Original Research

The development of *Trypanosoma brucei* within the tsetse fly midgut observed using green fluorescent trypanosomes

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Abstract

Background: The protozoan pathogen, *Trypanosoma brucei*, undergoes complex cycles of differentiation and multiplication in its vector, the tsetse fly, genus *Glossina*. Flies are refractory to infection and resistance mechanisms operate at a number of levels and timepoints. Here we have used highly conspicuous green fluorescent trypanosomes to study the early events in establishment of infection in the fly midgut.

Results: Less than 10% of the bloodstream form trypanosomes in the infected feed differentiated into viable procyclics. Up to day 3, trypanosomes were found in the bloodmeal in every fly examined, and increased in number between days 1 and 3. Flies dissected on days 5 and 6 fell into 2 clearly distinct groups: those with high numbers of trypanosomes and those with undetectable infection. Trypanosomes were found in the ectoperitrophic space and proventriculus from 6 days following the infective feed.

Conclusion: Trypanosomes that have undergone successful differentiation appear to experience an environment within the midgut suited to their unrestricted growth for the first 3 days. After this time, a process of attrition is evident in some flies, which leads to the complete elimination of infection. By day 5, flies fall into 2 groups according to the level of infection: high or undetectable. This timecourse coincides with lectin secretion, development of the PM and the digestion and movement of the bloodmeal along the gut. Further experiments are needed to discriminate between these factors.

Background

The protozoan pathogen, *Trypanosoma brucei*, undergoes complex cycles of differentiation and multiplication in its vector, the tsetse fly, genus *Glossina*. The infection is first established in the midgut and the trypanosomes migrate anteriorly from this stronghold on a tortuous journey to the paired salivary glands, where they mature into infective metacyclics secreted in the saliva. The essential facts of the lifecycle were described by [1], but others have contributed important details missed in the early work, for ex-

ample the role of the peritrophic membrane or matrix (PM) [2–5]. It takes a minimum of 2 weeks for *T. brucei* to complete the cycle, but the fly then continues to produce metacyclics for the rest of its life, potentially infecting a new host every few days when it takes a bloodmeal or even just pierces the skin with its proboscis. The cycle in the fly effectively amplifies the typically scanty parasitaemia found in the mammalian host, giving an immense advantage over simple mechanical transmission by contamination of the mouthparts with infected blood. In

addition, *T. brucei* has the opportunity for sexual reproduction in the fly [6].

From the above, it would appear that the lifecycle of *T. brucei* is well understood. However, there remain several puzzling and contentious issues. Foremost of these is the poor efficiency of the process both in field and laboratory flies, leading some authors to conclude that the trypanosomes are poorly adapted to the tsetse vector, perhaps reflecting recent evolution, e.g. ref. [7]. Typically far fewer than 1% of wild flies have salivary gland infections, e.g. [8], and even under laboratory conditions, flies are refractory to infection [9]. For example, in experimental transmissions of various clones of *T. b. brucei* through our laboratory colony of *Glossina morsitans morsitans*, a reasonable proportion (typically one third) of flies will develop a midgut infection if given trypanosomes in their first bloodmeal, but only a proportion of these infections then go on to mature (typically less than half).

The nature of the barriers firstly to establishment of trypanosomes in the midgut, and secondly, to onward progression to maturation in the salivary glands, are uncertain. Many factors are known to influence fly infection rates, including variables specific to the trypanosome, infected host or fly. In experimental transmissions in the laboratory, trypanosome and host-specific factors can be held constant, allowing the influence of environmental or fly factors, such as immunity, to be studied. Here we have used highly conspicuous green fluorescent trypanosomes to study the early events in establishment of infection in the fly midgut.

Results and discussion
24 hour dissections of infected flies
Groups of flies were fed a single bloodmeal containing trypanosomes and were then dissected at intervals of approximately 24 hours. The guts were examined immediately as live, wet preparations by fluorescence microscopy and the position and estimated density of fluorescent trypanosomes were recorded for each fly. Samples of the infected bloodmeal from two replicate experiments were cultured *in vitro* to follow the timing of appearance of fluorescent trypanosomes as the bloodstream forms differentiated to procyclics, with concomitant expression of green fluorescent protein (GFP). The trypanosomes were not visible at first, but faintly fluorescent trypanosomes were just visible after 2 hours of incubation and trypanosomes were brightly fluorescent after 4 hours in culture. Fluorescent trypanosomes therefore represent only the proportion of bloodstream form trypanosomes that were viable and capable of differentiation.

Between 24 and 72 hours (1–3 days) after feeding, trypanosomes were found in every fly examined (total 21 from 3 separate experiments). The trypanosomes were always exclusively in the bloodmeal, which was held confined within the PM in the midgut (Fig 1). The trypanosomes were very active, swimming freely in the bloodmeal (see additional file 1: Movie 1), and appeared to increase in numbers over time. In later infections (48–72 hours), some trypanosomes could be seen lying adjacent to the PM, with the length of their body apparently in contact with it (Fig 2; see additional file 1: Movie 1).

By 96–120 hours (4–5 days) the bloodmeal was almost completely digested and the midguts of most flies were empty except for a brownish residue towards the posterior end, at the junction of the Malpighian tubules. In some flies (4), trypanosomes were only found in this region, sometimes just beyond the junction of the Malpighian tubules in the hindgut (Fig 3). Although it is possible that small numbers of non-fluorescent or weakly fluorescent trypanosomes, especially sluggish ones, might have been missed in these flies, large numbers would have been seen. In flies where digestion was less advanced (3), scattered trypanosomes were still observed in the bloodmeal. However, in two flies trypanosomes were found in the empty, middle portion of the midgut, well anterior to the digested bloodmeal. No trypanosomes were detected in a proportion of flies dissected between 96 and 120 hours after infection (10 of 19 flies, or 53%, in 3 separate experiments).

A dramatically different picture was seen in flies dissected after 144 hours (6 days). Flies were either completely negative or contained large numbers of highly active trypanosomes in the anterior region of the midgut; importantly, these trypanosomes were no longer associated with the residue of the bloodmeal. For flies dissected between 144 and 168 hours (6–7 days), 8 of 12 flies (67%) from 2 separate experiments were negative. 144 hours was the earliest timepoint at which trypanosomes were found in the ectoperitrophic space, ie. between the PM and the gut wall (Fig 4; see additional file 2: Movie 2).

Two routes of migration into the ectoperitrophic space have been suggested previously: direct penetration of the PM or migration around the open, posterior end. EM analysis has provided convincing evidence of direct penetration through the PM in the middle region of the midgut for *T. brucei* [5,10]. In agreement with this, we observed trypanosomes lying parallel to the PM in the middle portion of the midgut (e.g. Fig 2), and sometimes found large numbers of trypanosomes associated with fragments of PM during counting of homogenized guts (see below). On the other hand, the existence of a posterior route could be indicated by the flies dissected on days 4–5 after infection, in which trypanosomes were found only at the posterior end of the midgut (e.g. Fig 3). In these flies,
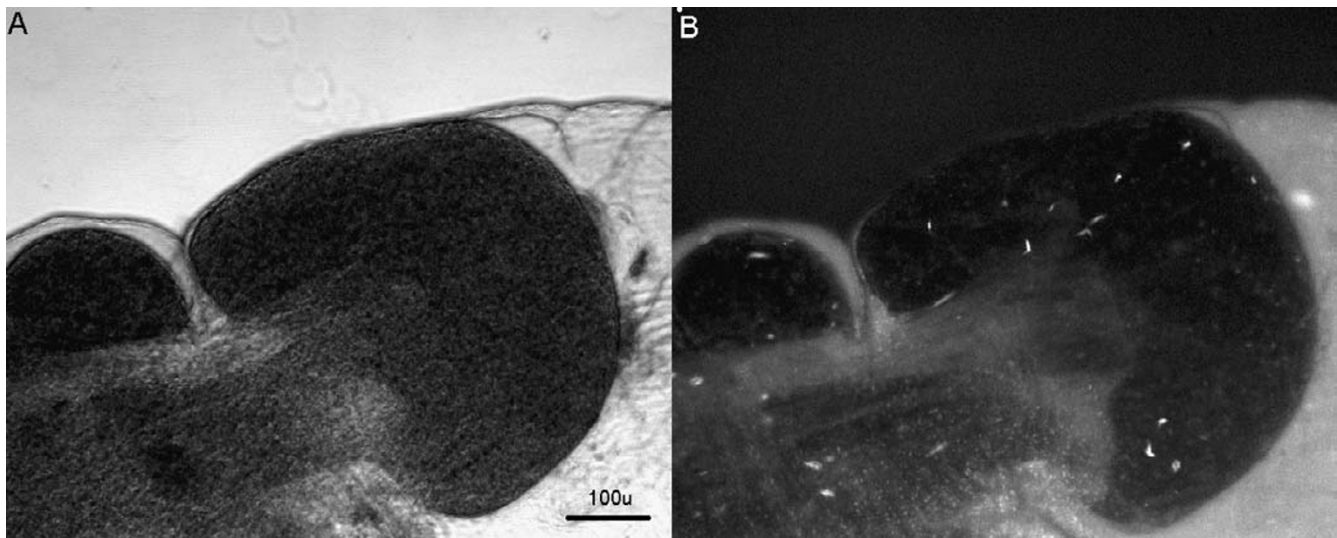
Figure 1
Trypanosomes in the midgut 48 hours after the infective feed. A, brightfield image showing top of bloodmeal within the anterior midgut 48 hours after the infective feed. B, UV image revealing scattered green fluorescent trypanosomes within the bloodmeal.
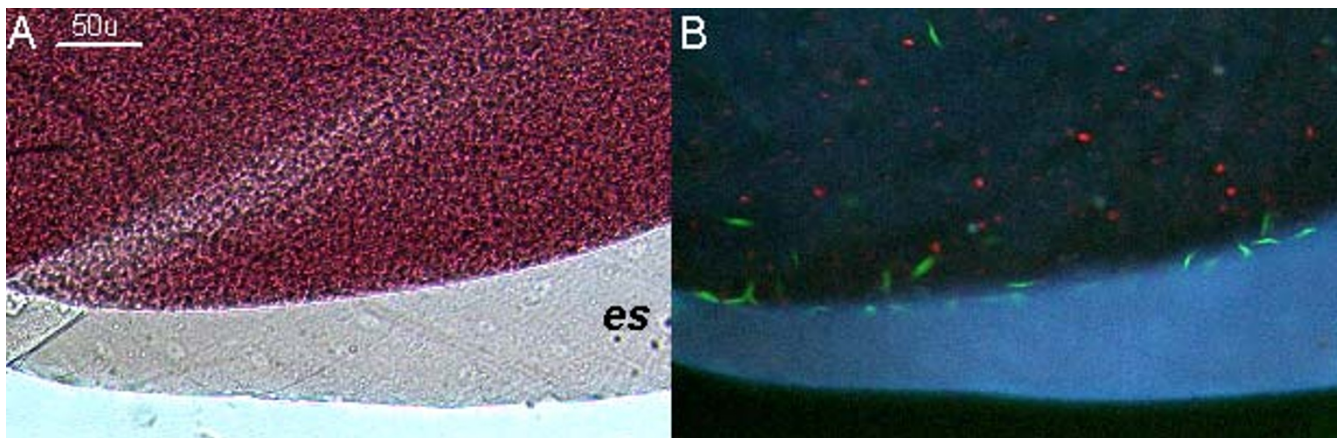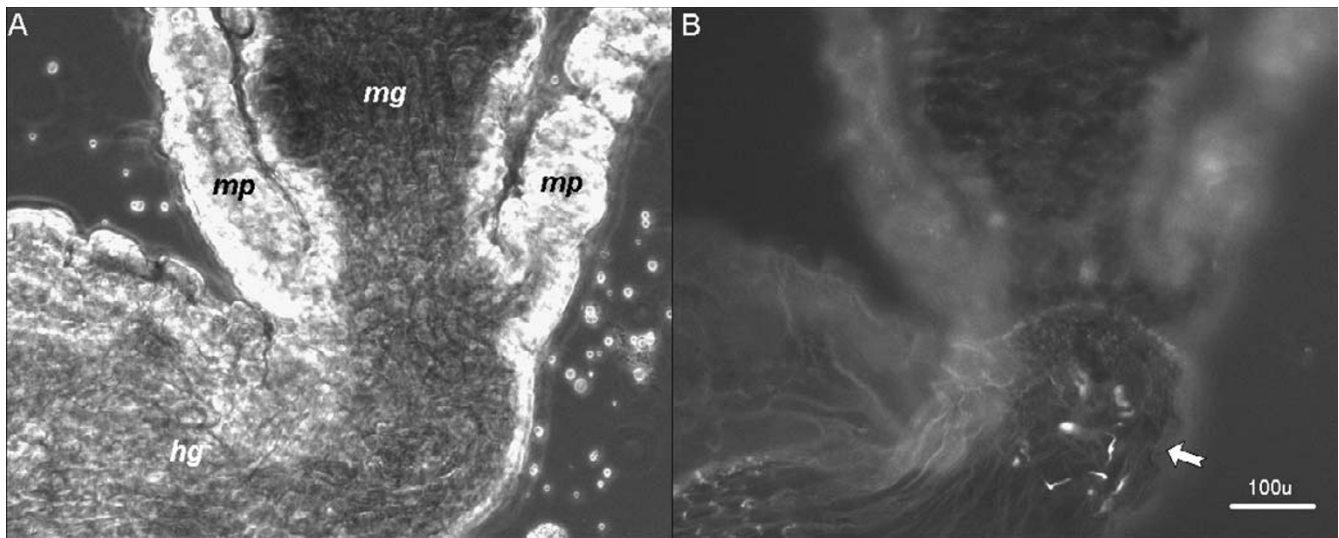

Figure 2
Trypanosomes in the midgut 72 hours after the infective feed A, brightfield image showing part of the bloodmeal bounded by the PM within the midgut 72 hours after the infective feed. B, UV image revealing green fluorescent trypanosomes within the bloodmeal. Some trypanosomes are in close proximity to the PM, but none are in the ectoperitrophic space (es).

however, trypanosomes were present in small numbers and it is difficult to see how these scanty trypanosomes could build up to the great numbers seen in infected flies from day 6 onwards. It seems more likely that the small numbers of trypanosomes found at the very end of the midgut represent a terminal stage of infection.

Observations after day 7 had to be carried out on flies that had received a second bloodmeal. Of 12 flies dissected between 9 and 14 days after the infective feed in 2 separate experiments, 4 (33%) were completely negative and 8 (67%) had very heavy infections. Trypanosomes were seen in large numbers, extending from the proventriculus through the middle portion of the midgut and becoming
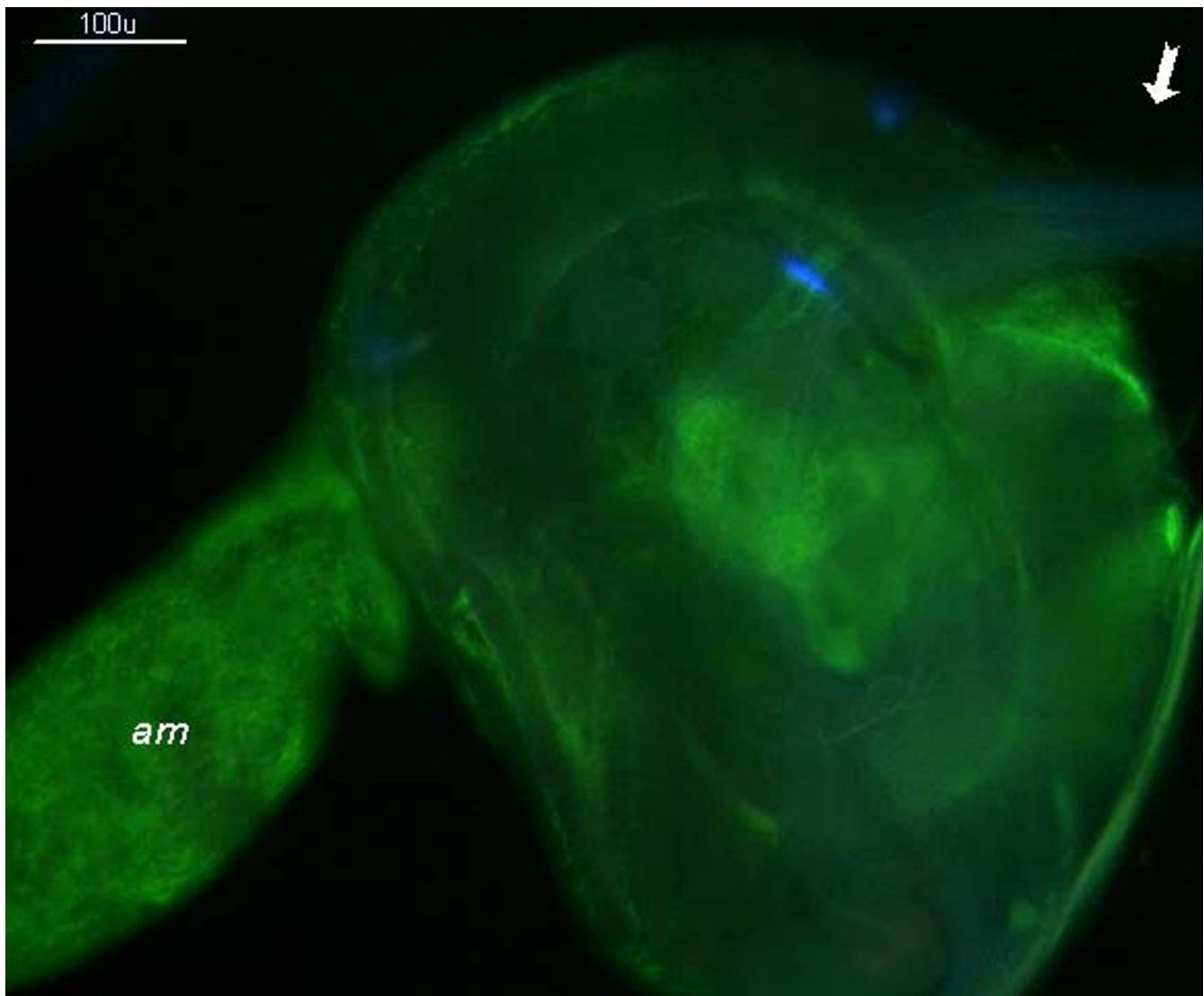
Figure 3
Trypanosomes in the residual bloodmeal at the midgut/hindgut junction A, brightfield image. mg, posterior part of midgut; hg, anterior end of hindgut; mp, Malpighian tubule. Division between the mid- and hindgut is marked by the junction of the Malpighian tubules. B, UV image revealing scanty green fluorescent trypanosomes (arrowed).
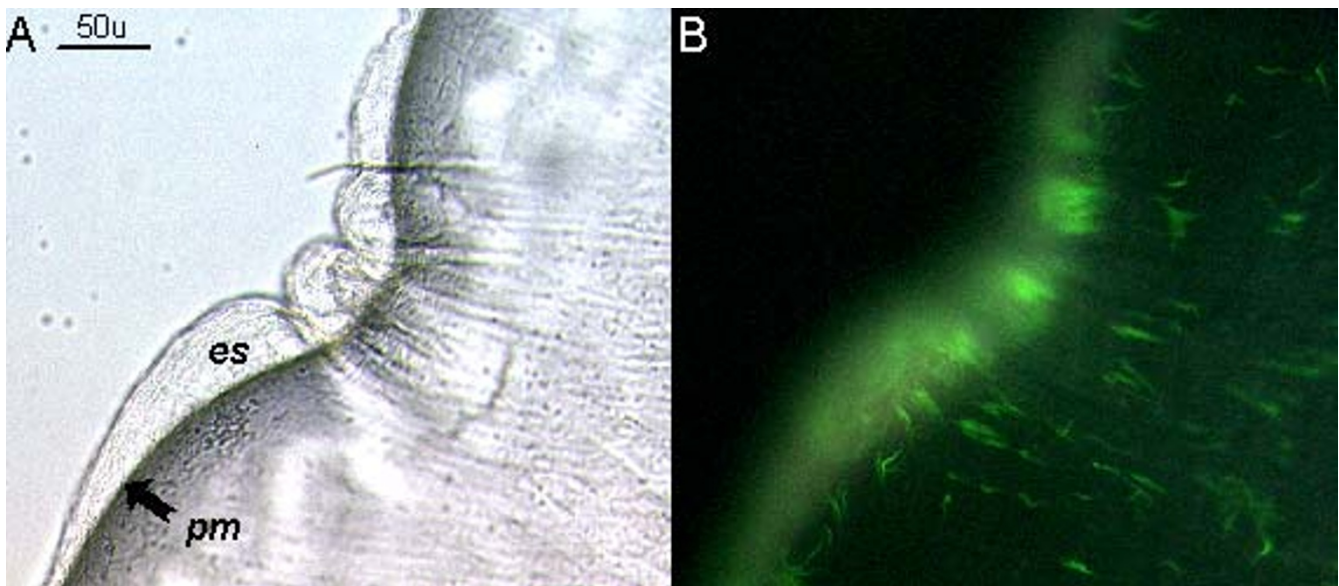
Figure 4
Trypanosomes in the ectoperitrophic space A, brightfield image of part of the anterior midgut 6 days after the infective feed. The midgut is now empty of blood; the dark line (arrowed) shows the boundary of the peritrophic matrix (pm). B, UV image revealing green fluorescent trypanosomes within the ectoperitrophic space (es).
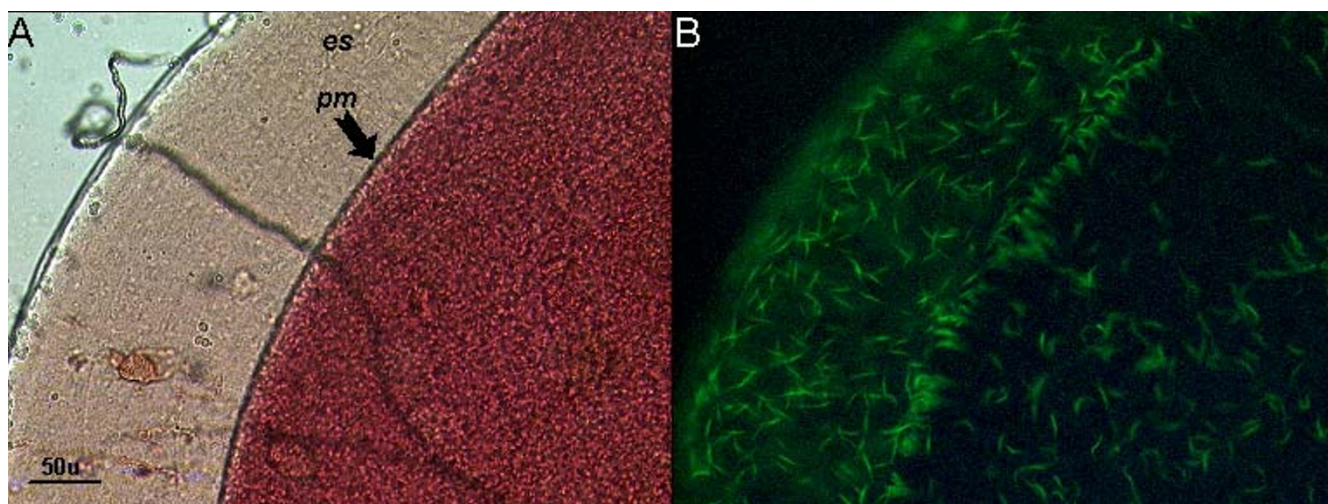
Figure 5
Proventriculus packed with trypanosomes UV image of proventriculus from an infected fly 2 weeks after the infected feed. Numerous green fluorescent trypanosomes swarm in the anterior midgut (am) and at the rim of the proventriculus, such that these organs appear bathed in fluorescence. All the green fluorescence seen is from trypanosomes; these tsetse tissues show little if any autofluorescence. The oesophagus is faintly visible, emerging from the proventriculus (arrowed); no trypanosomes were observed within it.

less dense towards the posterior end. The proventriculus was often packed with trypanosomes (Fig 5; see additional file 3: Movie 3), as was the ectoperitrophic space (Fig 6). The density of infection made it impossible to see whether trypanosomes were in the ectoperitrophic space only or were also in the endoperitrophic space. Infection of the ectoperitrophic space in the region of the midgut with columnar epithelium gave a tessellated or honeycomb appearance (Fig 7). Despite the very great numbers in the

anterior midgut and proventriculus, trypanosomes were not observed in the oesophagus or crop duct at this stage of infection.

In some infections cyst-like forms were observed, apparently associated with or imbedded in the PM (Fig 8). Such cyst-like forms were observed from day 5 onwards, but were particularly evident in flies dissected on days 9–11 after infection. Some cysts contained highly motile

Figure 6
Ectoperitrophic space packed with trypanosomes A, brightfield image of part of the anterior midgut in a fly fed a second bloodmeal; the dark line shows the boundary of the peritrophic matrix (pm) containing the bloodmeal. B, UV image revealing large numbers of green fluorescent trypanosomes within the ectoperitrophic space (es).

trypanosomes with flagella; the internal structure of other cysts was indeterminate and they appeared to consist of aggregates of rounded-up trypanosomes. Similar cyst-like forms were first noted by Robertson [1], who eloquently described 3 types of cyst ("confused motile forms with several flagella, amoeboid forms, wriggling masses of half-fused trypanosomes"), and have been reported by several observers [5]. Whether these cyst-like forms represent degenerating forms, trypanosomes engulfed by phagocytic cells or a stage in the lifecycle is not known. However, there is a striking similarity to the forms produced by treating procyclics with the lectin ConA in vitro to induce cell death [11].

Quantitation of infection
The concentration of trypanosomes in the infective bloodmeal was estimated to be 4×10^3 μl^{-1}, based on the level of parasitaemia in the blood before cryopreservation. A fly taking a typical 20 μl bloodmeal would therefore imbibe an estimated 8×10^4 trypanosomes.

A sample of the infected bloodmeal from one experiment was cultured *in vitro* to establish the efficiency of procyclic transformation and growth rate (Fig 9). The density of fluorescent trypanosomes after 24 hours in culture represented a concentration of approximately 10^2 trypanosomes μl^{-1} in the infected bloodmeal, assuming that transformation to procyclics, but not division, had occurred by this time. This indicates that less than 10% of the original population of bloodstream form trypanosomes were viable and able to transform into procyclics. The total number of fluorescent trypanosomes counted in individual fly guts after 24 hours in this experiment was in the range 1.9×10^2 to 20.6×10^2, which tallies with the number expected after a 20 μl bloodmeal containing 10^2 trypanosomes μl^{-1}. This suggests that any immediate fall in trypanosome numbers on entering the midgut in this experiment is due to trypanosome rather than fly factors, ie. poor viability or inability to differentiate.

We do not routinely assess the recovery of bloodstream forms after cryopreservation, so reduced viability cannot be ruled out. Neither can inability to differentiate, as it is assumed that only a proportion of bloodstream forms (the short stumpy forms) can readily transform into procyclics, because they are metabolically preadapted for transformation [12]. It is not unrealistic that a combination of these factors might have resulted in the initial tenfold drop in numbers observed. Similarly, a drastic initial drop in numbers within 48 hours of the infected feed was recorded for experimental infection of *G. m. morsitans* with *T. b. brucei* [3], and about 80% of bloodstream form *T. b. rhodesiense* had abnormal motility and/or morphology after 10 hours in the midgut of *G. m. morsitans* [13]. Evans [14] concluded from his successful attempts to infect flies using procyclics, that many infections were probably lost by failure of bloodstream forms to transform into procyclics.
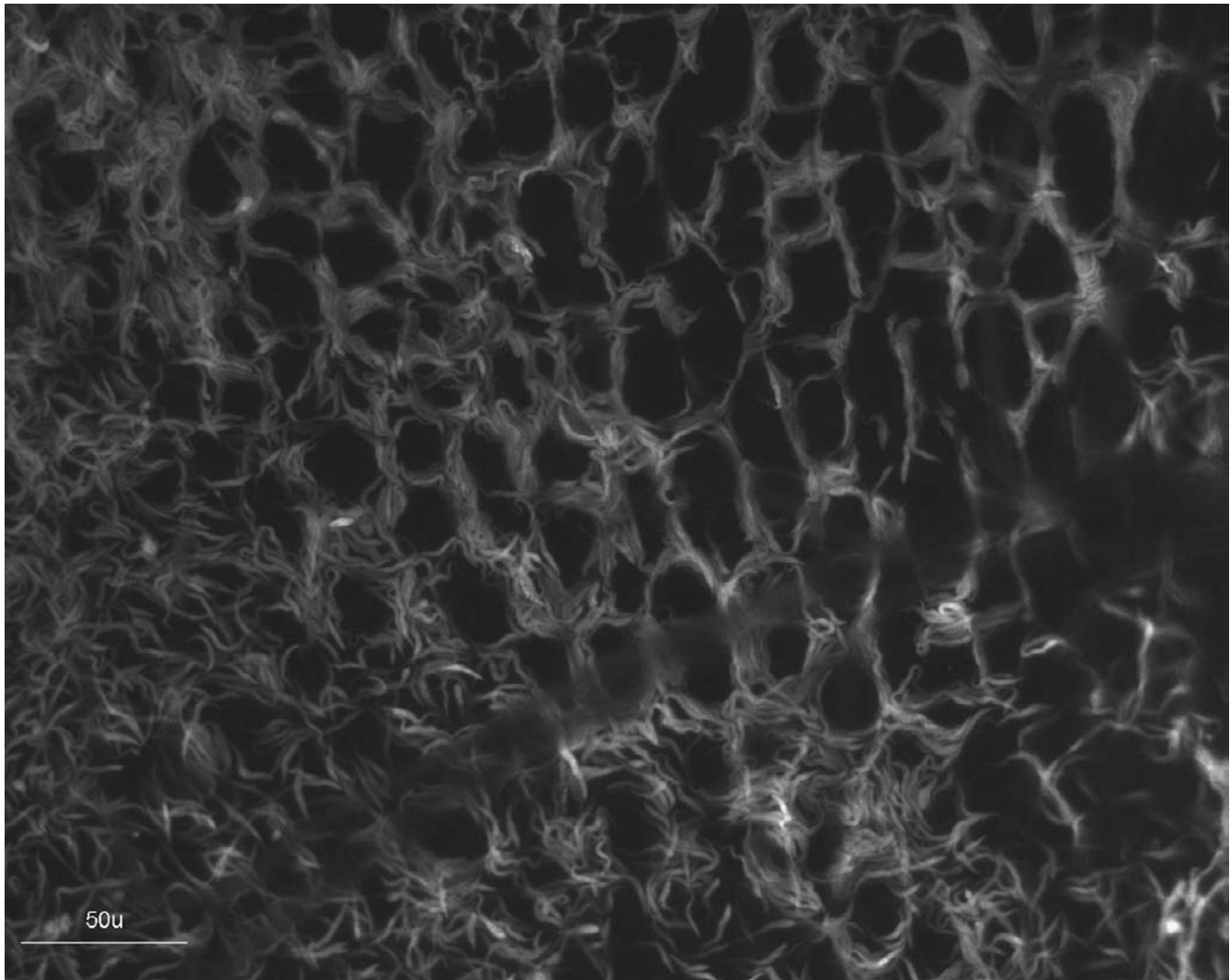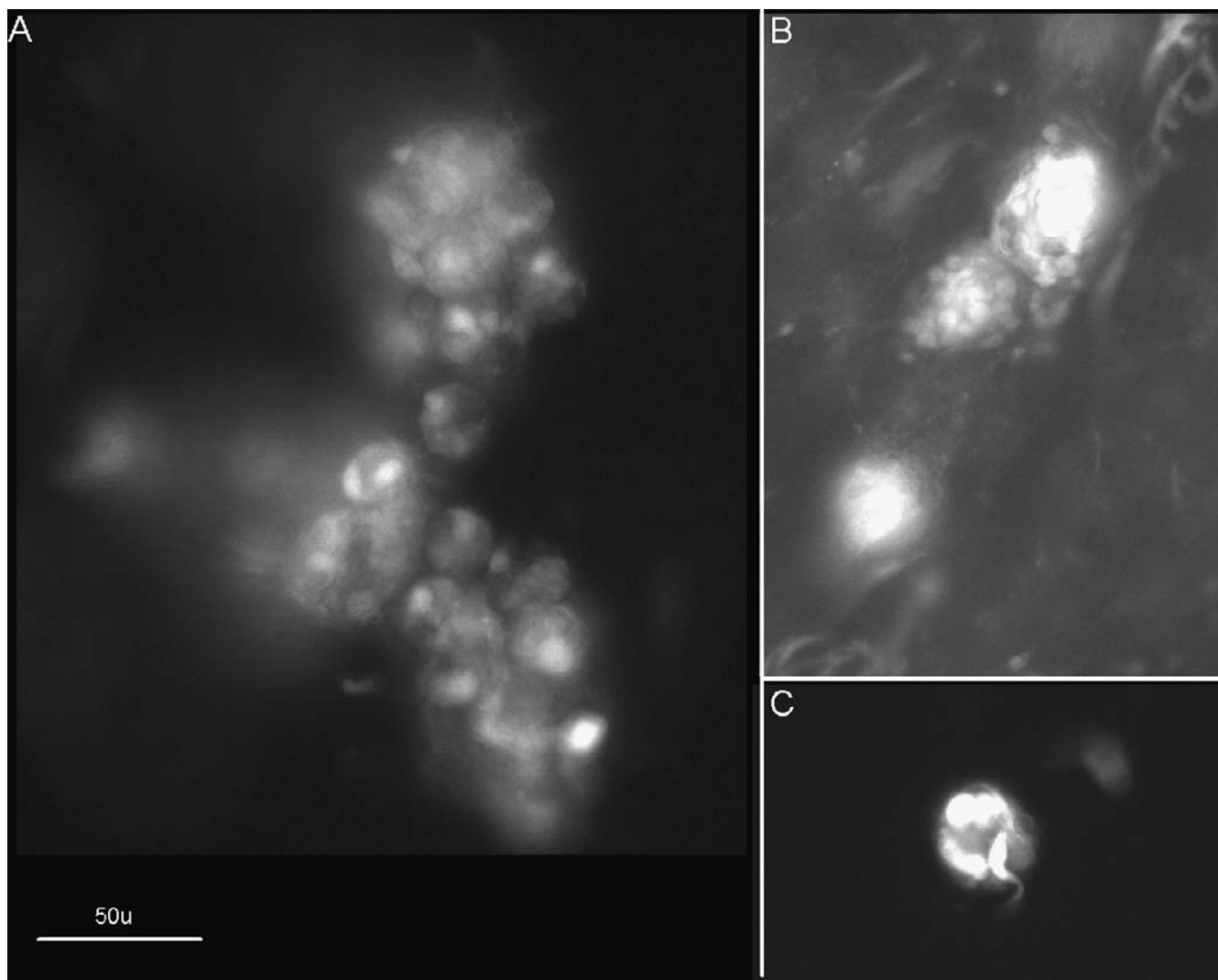
Figure 7
Honeycomb appearence of midgut trypanosomes UV image of part of midgut showing the "honeycomb" appearance of trypanosomes confined between the peritrophic matrix and columnar cells of the midgut epithelium 9 days after the infective feed

After transformation, the trypanosomes grew exponentially in culture as expected (Fig 9), and trypanosomes within the fly midgut also multiplied exponentially during the first 24 to 72 hours (Fig 10). In some flies, trypanosome numbers continued to increase or remained at a high level up to day 6 when the experiment ended. However, in other flies trypanosome numbers started to fall after 3 days of infection, leading to the complete elimination of infection in some flies. The quantitative results are in agreement with our microscopy observations above.

Statistical analysis showed that the frequency distribution of number of trypanosomes in the flies was essentially normal on days 1 and 2, indicating that infected flies were a single population (Table 1). By day 5, however, the frequency distribution was significantly different from normal, showing a clear split into 2 populations of flies: those with heavy infections and those with undetectable infections (Fig 10; Table 1). Although not significant, evidence for this was also seen on days 3 and 4 (Table 1); a larger sample size might increase statistical support for this trend. These results confirm previous observations also made using *G. m. morsitans* from the Bristol colony
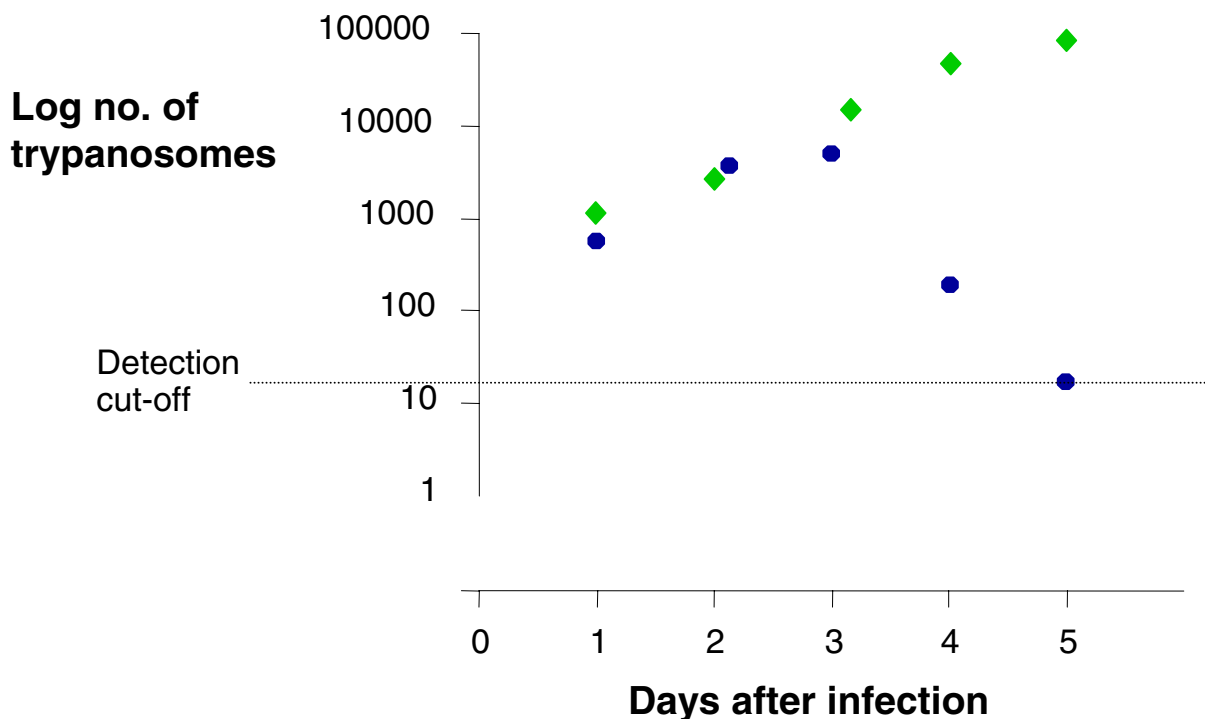
Figure 8
Cyst-like bodies associated with the PM in the midgut UV images of cyst-like bodies found associated with the peritrophic matrix in flies dissected 10–11 days after the infective feed. Images A and B show aggregations of rounded up trypanosomes; C shows a cyst-like body containing highly motile trypanosomes.

infected with *T. brucei*, that the number of infected flies fell between days 3 and 5 after infection [15], and that the proportion of negative flies increased from 48 hours after infection [16].

The cause of elimination of some trypanosome infections from day 3 onwards remains to be determined. Trypanocidal lectins may play a role. There is indirect evidence that some infections are terminated by the action of trypanocidal lectins in the midgut, since the proportion of infected flies can be significantly increased by feeding glucosamine, a lectin-binding sugar [17,18]. However, glucosamine has other effects, notably the inhibition of tsetse midgut trypsin: the same concentration of glucosamine used to enhance midgut infection rates inhibited about 40% of the proteolytic action of trypsin in vivo [19]. In preliminary experiments, we found that feeding glucosamine in the first bloodmeal visibly slowed the rate of digestion (unpublished results). Nonetheless, the timing of lectin secretion fits with the timecourse of trypanosome elimination determined here. A slow rise in lectin levels between days 0 and 8 after eclosion was demonstrated by haemagglutination assays, with significant amounts appearing after day 3; lectin levels rose exponentially after
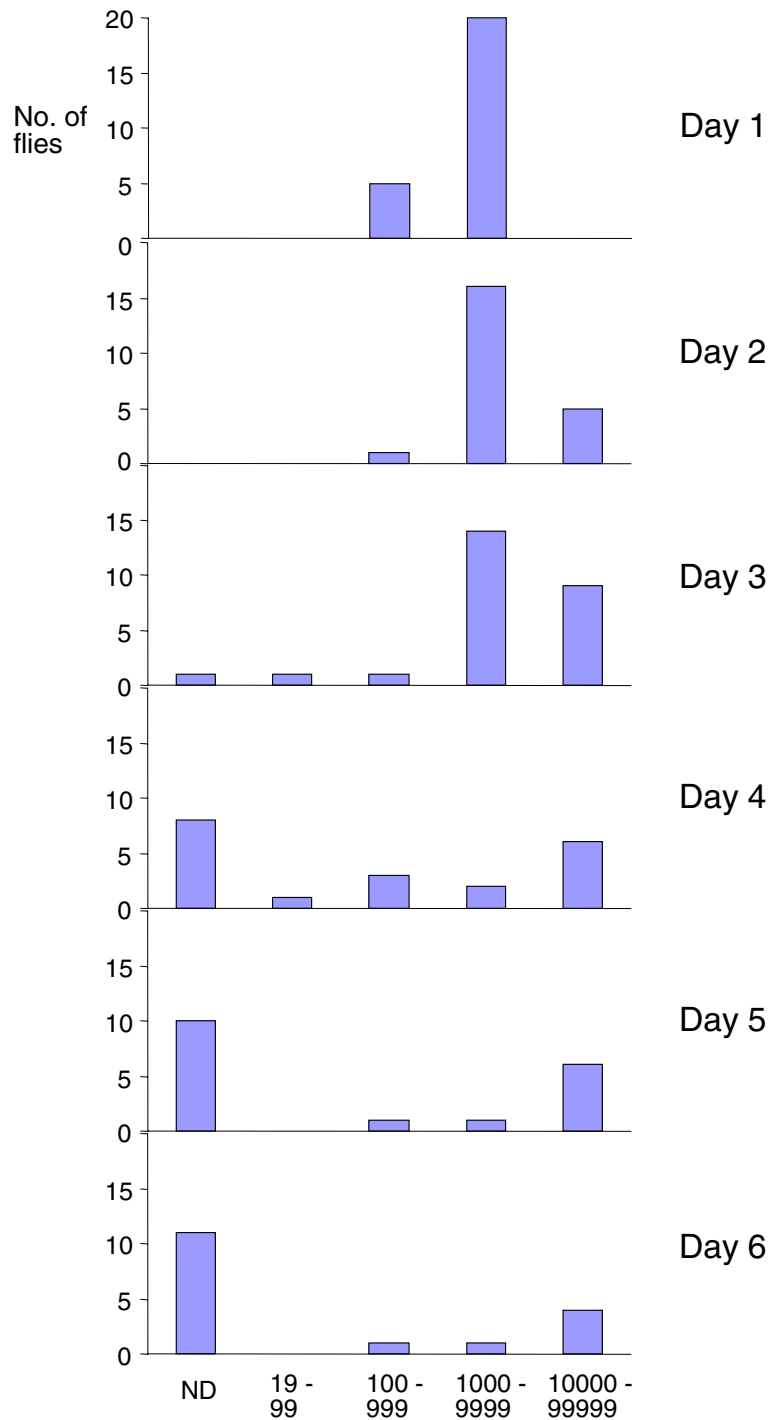
Figure 9
Growth of trypanosomes in in vitro culture compared to fly midgut Graph showing the growth of trypanosomes in culture (diamonds; geometric mean of 2 separate cultures) compared to the fly midgut (circles; geometric mean of pooled flies). The numbers in culture reflect growth of 10 ul of the same trypanosome-containing bloodmeal used to infect the flies.

day 8 and reached very high levels by day 14 when the experiment terminated [20]. Interestingly, the lectin was also found to be associated with the PM, which developed to fully line the midgut within 80–90 hours of eclosion [20].

Exploration of the interactions of trypanosomes with components of the tsetse anti-microbial immune system is at an early stage. Flies are able to detect the presence of pathogens, including trypanosomes, in the midgut, as shown by upregulation of transcription of the anti-microbial peptides, attacin and defensin, in the fat body within a few days of infection [21]. In addition, flies that were immune-stimulated by prior injection with bacteria showed significantly reduced infection levels with trypanosomes compared to controls when dissected 20

days after infection [21]. Although there is clearly molecular communication between the midgut and the immune organs of the fly, the nature of the effector molecules is as yet unknown.

The timecourse of trypanosome elimination also coincides with digestion and movement of the bloodmeal along the gut: by day 4 flies held at 25°C have only a brown residue from the bloodmeal remaining at the posterior end of the midgut, and by day 5, the midgut is completely empty of any residual bloodmeal. Digestive enzymes are largely absent from the anterior midgut, being secreted in the posterior half [22–24], perhaps explaining why trypanosome survival is correlated with migration towards the anterior [15].

Figure 10
Numbers of trypanosomes in individual flies Counts of trypanosomes in individual flies on days 1 to 6 following the infected feed. Infections have been divided into 5 categories according to the number of trypanosomes; ND, not detectable.

Table 1: Frequency distribution of trypanosome numbers per fly P-values from Kolmogorov-Smirnov test. * significantly different from normal distribution (p < 0.05).

Day	No. of flies	p value
1	25	0.904
2	21	0.307
3	27	0.092
4	20	0.188
5	18	0.021*
6	17	0.010*

For infection to endure after day 5, trypanosomes apparently need to transfer to the ectoperitrophic space. It is possible that conditions in the digesting bloodmeal become inimical to trypanosome survival and the ectoperitrophic space offers some kind of refuge. The PM may serve to compartmentalize regions of high enzyme concentrations and intensive digestion, perhaps by binding certain molecules to its surface [25].

Conclusions

We now have a clearer picture of the early events in establishment of *T. brucei* in the tsetse midgut. By using GFP expression as an indicator of transformation, we were able to record a greater than tenfold disparity between the estimated number of bloodstream form trypanosomes in the infective bloodmeal compared to the number of procyclics in the midgut or *in vitro* culture 24 hours after infection. Therefore the initial factors inhibiting establishment of infection in the midgut are trypanosome-specific, depending on viability and ability to differentiate.

The trypanosomes that have undergone successful differentiation appear to experience an environment within the midgut suited to their unrestricted growth for the first 3 days. After this time, a process of attrition is evident, which leads to the complete elimination of infection in some flies. By day 5, flies fall into 2 groups according to the level of infection: high or undetectable. This timecourse coincides with lectin secretion, development of the PM and the digestion and movement of the bloodmeal along the gut.

Maintaining a foothold in the fly appears to depend on reaching the ectoperitrophic space. The earliest timepoint that trypanosomes were found in the ectoperitrophic space was day 6 and they were then present in great numbers. Infection at this level was maintained until the end of observations (day 14), with trypanosomes swarming in the proventriculus and midgut, but becoming less dense towards the posterior end.

Methods
Trypanosomes
The group 2 *Trypanosoma brucei gambiense* stock TH2 (MHOM/CI/78/TH2 [78E], [26]) was transfected with a construct containing the gene for green fluorescent protein under the control of the procyclin promotor as described by [27]. Clone E21 was shown to carry the *GFP* gene in the ribosomal locus [27]. Aliquots of E21 bloodstream forms in log phase growth were cryopreserved for fly infection. E21 bloodstream forms were not visibly fluorescent, but became fluorescent on transformation to procyclics due to the concomitant upregulation of the procyclin promotor.

Fly infection and dissection
The experimental flies were from the long-established Bristol laboratory colony of *Glossina morsitans morsitans*, originally from Zimbabwe. Male flies, approximately 18–24 hours post-eclosion, were fed a single bloodmeal of sterile horse blood containing a thawed aliquot of bloodstream form E21 trypanosomes; the final concentration of trypanosomes in the bloodmeal was estimated as 4×10^3 μl^{-1}. Flies were subsequently maintained in small cages containing 3 – 5 flies without feeding for up to 6 days and longer if given a second bloodmeal of sterile horse blood. Dissection took place at approximately 24 hour intervals. Flies were briefly chilled before dissection of the whole posterior gut from the proventriculus to the rectum.

Imaging
Whole tsetse midguts were dissected into a drop of PBS and viewed as wet mounts. A DMRB microscope (Leica) equipped with either a 24 bit Colour Coolview camera (Photonic Science) or 10 bit monochrome Megaview II camera (Norfolk Analytical) was used for fluorescence and brightfield microscopy, with ImagePro Plus software (Photonic Science) or analySIS software (Norfolk Analytical) respectively. Multiple images captured with a minimum timelapse were assembled to create video sequences (ImagePro Plus). Results were compiled from 5 batches of flies infected in separate experiments.

Counting and statistics

For counting trypanosomes in individual flies, the whole abdomen, including the posterior gut with the proventriculus, was placed in 50 µl of PBS in a microcentrifuge tube and thoroughly disrupted using a teflon pestle. The trypanosomes were fixed by adding paraformaldehyde to a final concentration of 0.1 % w/v in PBS and counted immediately using a haemocytometer. Only fluorescent trypanosomes were counted. The lower limit of detection was 18 trypanosomes per fly, based on the number of trypanosomes present if only 1 trypanosome was recorded in 6 haemocytometer counts. Seven replicate experiments were carried out to give adequate numbers of flies at each time point.

The frequency distribution of trypanosome numbers in flies was analysed for conformity to a normal distribution using a single-sample Kolmogorov-Smirnov test (SPSS).

Competing interests

None declared.

Authors' contributions

WG carried out the tsetse transmission experiments and drafted the manuscript; MB carried out the imaging and statistical analyis. Both authors contributed to the design of the study, read and approved the final manuscript.

Additional material

Additional File 1

Movie showing live green fluorescent trypanosomes in bloodmeal in tsetse midgut
Click here for file
[http://www.biomedcentral.com/content/supplementary/1475-9292-2-1-S1.avi]

Additional File 2

Movie showing live green fluorescent trypanosomes in ectoperitrophic space in tsetse midgut
Click here for file
[http://www.biomedcentral.com/content/supplementary/1475-9292-2-1-S2.avi]

Additional File 3

Movie showing live green fluorescent trypanosomes in tsetse proventriculus and anterior midgut. All the green fluorescence seen is from trypanosomes; these tsetse tissues show little if any autofluorescence.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1475-9292-2-1-S3.avi]

Acknowledgements

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