it is highly probable that the above described studies cannot be simply compared and that two different mechanisms are at play. Indeed, besides the specific physiological and biochemical status of pregnant females (Langley and Pimley 1979, Attardo et al. 2006a), it can be assumed that the continuous intrauterine nourishment of the larva may result in a decrease of specific nutrients whereas the extensive starvation of the male flies will result in a general and substantial decrease of all free nutrients. Other studies have shown that specific immune responses of the tsetse fly against the trypanosome parasite interfere with the development of the trypanosome in the fly (Boulanger et al. 2002, Aksoy et al. 2003, Lehane et al. 2004, Attardo et al. 2006b). Because nutritional stress affects the immune status of *G. morsitans* flies (Akoda et al. 2009), this also could be a contributing factor to the enhanced maturation of the established procyclic trypanosomes.

The findings of this study, together with those from Kubi et al. (2006), contribute to a better understanding of the dynamics of *T. brucei* transmission in a natural tsetse population. Indeed, it seems that a range of environmental factors that cause nutritional stress not only make young and old tsetse flies more susceptible to establish a trypanosome midgut infection (Kubi et al. 2006) but also boost the maturation of a midgut infection to the infectious stage that would not have matured under normal circumstances. Because the *T. brucei* spp. infection rates in natural tsetse fly populations are usually very low (Hide 1999, Waiswa et al. 2006), any significant increase in the overall mature infection rate may result in the enhanced transmission of this parasite within a susceptible host population. As such, factors enhancing trypanosome development may contribute to the maintenance and/or activation of a sleeping sickness focus in an endemic area.

In the view of tsetse females higher longevity and their ensuing role in trypanosome transmission (Welburn and Maudlin 1999), it would be interesting to determine whether the ability to transmit trypanosomes of female *G. morsitans* flies is similarly affected by starvation. Moreover, similar experimental studies using other tsetse fly–trypanosome transmission models are required to broaden our appreciation of the impact of nutritional stress on the vector competence of other tsetse fly species.

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