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Effect of Aflatoxin B-1 on Transmissibility of Trypanosoma Congolense in Mice

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Abstract
Mycotoxins as secondary metabolites are known to be common contaminants of both human food and animal feed. If ingested in minute but regular doses, they are known to cause suppression of the immune system and therefore, alter pathogenesis of many infectious diseases. *Trypanosoma congolense* an intravascular parasite is the most important cause of African animal trypanosomosis. The aim of this work was to investigate the effect of aflatoxin B-1, a common mycotoxin on transmissibility of *T. congolense*. Female Swiss white mice were intra-peritoneally injected with 0.05mg/kg body weight aflatoxin B-1 every after 3 days upto 10 times and on the 21st day were infected with *T. congolense*. Parasitological parameters including weight, packed cell volume and parasitemia levels of aflatoxin B-1-injected-*T. congolense*-infected mice were compared with those of *T. congolense*-infected mice. In a separate study, aflatoxin B-1-injected-*T. congolense*-infected and *T. congolense*-infected mice (12 each) were fed on by 400 tsetse flies. Some of these flies were used to cyclically infect 100 uninfected mice. ANOVA and mean separation were used to determine differences between the test and control mice. It was observed that there was significant difference ($p<0.05$) in body weight but no significant difference in packed cell volume, establishment of infection within the tsetse flies and subsequent transmission to uninfected mice. It was concluded that aflatoxin B-1 has an effect on pathogenesis and hence transmissibility of *T. congolense*.
Keywords: Aflatoxin B-1, *Trypanosoma congolense*, pathogenesis, transmissibility

1.0 INTRODUCTION

Trypanosoma congolense causes African animal trypanosomiasis (AAT), which is a severe and fatal disease of cattle and other animals. AAT is a wasting disease in which there is a slow progressive loss of condition accompanied by increasing anaemia and weakness to the point of extreme emaciation, collapse and death. Therefore, it can be said categorically that the tsetse-infested belt of Africa cannot be utilized for animal farming because of AAT. Countries in this region lose large sum of revenue for failing to exploit this potential rangeland. Epidemiology of AAT is determined by various intrinsic and extrinsic factors, which range from nutritional, ecological and socio-economic to man-made factors (Maudlin, *et al*., 1998). Ideal farm practice has been developed by people in the tsetse belt, which involves crop-livestock interactions. It is deemed necessary to apply manure to fertilize crops and use crop residues to feed livestock (Tittonell *et al*., 2009). In doing this, the farmer is able to supplement one agricultural practice with the other hence improving on returns from the ever decreasing land/farm. The use of spoiled farm produce, particularly crop residues like maize stover, is a likely source of mycotoxins. Investigation of effect of mycotoxins on progression of trypanosomiases is yet to be studied (Kibugu *et al*., 2009).

Trypanosomiases compromise the immune system's haemopoietic system(Williams *et al*., 2004). The disease interferes with functions of essential nutrients like vitamins (Anyanwu *et al*., 2004) and exerts pathological effects on vital organs like spleen, liver, kidney, heart, lymphoid tissue and central nervous system (Kibugu, 2008) and may influence the direction of infections of various diseases. Aflatoxin B-1 (AFB-1) on the other hand is a common mycotoxin of the tropics, which contaminates farm produce, food products and feeds (Azziz-Baumgartner *et al*., 2005). Mycotoxic contamination occurs during pre-harvest, harvest and post-harvest activities and is favoured by plant (or crop) genotype, handling, ecological characteristics and edaphic factors (Williams *et al*., 2004). AFB-1 enters the body through ingestion of contaminated food, inhalation, skin contact or vertical transfer and its symptoms among others depends on state of health of the exposed individual (Bennett and Klich, 2003). AFB-1 is carcinogenic, mutagenic, immunotoxic, and is a growth impairment factor particularly for protein calorie malnutrition (Gong *et al*., 2004). Besides these, AFB-1 causes gastrointestinal dysfunction, reduced food utilization and efficiency, anaemia, jaundice, ascites and high death rate. Just like other mycotoxins, AFB-1 may contribute to pathogenesis of many diseases and conditions (Thrasher, 2007). Endemicity of AAT and AFB-1 to the tropics and their immunosuppressive properties forms the quest for this research work.

2.0 MATERIALS AND METHODS

2.1 Ethical approval

All protocols and procedures that were used in this study were reviewed and approved by KARI-TRC Institutional Animal Care and Use committee (IACUC). Use and handling of mycotoxin and its detoxification process was done as described by Scott (1995) and Karlovsky (1999).

2.2 Mice

One hundred and fifty (2 donor and 148 experimental), 30-day old female and male (in the ratio of 2 female: 1male as per the availability at KARI-TRC facility) Swiss white mice weighing between 20-30g from the small animal breeding unit colony at KARI-TRC were used. The mice were acclimatized for 14 days before commencement of the experiment during which were dewormed using ivermectin at the rate of 20mg/kg. The mice were housed in groups of 12 per cage and maintained on mice pencils, (Unga Feeds Limited, Kenya) and water *ad libitum* at a temperature of 21-25°C and on wood shavings as bedding material (Seed and Sechelski, 1988).

2.3 Trypanosome

A cryo-preserved *T. congolense* stabilate KETRI 2409, from KARI-TRC trypanosome bank was used to infect the experimental mice. The stabilate was isolated by Dr. George Losos in 1978 from a bovine host at Galana, Malindi, Kenya (Losos, 1986).

2.4 AFB-1

Analytical column purified extracts of AFB-1 from BoraBiotech Limited, Cooper centre, Nairobi, Kenya were used during the experiment.

2.5 Tsetse flies

Four-hundred male teneral *Glossina pallidipes* Austen, between 28-48 hours old, originating from the colony maintained at KARI-TRC were used in transmission studies (according to availability at KARI-TRC facility).

2.6 Experimental design

Six groups of mice were used in the study. They were divided and treated as indicated in Table 1. Group A mice were injected with AFB-1 intraperitoneally (ip) at rate of 0.5mg/kg (Gathumbi *et al*., 2001) every third day and then infected with *T. congolense* 21 days post exposure to AFB-1. Group B mice were not injected with AFB-1, but only infected with *T. congolense*. Group C mice were injected with AFB-1 ip at rate of 0.5mg/kg every third day and then were infected with *T. congolense* 21 days post exposure to AFB-1. At peak parasitaemia, this group of mice was fed on by tsetse in batches of 10 flies. Group D mice were not injected with AFB-1, but were only infected with *T. congolense* then at peak parasitaemia was fed on by tsetse in batches of 10 flies. Group G and H mice were uninfected mice (clean mice) which were infected by tsetse which fed on group C and D mice.

Table 1: Summary of mice groups and treatments

Group	Number of mice	Treatment
A	12	Injected with AFB-1 ip at rate of 0.5mg/kg every after 3 days then infected with *T. congolense*
B	12	Infected with *T. congolense*
C	12	Injected with AFB-1 then infected with T. congolense and each fed on by tsetse once in batches of 10
D	12	Infected with T. congolense and then each fed on by tsetse once in batches of 10
E	50	Uninfected mice to be infected by tsetse from group C
F	50	Uninfected mice to be infected by tsetse from group D

2.7 Determination of body weight

Body weight of experimental mice was determined twice a week using an electronic analytical balance (Mettler PM34, DoltaRange®) (Kibugu *et al*., 2009).

2.8 AFB-1 injection in mice

AFB-1 was dissolved in distilled de-ionized water at the rate of 0.05mg/ml (El-Arabi *et al*., 2006) and ip injected in the mice at the rate of 0.5mg/kg body weight every third day upto 10 times.

2.9 Infecting mice with trypanosomes
Two donor mice were immunosuppressed for three consecutive days with cyclophosphamide at rate of 8.3mg/kg body weight (Mdachi personal communication) and then on the third day injected with *T. congolense* KETRI 2409 stabilate.

2.10 Determination of parasitaemia
Blood of all infected mice was microscopically examined for parasites daily by a tail clip and a drop of blood was put on a clean slide then coverslip placed and observed at x400 magnification (Gichuki and Brun, 1999) starting second day post-infection for the first two weeks and twice weekly for subsequent weeks. Parasitemia levels were assessed by matching technique of Herbert and Lumsden (1976).

2.11 Determination of packed cell volume
Taking of packed cell volume (PCV) was done for each experimental mouse twice weekly by a tail snip and about 75µl of blood collected in EDTA capillary tubes and centrifuged at 10,000 revolutions for 5 minutes. The PCV was then measured by haemacytometer (hawksley® micro-haematocrit reader, England) and expressed as a percentage of the total blood volume as described by Naessens *et al*. (2005).

2.12 Feeding success of tsetse flies
Male teneral tsetse in batches of 10 flies per cage, were fed onto individual group C and D mice. During feeding, the duration to full engorgement of each fly batch and number of engorged and non-engorged flies was recorded. Fully engorged and non-fed flies were separated and kept in separate holding cages. These flies were then kept in the experimental insectary in which temperature and humidity were maintained at between 25-30°C and 75-80% respectively while non-fed flies were discarded. The flies were maintained on defibrinated bovine blood by *in vitro* silicon membrane feeding (Masumu *et al*., 2006) on Mondays, Wednesdays and Fridays for upto 33 days post-feeding on parasitaemic mice.

2.13Fly mortality
The number of dead flies were counted and recorded once every week during the study period and comparisons made between controls and flies fed on group C and D mice.

2.14 Dissection of flies
Starting day 13 post-feeding on parasitaemic mice and on alternate days flies in groups of 20 (10 from each group) were dissected in normal saline as described in FAO/UN (1982) training manual. The midguts and proboscis were examined microscopically as wet mounts under phase contrast at x400 magnification for trypanosomes (both procyclics and metacyclics) (Njiru *et al*., 2010).

2.15 Probing tsetse flies for trypanosomes in their saliva
On day 24 post feeding on parasitaemic mice, 100 flies (50 each from group C and D as in table 1) were probed for presence of trypanosomes in their saliva. Probing slides were pre-warmed to a temperature of 37-40°C and individually caged flies allowed to spit saliva onto the slides. While still fresh, the saliva was examined at x400 magnification (Thuita *et al*., 2008).

2.16 Transmitting trypanosomes to uninfected mice
After probing, the individually caged flies were fed on a single uninfected mouse each (group E and F mice as indicated in table 1) to mimic natural event of xenodiagnosis. Each of the uninfected mice was restrained by hands and the caged fly used for cyclical transmission as was described by Okoth *et al*. (2006).

2.17 Detoxification of mycotoxic wastes
All safety precautions of handling and storage of mycotoxins and their wastes as described by Gathumbi *et al*. (2001) were adhered to. The housing cages holding AFB-1-injected mice were well labeled. Glassware and equipment used were rinsed in methanol then immersed in 1% NaOCl solution for 2hours and then rinsed in acetone, (Scott, 1995). The beddings and droppings of AFB-1-injected mice were put in a well labeled eco-garb® heavy duty plastic bag and drenched in diesel and incinerated (Kibugu *et al*., 2009).

2.18 Data analysis
Data was collected in data sheets and entered into spreadsheets (Microsoft excel) and analyzed using Graph pad Prism ver. 5.01 statistical software package (Graph pad Prism Inc. USA). All the tests were two-tailed with critical limit being equal to a p value of 0.05 for significance.

3.0 RESULTS

3.1 Effect of AFB-1 on weight of mice

The weekly mean weight of infected mice was as shown in Figure 1. The mean weight for AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected mice was 27.47 ± 2.076 and 30.31 ± 2.417 grams respectively. This was significantly different ($p<0.05$) for the two groups of mice.

3.2 Effect of AFB-1 on PCV of mice

The mean PCV of mice was as shown in Figure 2. The mean PCV of AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected mice was 49.95 ± 3.07 and 51.8 ± 3.6 percent respectively. This was not significantly different ($p>0.05$) for the two groups of mice.

3.3 Feeding success of tsetse flies.

Tsetse flies were considered to have successfully fed upon observation of an engorged and reddened abdomen. The number of fully engorged flies and the time they took to engorgement was as indicated in Figure 3 below. Tsetse that fed on AFB-1-injected-*T. congolense*-infected mice had a mean feeding duration of 15.64 ± 2.494 minutes and those that fed on *T. congolense*-infected mice had a mean feeding duration of 13.36 ± 2.139 minutes. Time to full engorgement of tsetse was significantly different ($p<0.05$) between the two groups of tsetse. During transmission studies when tsetse flies from the two groups were used to infect uninfected mice, the mean time flies from each group took to full engorgement was not significant ($p>0.05$). Tsetse that initially fed on AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected mice took a mean time of 3.6 ± 2.143 and 4.1667 ± 2.832 minutes respectively to feed on uninfected mice.

3.4 Tsetse fly mortality

The death pattern of tsetse flies that fed on AFB-1-injected-*T. congolense*-infected, *T. congolense*-infected mice and control tsetse flies was as indicated in Figure 4 below. Death in flies fed on AFB-1-injected-*T. congolense*-infected, *T. congolense*-infected mice and control tsetse flies was 41.4, 29.3 and 38.75 percent respectively. The mean death rate for tsetse fed on AFB-1-injected-*T. congolense*-infected, *T. congolense*-infected mice and control tsetse flies was 4.8, 3.6 and 9.3 flies/day respectively. Mortality rate was not significant ($p>0.05$) between any two groups of flies. However, the death rate was significant within tsetse fed on AFB-1-injected-*T. congolense*-infected mice and control tsetse flies.

3.5 Presence of trypanosomes in proboscis and midgut

Upon dissection of tsetse flies for observation of trypanosomes in their midgut (procyclics) and proboscis (metacyclics), there was no significant difference ($p>0.05$) in the presence of procyclics and metacyclics in the tsetse that fed on AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected mice.

3.6 Ability of tsetse flies to probe

A tsetse fly was considered to have successfully probed if it spat saliva on the pre-warmed probing slide. While the saliva was still fresh the slide was observed for trypanosomes at ×400. The result was as shown in the Table 2 below:

Table 2: Result of probing of tsetse flies before cyclic transmission

	Tsetse fed on AFB-1 + *T. congolense* mice	Tsetse fed on *T. congolense* mice	Total
Number of tsetse that probed	16	33	49
Number of tsetse that did not probe	36	20	56
Total number of tsetse	52	53	105
Percent success	30.77	62.26	46.67

It was observed that tsetse flies fed on *T. congolense*-infected mice had a higher percentage of probing success than tsetse fed on AFB-1-injected-*T. congolense*-infected mice. However, there was no significant difference ($p>0.05$) in the probing success between the two groups of flies.

3.7 Ability of tsetse flies to transmit *T. congolense* to uninfected mice

This was based on data available from dissected tsetse and the number of uninfected mice that successfully became infected after cyclic transmission. As observed in this study, there was no significant difference in the presence of procyclics and metacyclics in the two groups of tsetse. Uninfected mice that ultimately became positive for *T. congolense* was 6 and 8 percent respectively for the tsetse that fed on AFB-1-injected-*T.*

congolense-infected and *T. congolense*-infected mice. This was significantly not different ($p>0.05$) between the two groups of tsetse.

4.0 DISUSSION

Any species of *Glossina* can act as a vector for trypanosomiasis and some species seem inherently more efficient than others. These tsetse flies can become infected at any age with AAT causing trypanosomes and will remain infected for life. It has been suggested that the development of trypanosome infections in tsetse depends in equal measure on the genomes of tsetse and trypanosomes (Maudlin, *et al*., 1998) and that a range of biochemical and immunological processes play a crucial role in the establishment and maturation of the trypanosome in the tsetse fly (Aksoy *et al*., 2003). These properties confer an advantage to *Glossina morsitans morsitans* and *Glossina pallidipes* to be better vectors of *T. congolense* and *T. b. brucei*. The *T. congolense* in this study was a moderately virulent strain and as the results showed the establishment and development of the trypanosomes in the two groups of tsetse was not significant though the feeding duration was. The 28-48 hour old teneral flies fed on parasitaemic mice and the percentage development of the trypanosomes within them is indicative of the discrepancies observed by other scientists (Aksoy *et al*., 2003). A teneral fly has to get a blood meal soon after emerging to raise chances of survival. A delay in acquisition of a meal leads to fatigue and when the fly lands on an animal host, it is unable to probe and feed effectively (Secundino *et al*., 2012).

In the laboratory, tsetse flies are fed on trypanosome-infected donor animals in this case mice. During the process of feeding the failure of some flies to feed was a sign of poorly developed mouthparts and/or lack of energy (Okoth *et al*., 2006). The variation in the time taken by the tsetse to feed on the two groups of mice was indicative of the severity of anaemia in the infected mice. Flies that fed on AFB-1-injected-*T. congolense*-infected mice took relatively longer than those that fed on *T. congolense*-infected mice. This could be attributed to lower mean PCV value which had a bearing on the availability of blood in peripheral circulation or differences in feeding habits of individual flies. Tsetse flies naturally feed on the blood of numerous species of vertebrates and exhibit a remarkable range of host preferences and other behavioural characteristics. During feeding tsetse flies discharge saliva which contains powerful anticoagulant and vasodilatory properties and platelet aggregation inhibitors. The quantity of saliva secreted by tsetse appears to increase as they become hungrier and therefore expected the 28-48 hour teneral flies to feed voraciously. Additionally, this formed the basis of starving the tsetse flies for 40 hours before probing on the 24th day post-infection on *T. congolense* infected mice and before cyclical transmission of trypanosomes to uninfected mice. Female tsetse have a likelihood of 2.5 of infection more than male flies and this is attributed to the higher volume of blood they ingest (Njiru *et al*., 2004). However, Macleod *et al*. (2007) observed that male flies have a higher chance of developing mature midgut infections than females. The use of male *Glossina pallidipes* and a moderately virulent strain of trypanosome could have contributed to the low infection rates observed in this study. The presence of trypanosomes in the midgut and proboscis of dissected flies was not significantly different. Similarly the number of uninfected mice that became positive for *T. congolense* was not significantly different for the two groups of tsetse flies. This indicated that AFB-1 might not have a direct effect on the development of infection within the tsetse vector.

Glossina pallidipes and *Glossina morsitans morsitans* are efficient transmitters of AAT and as indicated above, the establishment and development of trypanosomes in the tsetse is determined by exogenous and endogenous factors (Masumu *et al*., 2006). However, the percentage infection in the tsetse obtained was way too low compared to those obtained by other researchers (Masumu *et al*., 2009). Their findings indicate that establishment and development of infection in the tsetse is a function of the virulence of the strain, the genome and the route of development of the trypanosome and the tsetse vector. Van den Abbeele *et al*. (1999) hypothesized that given the experimental flies fed on the same parasitaemic mice some trypanosome strains may be less susceptible to the elimination process in the tsetse's midgut immediately after the bloodmeal had been ingested. *T. congolense* is a high parasitaemic trypanosome to susceptible hosts and the subsequent high levels of host damage require higher transmission rates and thus increased parasite fitness for the strain to survive (Masumu *et al*., 2006a). And this explains the maintainance of the trypanosome in a natural vertebrate population. Studies done by other researchers indicate that to increase the infection rates of tsetse flies that feed on bloodmeal once, the sterile blood should be supplemented by components like N-acetyl-glucosamine (NAG) or L-glutathione (Peacock *et al*., 2012).

Environmental factors have been shown to affect survival of the tsetse and development of trypanosomes within the tsetse vector. Trypanosomiasis has a strong link to seasonal temperature periodicity (Macleod *et al*., 2007). This study showed a significant death in the tsetse fed on AFB-1-injected-*T. congolense*-infected mice and a higher mortality rate in the first few days post-infection of the tsetse. This is attributable to acclimatization of the tsetse to the experimental insectary conditions. Differentiation of trypanosomes from BSF to PF in the laboratory is dependent on temperature fall from 37°C to 26°C (Bass and Wang, 1991). To raise midgut infection chilling of tsetse at 0-5°C for 30 minutes post-infection is done. However, in nature tsetse

develop heat shock proteins or the cold shock which may have some effect on the trypanosome and hence potentiates transmission (Macleod *et al*., 2007). Principal environmental factors that influence tsetse population are host availability, climate and vegetation (Munang'andu *et al*., 2012).

5.0 CONCLUSION

The depressed mean weight of AFB-1-injected mice was an indication of inhibition of protein synthesis leading to muscle wasting and energy deficit. Therefore, it can be argued that AFB-1 would aggravate weight loss observed in *T. congolense* infections. Secondly, the lengthened time taken by tsetse to feed on AFB-1-injected mice could be associated with effect of AFB-1 on PCV of the mice. This would mean that the tsetse flies would need longer time to probe and feed on infected animals in the field. This in turn enhances disease transmission and incidence.

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FIGURES

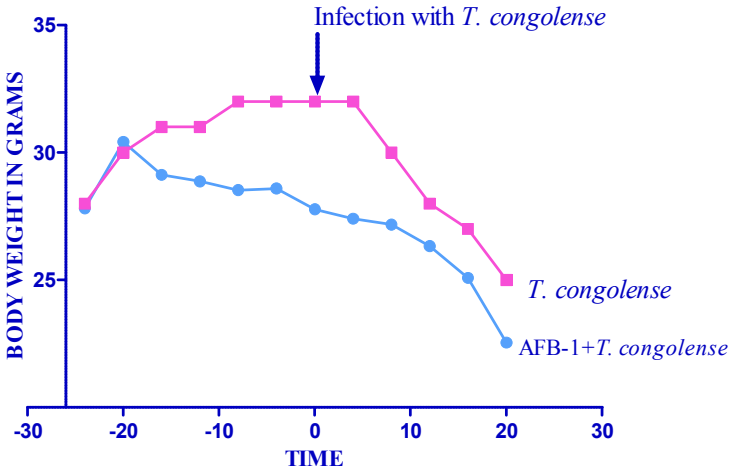

Figure 1: Changes in body weight over time in days of AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected mice.

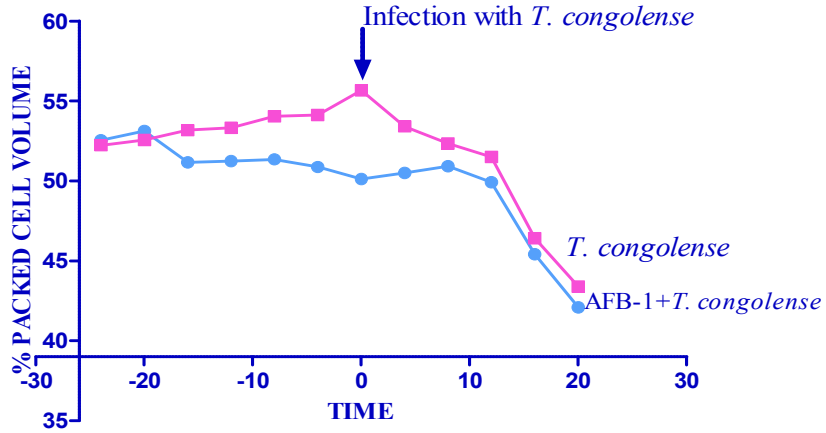

Figure 2: Variations in percentage packed cell volume against time in days of AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected mice.

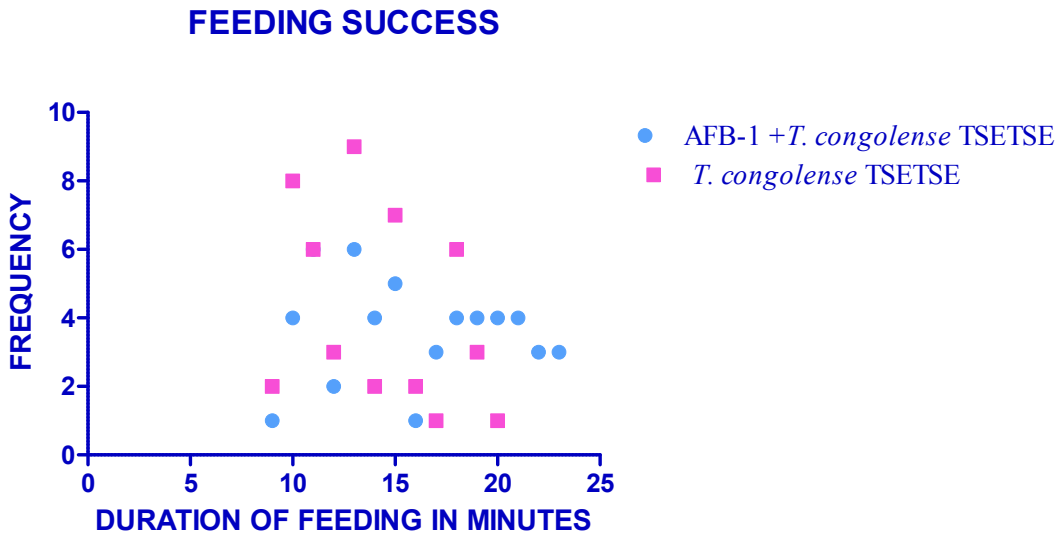

Figure 3: Time in minutes taken by teneral tsetse flies to feed on parasitaemic mice.

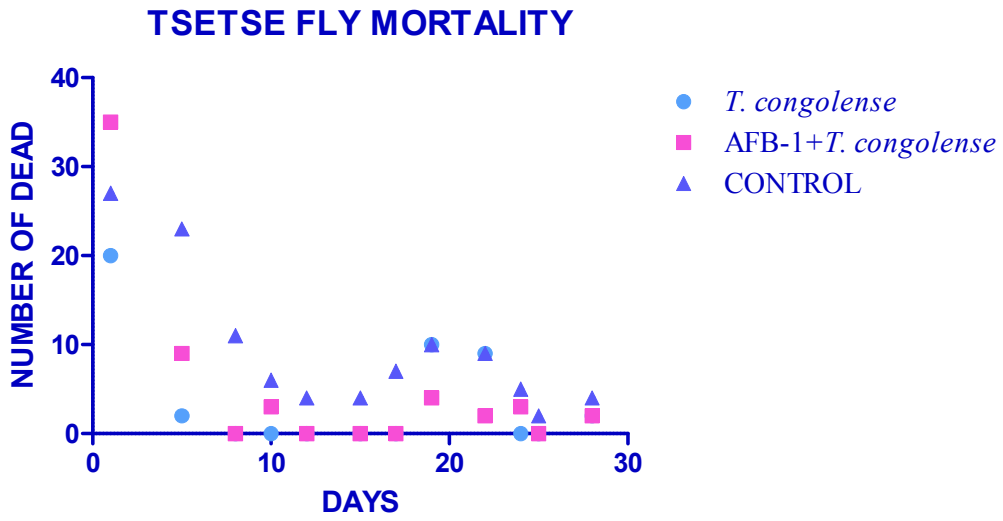

Figure 4: Tsetse fly deaths over time

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