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Infection With the Secondary Tsetse-Endosymbiont *Sodalis glossinidius* (Enterobacteriales: Enterobacteriaceae) Influences Parasitism in *Glossina pallidipes* (Diptera: Glossinidae)

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Subject Editor: Michael Strand

J. Insect Sci. 14(272): 2014; DOI: 10.1093/jisesa/ieu134

ABSTRACT. The establishment of infection with three *Trypanosoma spp* (Gruby) (Kinetoplastida: Trypanosomatidae), specifically *Trypanosoma brucei brucei* (Plimmer and Bradford), *T. b. rhodesiense* (Stephen and Fatham) and *T. congolense* (Broden) was evaluated in *Glossina pallidipes* (Austen) (Diptera: Glossinidae) that either harbored or were uninfected by the endosymbiont *Sodalis glossinidius* (Dale and Maudlin) (Enterobacteriales: Enterobacteriaceae). Temporal variation of co-infection with *T. b. rhodesiense* and *S. glossinidius* was also assessed. The results show that both *S. glossinidius* infection ($\chi^2 = 1.134$, df = 2, $P = 0.567$) and trypanosome infection rate ($\chi^2 = 1.85$, df = 2, $P = 0.397$) were comparable across the three infection groups. A significant association was observed between the presence of *S. glossinidius* and concurrent trypanosome infection with *T. b. rhodesiense* ($P = 0.0009$) and *T. congolense* ($P = 0.0074$) but not with *T. b. brucei* ($P = 0.5491$). The time-series experiment revealed a slight decrease in the incidence of *S. glossinidius* infection with increasing fly age, which may infer a fitness cost associated with *Sodalis* infection. The present findings contribute to research on the feasibility of *S. glossinidius*-based paratransgenic approaches in tsetse and trypanosomiasis control, in particular relating to *G. pallidipes* control.

Key Words: *Glossina*, *Sodalis glossinidius*, trypanosome, co-infection, vector competence

Glossina pallidipes (Austen) (Diptera: Glossinidae) is one of the most important tsetse fly vectors in Eastern Africa because of its widespread distribution (Ouma et al. 2011). This species has been implicated in the spread of Human African Trypanosomiasis and is a key vector for animal trypanosomes in this region (Ohaga et al. 2007, Malele et al. 2011, Peacock et al. 2012). Tsetse flies are considered to be naturally refractory to trypanosome infection and only a few of the trypanosomes introduced into a fly via an infective feed are able to overcome the immune system response and thus establish an infection (Welburn and Maudlin 1999). The establishment and maturation of trypanosomes in the tsetse gut is dependent on many variables and involve complex interactions between the fly, endosymbionts, and the parasite itself (Welburn and Maudlin 1999). Tsetse flies harbor at least three gut endosymbionts namely; the obligate *Wigglesworthia glossinidae*, the facultative *Sodalis glossinidius* and the ricketssia-like *Wolbachia pipientis* (Cheng and Aksoy 1999). *Wigglesworthia* and *S. glossinidius* are transmitted through maternal milk gland secretions to the intra-uterine developing larva, whereas *Wolbachia* is transmitted transovarially. Symbionts are therefore present at eclosion in the teneral fly, whereas trypanosome infection is acquired mainly at the first feed in the presence of an infected blood meal source. In various arthropods, gut microbiota have been shown to increase insect immunity to pathogens such as viruses and parasites (Teixeira et al. 2008, Moreira et al. 2009, Koch and Schmid-Hempel 2011). In the adult tsetse fly, responses to parasite infection are indirectly modulated by symbionts (Weiss et al. 2013). The possible influence of secondary symbionts on tsetse vectorial capacity has been investigated primarily using homogeneous laboratory populations of *Glossina morsitans morsitans* (Rio et al. 2006) and *Glossina palpalis gambiensis* (Geiger et al. 2007), whereby all individuals are *S. glossinidius* infected. This study utilized by using a naturally heterogeneous population of *G. pallidipes* to establish the correlation between *S. glossinidius* infection in *G. pallidipes* and experimental infection with *T. b. brucei*, *T. b. rhodesiense*, and *T. congolense.* The temporal variation of *T. b. rhodesiense* and *S. glossinidius* co-infection was also investigated.

Materials and Methods

Infection of Tsetse Flies. Male teneral *G. pallidipes* of age 0–2-d-old from the Trypanosomiasis Research Centre (TRC) colony were used. Details of the three trypanosome isolates used for the fly infection are presented in Table 1. The stabilates were expanded in two donor Swiss white mice that had been immune-suppressed with cyclophosphamide at a dose of 300 mg/kg body weight. Disease progression in the mice was monitored by collection and microscopic examination of blood obtained through tail snipes on alternate days. At the peak of parasitemia, the mice were euthanized using concentrated carbon dioxide. Blood from the heart was then collected by cardiac puncture into a tube containing ethylene diamine tetra acetic acid (EDTA). The level of parasitemia was estimated using the matching method (Herbert and Lumsden 1976) and subsequently, an inoculum dose of 1×10^6 trypanosomes/ml was prepared in phosphate saline glucose pH 8.0. Two milliliters of this inoculum used to infect 12 recipient mice. At peak parasitemia, teneral flies in 4″ diameter cages were allowed to feed on the belly of the infected mice. Feeding success was confirmed by visual observation of engorged fly abdomens. Flies that did not feed were excluded from the experiment. After 10–15 min, feeding was interrupted and the engorged flies transferred to the insectary which is maintained at a temperature of 24 ± 1°C and 70 ± 5% relative humidity. These flies were fed on defibrinated bovine blood on alternate days using the in vitro feeding system (Feldmann 1994).

Experimental Design. Experimental flies were assigned to four infection groups: (1) *T. b. rhodesiense* infection time series experiment (TBR^{ts}), (2) *T. b. rhodesiense* infection (TBR^{35}), (3) *T. b. brucei* infection (TBB), and (4) *T. congolense* (TC) infection groups were constituted as detailed in Table 2. The assays for groups 2–4 were conducted after completion of the respective trypanosome maturation period.

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Table 1. Trypanosome isolates used to infect teneral *G. pallidipes*

Parasite	*T. b. rhodesiense*	*T. b. brucei*	*T. congolense*
Isolate code	KETRI2537	KETRI3386	EATRO993
Host	Human	*G. pallidipes*	*G. pallidipes*
Origin, year of isolation	Busoga, 1972	Kibwezi, 1979	South Nyanza, 1962

Table 2. Details of the experimental groups used

Group no.	Experimental group	*N*	Parasite	Dissection performed at dpi
1	TBR^{ts}	100 (25, 22, 26, 27)	*T. b. rhodesiense*	7, 14, 21, 28
2	TBR^{35}	18	*T. b. rhodesiense*	35
3	TBB	77	*T. b. brucei*	40
4	TC	98	*T. congolense*	30

TBR, *T. b. rhodesiense*; TBB, *T. b. brucei*; TC, *T. congolense*; dpi, days post-infection; TBR^{ts}, TBR infection time series experiment; TBR^{35}, TBR infection with dissection at 35 dpi; numbers of *n* in brackets represent number of flies dissected the different time points of 7, 14, 21, and 28 dpi for the group TBR^{ts}.

Dissections and DNA Extraction. Dissections were performed on a microscope slide using phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). Following the method of Lloyd et al. (1924), the mouthparts, gut, and the salivary glands (in the *brucei* infection groups only) were isolated and examined microscopically. Subsequently, individual midguts from the dissected tsetse flies were placed in a 1.5-ml microfuge tube. Total genomic DNA was isolated from the midgut samples using the DNeasy Blood and Tissue Kit (Qiagen Sciences, Gaithersburg, MD, USA), with a minor modification to the manufacturer's instructions being that the final elution step was performed with 50 µl instead of 100 µl of elution buffer.

PCR Detection of Trypanosome and *S. glossinidius* Infections. Midgut trypanosome infection in the *T. congolense* treatment group was determined using the primers specific for *T. congolense* savannah previously described by Masiga et al. (1992) while infections in the *T. b. brucei* infection groups were determined using *T. b. rhodesiense* specific primers (TBR) 1 and TBR2 (Moser et al. 1989). The presence of *S. glossinidius* in the gut tissues was determined using the primers *GPO1*F/R which amplify a 1.2-kb product of the extra-chromosomal plasmid (Dale and Maudlin 1999). The 20 -µl final PCR reaction contained 2 µl of 10× PCR reaction buffer, 2.5 mM $MgCl_2$, 0.5 mM dNTPs, 500 nM of each primer, and 0.3 µl of GoTaq Flexi DNA polymerase 5 units/µl (Promega, Madison, WI, USA). For each PCR run, a negative control (water) and the respective positive controls were included. After completion of the PCR run, 10 µl of the amplification products was analyzed by electrophoresis in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) on a 1.5% agarose gel together with a 100-bp DNA ladder size standard (Invitrogen, Carlsbad, CA, USA) and visualized using ethidium bromide staining.

Statistical Analysis. Fisher's exact test was used for analysis of categorical data using the online program GraphPad found at http://www.graphpad.com/quickcalcs/contingency1.cfm.

Results

***S. glossinidius* Infection.** *S. glossinidius* was detected in 38.9, 36.4, and 32.7% of the TBR^{35}, *T. b. brucei*, and *T. congolense* group flies, respectively. There was no significant difference in *S. glossinidius* prevalence among these three groups ($\chi^2 = 1.134$, df = 2, $P = 0.567$). However, *S. glossinidius* infection prevalence in the TBR^{ts} was higher and ranged between 65 and 86% depending on the period after the infective blood meal, with an average infection rate of 76.5 ± 8.9%.

Trypanosome Infection. Dissections of TBR^{ts} group flies detected three midgut infections, two of which were identified at 7-d post-infection (dpi) and one at 14 dpi (Table 3). No parasites were observed microscopically in the TBR^{35} treatment group. In the *T. b. brucei* group, 7.8% ($n = 77$) of the dissected flies were infected with trypanosomes in the mouthparts, the midgut, or both. No salivary gland infections were observed, indicating the absence of mature infections. In total, 13.3% ($n = 98$) of *T. congolense* experimental group flies dissected harbored trypanosomes. In the latter group, out of 13 infected flies, 12 had parasites in both mouthparts and the midgut, whereas only 1 fly had an immature infection with no trypanosomes found in the midgut. PCR analysis detected 74% trypanosome infection rate in the TBR^{ts} group, but only 50% infections at 35 dpi (TBR^{35} group). Trypanosome infection was detected in 52 and 61% of the *T. b. brucei* and *T. congolense* treatment groups, respectively. Comparing infection rates at trypanosome maturity, these were not significantly different between the TBR^{35}, TBB, and TC infection groups as determined by both dissection ($\chi^2 = 3.62$, df = 2, $P = 0.163$) and PCR methods ($\chi^2 = 1.85$, df = 2, $P = 0.397$).

S. glossinidius and Trypanosome Co-infection

T. b. rhodesiense Infection Group. The temporal variation of parasite and *Sodalis* infections in the time-series TBR^{ts} experiment is detailed in Table 4. An apparent decrease in the prevalence of *S. glossinidius* with increasing number of dpi and hence with the age of the assayed flies ($r = -0.56$) was noted. This may infer a negative fitness cost associated with *Sodalis* infection, whereby infected flies have reduced longevity.

In the *T. b. rhodesiense*^{ts} group, 76% of flies were infected with the symbiont and out of these, 82.9% had established the trypanosome infection by 28 dpi. In contrast, only 45.8% of those flies lacking the symbiont (S^-T^+) were able to establish trypanosomes by this time point (Table 5). Overall, in this group there was a highly significant association between infection with *S. glossinidius* and *T. b. rhodesiense* infection ($P = 0.0009$, Fisher's exact test). Analysis of temporal infection reveals that at 7, 14, 21, and 28 dpi, the proportion of *S. glossinidius*-positive flies that were infected by trypanosome parasites (S^+T^+) was constantly higher (76.5 ± 8.9%) than those in which the parasite did not establish (S^+T^-) (17.9 ± 6.3%). Infection prevalence in the TBR^{35} group was 38.9% for *S. glossinidius* and 50% for trypanosomes. In this group as well, this association was statistically significant ($P = 0.0023$).

T. b. brucei and T. congolense Infection Groups. In *S. glossinidius*-infected flies, an infection rate of 61.5 and 74.3% was detected with *T. b. brucei* and *T. congolense*, respectively. In comparison, with *S. glossinidius*-negative flies, infection rates of 50 and 53.1% were detected with *T. b. brucei* and *T. congolense*, respectively. Fisher's exact test revealed that the association between the presence of *S. glossinidius* and concurrent trypanosome infection was statistically significant in *T. b. rhodesiense* and *T. congolense* but not in *T. b. brucei* (Table 5).

Discussion

This study provides a significant insight into the contribution of endosymbiont *S. glossinidius* to the outcome of exposing tsetse flies to trypanosome-infected mice. Tsetse flies reproduce by adenotrophic viviparity, whereby the developing larva is nourished in utero by secretions from the milk gland. It is through these secretions that *S. glossinidius* is transferred from the mother to offspring (Balmand et al. 2013). *S. glossinidius* is therefore present at eclosion of the teneral fly, whereas trypanosomes are ingested by the fly at its first and/or subsequent infective feeds (Welburn and Maudlin 1992). Previous researchers have fed flies with various antibiotics to eliminate gut endosymbionts before performing similar comparative experiments (Weiss et al. 2013). However, antibiotic treatment has been shown to have negative effects on fly

Table 3. Trypanosome infections in *G. pallidipes*

Parameter	*T. b. rhodesiense*^{ts}	*T. b. rhodesiense*^{35}	*T. b. brucei*	*T. congolense*
Dissection	3/100 (3)	0/18 (0)	6/77 (7.8)	13/98 (13.3)
Mature infections[a]	0/3 (0)	0/0 (0)	0/6 (0)	12/13 (92.3)
Trypanosome infection (PCR)	74/100 (76)	9/18 (50)	40/77 (51.9)	60/98 (61.2)

[a]Number of mature trypanosome infections out of total infections; Percentages are in brackets; *T. b. rhodesiense*^{ts} TBR infection time series experiment; *T. b. rhodesiense*^{35} TBR infection with dissection at 35 dpi.

Table 4. Temporal variation of *T. b. rhodesiense* and *Sodalis* infections in *G. pallidipes* midgut

Dpi	*n*	S+	T+	S+T−	S−T−	S+T+	S−T+	*P*-value
7	25	20 (80.0)	15 (60.0)	6 (24.0) [30]	4 (16.0)	14 (56.0) [70.0]	1 (4.0)	0.1206
14	22	19 (86.4)	16 (72.7)	4 (18.2) [21.1]	2 (9.1)	15 (68.2) [78.9]	1 (4.5)	0.1688
21	26	17 (65.4)	18 (69.2)	3 (11.5) [17.6]	5 (19.2)	14 (53.8) [82.4]	4 (15.4)	0.0781
28	27	20 (74.1)	25 (96.6)	0 (0) [0]	2 (7.4)	20 (74.1) [100]	5 (18.5)	0.0598
Total	100	76 (76)	74 (74)	13 (13) [17.1]	13 (13)	63 (63) [82.9]	11 (11)	0.0009*

dpi, days post infection; *n*, number of flies tested; S+, total number of flies harboring symbiont; T+, total number of trypanosomes infected flies; S+T+, flies with both *Sodalis* and trypanosome infection; S+T−, *Sodalis*-infected without parasite; S−T+, parasite infected flies lacking *Sodalis* ; S−T−, flies with neither symbiont nor parasite. % prevalence indicated in brackets calculated with reference to total flies at the specific time period after infection. Values in square brackets represent parasite prevalence calculated with reference to the corresponding number of *Sodalis*-infected flies (s+). *P*-value, Fisher's exact test for association between *Sodalis* and trypanosome infection. *Statistically significant $P < 0.01$.

Table 5. *Sodalis* and parasite co-infection in experimentally infected *G. pallidipes*

	TBR^{ts} ($n = 100$)		TBR^{35} ($n = 18$)		TBB ($n = 77$)		TC ($n = 98$)	
	T+	T−	T+	T−	T+	T−	T+	T−
S+	63 (63%) **[82.9]**	13 (13%)	0 (0%) **[0]**	7 (38.9%)	8 (10.4) **[61.5]**	5 (6.5)	26 (26.5%) **[81.3]**	6 (6.1%)
S−	11 (11%)	13 (13%)	9 (50%)	2 (11.1%)	32 (41.6)	32 (41.6)	34 (34.7%)	32 (32.7%)
P-value	$P = 0.0009$		$P = 0.0023$		$P = 0.5491$ (NS)		$P = 0.0074$	

TBR^{ts}, TBR infection time series experiment; TBR^{35}, TBR infection with dissection at 35 dpi; TBB, *T. b. brucei*; TC, *T. congolense*; S+/−, *Sodalis* positive/negative; T+/−, trypanosome positive/negative; NS, not significant Fisher's exact test. Bold values in square brackets indicates % of *Sodalis*-positive flies that were also parasite-positive.

fecundity and longevity (Alam et al. 2011) and may ultimately have some effect on the development of trypanosome infection. In this study, we had access to a laboratory population of *G. pallidipes* that was naturally heterogeneous with respect to *S. glossinidius* infection, thereby eliminating the need for antibiotic treatment.

The *S. glossinidius* prevalence of the time series group (TBR^{ts}) was 76%, whereas for the TBR^{35}, TBB, and TC groups, it was ∼35%. This variation could be because of the fact that while flies in the last three groups were assayed when they were the same age, the time-series group was actually a composite group composed of four distinct groups that were assayed sequentially at different ages. In this study, we noted a negative correlation between *S. glossinidius* prevalence and the age of the specific group. This may have contributed to the much higher prevalence detected in the relatively younger composite group. It has been shown that although the relative density of *S. glossinidius* in individual flies may vary with age, the infection is permanent and is not lost in the tsetse's lifetime (Maudlin 1991, Rio et al. 2006). We therefore surmise that this apparent temporal decrease in prevalence is not due to reduced density or total symbiont loss, but is a complete absence of the bacteria. This result introduces the hypothesis that *S. glossinidius* infection may affect tsetse longevity. This phenomenon has been reported in other arthropods such as the pea aphid whereby the secondary symbionts *Hamiltonella*, *Regiella*, and *Spiroplasma* have caused negative effects on host longevity and fecundity (Maudlin 1991). A similar scenario in tsetse flies would add a new angle to the proposed use of *S. glossinidius*-based paratransgenic approaches in tsetse fly control. We intend to conduct further research to verify the effects, if any, of *S. glossinidius* infection on longevity of *G. pallidipes*.

In our study, a significant association between the presence of *S. glossinidius* and concurrent trypanosome infection was noted in *T. b. rhodesiense* and *T. congolense* but not in *T. b. brucei*. The findings related to the last parasite may be considered to be anomalous, given that the synergistic effect of *S. glossinidius* on trypanosome establishment and maturation is hypothesized to apply to trypanosome species that pass through a midgut stage in the fly including *T. congolense, T. b. brucei*, and *Trypanosoma simiae* but excluding *Trypanosoma vivax* (Welburn et al. 1993). The results we obtained agree with previously reported findings which postulate that *S. glossinidius* infection decreases the susceptibility of wild tsetse to infection with various trypanosomes (Farikou et al. 2010). However, the findings deviate from other studies which found no correlation between *S. glossinidius* infection and the ability of *G. p. gambiensis* to acquire *T. congolense* (Geiger et al. 2005). Studies using natural populations often reach contradictory conclusions, mainly because of the highly variable levels of *S. glossinidius* infection in wild flies. This prevalence varies depending on species and even populations, from apparently absent in *Glossina fuscipes fuscipes* (Lindh and Lehane 2011, Alam et al. 2012) to more than 50% in *Glossina palpalis palpalis* (Farikou et al. 2010). In the latter, *S. glossinidius* was detected in ∼55% of flies analyzed, and 59% of these were coinfected with various trypanosomes, primarily *T. congolense* and *T. b. brucei* sub-species (Farikou et al. 2010). Although a strong corelation was shown in *G. p. palpalis*, no corelation was noted between infection

with *S. glossinidius* and trypanosome establishment in Kenyan *G. austeni* and *G. pallidipes* with less than 2% of ~600 samples analyzed harboring both *S. glossinidius* and trypanosomes (Wamwiri et al. 2013). It is however evident that flies without *S. glossinidius* infection are also capable of developing trypanosome infections (Alam et al. 2012) as well. These divergent conclusions highlight the considerable influence of vector–trypanosome species pairings on the success of infection establishment (Moloo et al. 1992).

This study reinforces the current opinion that concurrent *S. glossinidius* infection increases susceptibility to trypanosome infection; however, the extent of this effect is depends on the fly species and parasite involved. We also postulate that *S. glossinidius* infection may have a negative effect on longevity in *G. pallidipes*, which could have important implications for the application of *S. glossinidius*-based tsetse control interventions. However, a greater understanding of the interplay between the effects of *S. glossinidius* infection on fly survival and trypanosome-susceptibility is required.

Acknowledgments

The authors thank members of the IAEA-CRP on Tsetse Symbiosis for valuable discussions on this subject and Patrick Abila for useful comments on the manuscript. The technical assistance of Purity Gitonga, James Murage, Patrick Obore and George Kimotho is gratefully acknowledged. This work was supported by the International Atomic Energy Agency (IAEA) Coordinated Research Project "Improving SIT for Tsetse Flies through Research on their Symbionts and Pathogens" (Contract No.14134).

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Received 14 October 2013; accepted 26 March 2014.