Parasitology International 70 (2019) 64–69

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Short Communication

Endosymbionts interaction with trypanosomes in Palpalis group of *Glossina* captured in southwest Nigeria

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ARTICLE INFO

Keywords:
Glossina
Endosymbionts
Trypanosomes
Nigeria

ABSTRACT

Glossina species epidemiological studies were conducted in "fly-belt" endemic zone of southwest Nigeria. Two major study areas were identified and four Nzi traps were set in each site for tsetse collection. This study was conducted to determine the prevalence of endosymbionts (*Wigglesworthia glossinidia*, *Sodalis glossinidius* and *Wolbachia*) in natural field-trapped populations of *G. p. palpalis* and *G. tachinoides* and investigate the corresponding interactions with African trypanosomes. A total of 64 tsetse flies were collected, these included *G. p. palpalis* ($n = 28$) and *G. tachinoides* ($n = 36$). Trypanosome infection and endosymbionts of these flies were determined using polymerase chain reaction (PCR) amplification. The infection rates of *W. glossinidia* was 100.0% in both species, no flies were positive for *Wolbachia*. *Sodalis glossinidius* prevalence was similar between the two-tsetse species, with *G. p. palpalis* and *G. tachinoides* showing prevalence of 35.7% (95%CI: 20.7–54.2) and 27.8% (95%CI: 15.9–44.0) respectively. No relationship was found between the endosymbionts and trypanosomes in trapped tsetse flies. More studies are needed to enhance the potential control interventions mediated by endosymbionts to reduce parasitic infections.

1. Introduction

Maternally-inherited endosymbiotic bacteria present in tsetse flies have been shown to be involved in interactions which affect the establishment and maturation of trypanosome infections in tsetse [1,2]. Tsetse flies traditionally have been thought to harbor three endosymbionts. These include the obligate or primary (P)-symbiont *Wigglesworthia glossinidia* is present in all tsetse flies and resides both in milk gland secretions and bacteriome [3,4], and is involved in the provision of B vitamins absent in tsetse bloodmeal [5]. *Wolbachia* are generally found in the reproductive organs of tsetse flies and are thought to be involved in cytoplasmic incompatibility [6], while secondary (*S*)-symbiont *Sodalis glossinidius* is thought to be involved in the susceptibility of tsetse flies to trypanosomes [1,7–9]. *S. glossinidius* has been observed in variety of tissues including midgut, fat body, milk gland, salivary glands and haemocoel [10]. There are complexities between endosymbiont and trypanosome interactions. Flies positive of *S. glossinidius* have been reported to show six times likelihood of becoming infected by *T. b. rhodesiense* in male *G. pallidipes*, while *T. b. brucei* showed no correlation with presence of endosymbiont in same study [11]. Hence, other intrinsic factors of tsetse populations found in different regions could be responsible of several variations in the endosymbiotic prevalence [12].

Wolbachia and *S. glossinidius* have been observed to show an infection rate of 100% in laboratory-reared tsetse [13–15], however, their prevalence in wild tsetse populations varies [16]. For example, *Wolbachia* heterogenous natural infections ranged from 0 to 100% in *G. austeni* and *G. brevipalpis* while *G. m. morsitans* were observed between 9.5 and 100% [15]. There have been several studies on identifying trypanosome species in tsetse flies using microscopy [17], and PCR assay [18] in Nigeria. The latter has been useful in identifying diverse trypanosome DNA species and tsetse with mixed infections, although, it may not differentiate between active infection and the passive presence of trypanosomes due to recent bloodmeals [19]. *S. glossinidius* positive flies have demonstrated significantly increased trypanosome infection rates in trapped *G. pallidipes* [20] and *G. palpalis* [21] in Kenya and Cameroon, respectively. However, Dennis et al. [16] showed no significant relationship between *Sodalis glossinidius* and trypanosome

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https://doi.org/10.1016/j.parint.2019.01.011
Received 24 November 2018; Received in revised form 23 January 2019; Accepted 31 January 2019
Available online 01 February 2019
1383-5769/

infections in *G. pallidipes*, *G. brevipalpis* and *G. m. morsitans* trapped in Zambia. Recently, another endosymbiont reported was *Spiroplasma* [22]. The understanding of the prevalent endosymbionts could help to ascertain the susceptibility and current status of the *Glossina* species present. Besides, there are current suggestions in using endosymbionts as elimination strategy against fly vector populations [23,24]. This could involve the use of transgenesis and paratransgenesis aimed at reducing tsetse vectorial capacity or eliminating the transmission of trypanosomes. In Nigeria however, no published study has attempted to examine the endosymbionts present in *Glossina* species and their associated interactions with trypanosomes.

2. Materials and methods

2.1. Fly survey

The trapping of the tsetse in this study was done between March and July 2016. Briefly Nzi traps were used and four traps at 500 m apart were deployed at each location (Idiroko and Adebayo) for five days/month (total days trapped at each location equals 25 days at each location). Idiroko study site is at the border between Nigeria and Benin Republic with dense forest and rivers present (Fig. 1). The coordinates of Idiroko traps were recorded (TID1: Longitude 2°90′67E and Latitude 7°60′63 N, TID2: Longitude 3°86′43E and Latitude 7°59′92 N, TID3: Longitude 3°90′13E and Latitude 7°60′12 N, TID4: Longitude 3°91′24E and Latitude 7°59′43 N). Adebayo is between Oyo and Ogun states and it is surrounded by primary forest and dense vegetation. Trap locations of Adebayo coordinates were reported (TAD1: Longitude 3°75′11E and Latitude 6°64′18 N, TAD2: Longitude 2°75′73E and Latitude 6°64′03 N, TAD3: Longitude 2°74′82E and Latitude 6°64′53 N, TAD4: Longitude 2°74′83E and Latitude 6°64′03 N). Tsetse were collected every 12 h and identified (species and sex) using FAO keys under the stereomicroscope [25]. The keys include the use of inferior and superior claspers of the genitalia, the width and length of the dorsal plates and coloration of the stermite.

2.2. PCR assay for endosymbionts and trypanosomes

Flies were washed with 5% sodium hypochlorite and phosphate buffered saline (Sigma-Aldrich, Germany) to remove surface

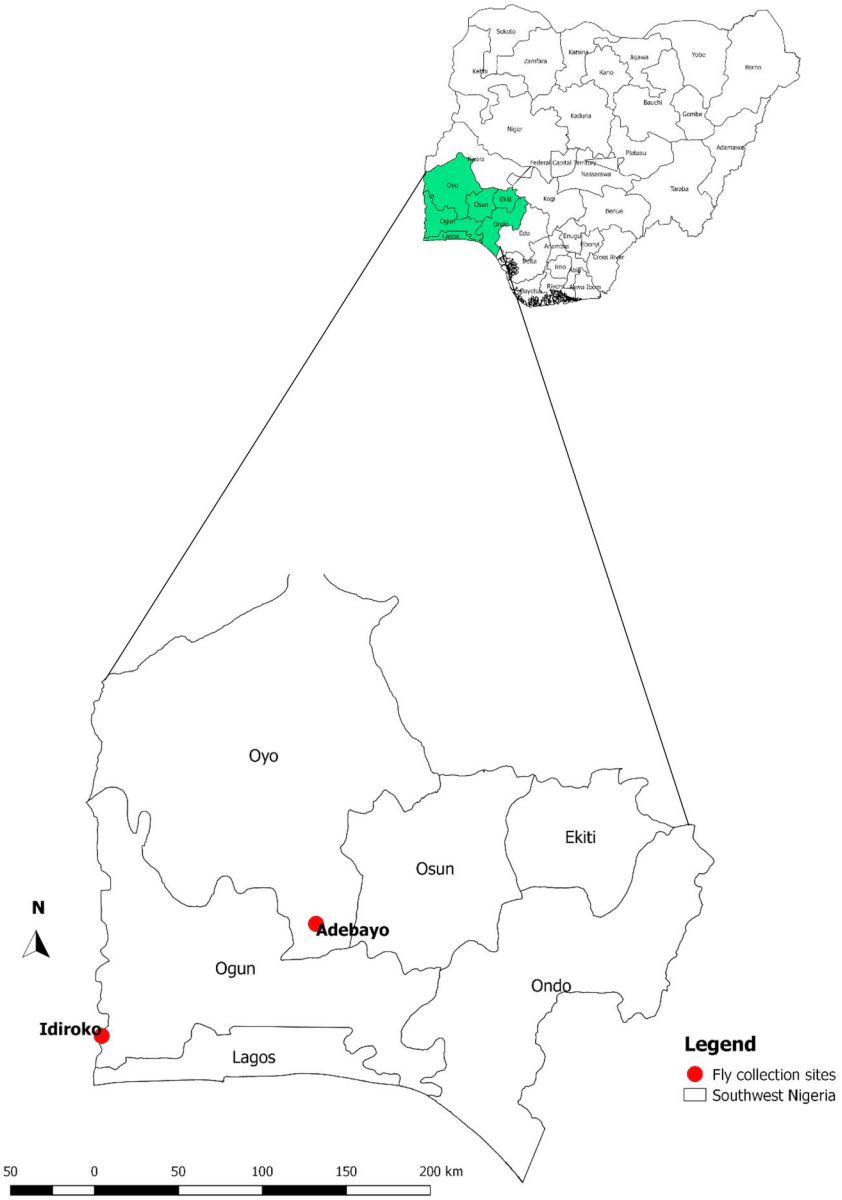

Fig. 1. Study sites for tsetse collections.

contaminants. Flies were cut into two parts namely, mouth part (MP) and thorax + abdomen (TA). Only the TA samples were used for endosymbiont assessment, while both parts (MP for *T. vivax*, TA for other *Trypanosoma* spp.) were examined for trypanosomes. Fly DNA was extracted separately using DNeasy tissue kit (QIAGEN, Germany) following manufacturer's instruction, while 50 µl elution buffer was used to elute the DNA.

All fly DNA extracts were first screened for *W. glossinidia*, the primary endosymbiont found in all tsetse flies. Primers were used to amplify a 129 bp fragment of the flagellin (FliC) gene of this endosymbiont as described by Soumana et al. [26] with the exception of using 1 U of BioTaq DNA polymerase (Bioline, UK). Standard PCR reactions for *W. glossinidia* were done in 25 µl volumes containing 0.2 µl of 5 × Mango Taq buffer, 1 µl of 50 mM $MgCl_2$, 0.3 µl of both primers, 0.2 mM of each dNTP (Rovalab, Germany), primers at a concentration of 0.1 µM and 1Unit of BioTaq DNA polymerase (Bioline, UK). Cycling conditions consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 40 s, 58 °C for 40 s, and 72 °C for 90 s with final extension at 72 °C for 5 min. *Wigglesworthia* DNA extracted from *G. palpalis* previously from West Africa in the laboratory was used as positive control.

Flies positive for *W. glossinidia* DNA were screened for *S. glossinidius* using primers that amplify a 95 bp fragment of the GroEL gene [27] as previously described by Dennis et al. [16]. Reaction volume was 25 µl and contained 1 µl of eluted DNA, 0.2 µl of 5 × Mango Taq buffer, 1 µl of 50 mM $MgCl_2$, 0.2 µl of 25 mM of each dNTP (Rovalab, Germany), 5 µl of 10 pmol/µl of forward and reverse primers, 0.2 µl of 1Unit of BioTaq DNA polymerase (Bioline, UK) and 12.6 µl of double distilled water. Cycling conditions were: initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 40 s, 58 °C for 40 s and 72 °C for 90 s. A final extension step was carried out at 72 °C for 5 min. *S. glossinidius* DNA extracted from *G. m. morsitans* previously from Zambia in the laboratory was used as positive control.

To screen for *Wolbachia* a 500 bp fragment of the *Wolbachia* surface protein (wsp) gene was amplified as described by Cheng et al. [28]. Reaction was carried out in 25 µl volumes containing 5 × Mango Taq buffer, 50 mM $MgCl_2$, 0.1 µM of both primers, 0.2 mM of each dNTP (Rovalab, Germany), primers at a concentration of 0.5 µM and 1Unit of BioTaq DNA polymerase (Bioline, UK) (Table 1). Cycling included an initial denaturation step at 94 °C for 5 min followed by 35 cycles of 94 °C for 40 s, 58 °C for 40 s, 72 °C for 90 s, and final extension at 72 °C for 5 min. *Wolbachia* DNA extracted from *G. austeni* from Kenya previously in the laboratory was used as positive control. For all the endosymbionts, a negative control (distilled water) was used to check for contamination.

Identification of trypanosome DNA was done with ITS PCR of Njiru et al. [29]. The protocol detects wide range of *Trypanosoma* species between 250 and 700 bp. DNA from *T. congolense* savannah from Ugandan cattle was used as positive control while tsetse larvae were used as negative control. All the experiments were done twice to confirm the results. PCR products were separated in 1.5% agarose containing GelRed™ nucleic acid stain (Biotium Inc., USA). Separated DNA products were viewed under ultraviolet light in a transilluminator (BIO-RAD), and band sizes were compared with exACTGene low range DNA ladder (Fisher Scientific International Inc.).

2.3. Ethical statement

The study was conducted with the approval of the University of Ibadan Animal Ethics Committee (UI-ACUREC/App/12/2016/05) in line with the guidelines of the committee.

2.4. Statistical analysis

Descriptive and inferential data analysis of fly distribution, trypanosomes and *S. glossinidius* abundance in tsetse flies were analysed with WINIPEPI statistic package. Fisher's exact test was used to analyse relationship between *S. glossinidius* and trypanosomes that inhabit the midgut (*T. brucei*, *T. congolense*, *T. simiae*), *T. vivax* was excluded as it completes its full lifecycle within the mouthparts of tsetse.

3. Results

3.1. Fly abundance

The relative apparent densities of tsetse flies trapped within the study period were 0.14 and 0.18/fly/trap/day for *G. palpalis* and *G. tachinoides*, respectively. More flies were trapped in Adebayo 53.1% (95%CI: 41.1–64.8) compared to Idiroko 46.9% (95%CI: 35.2–58.9), with no significant difference ($P > .05$) in abundance. In terms of sex, females 60.9% (95%CI: 48.7–71.9) were mostly trapped compared to males 39.1% (95%CI: 28.1–51.3).

3.2. Endosymbionts in tsetse flies

In total 64 tsetse flies examined for endosymbionts, all were positive for *Wigglesworthia glossinidia* (100.0%), however, none were positive for *Wolbachia*. Table 1 shows the results of molecular analysis for *S. glossinidius* and trypanosomes. In total 31.3% (95%CI: 21.2–43.4) were positive of *S. glossinidius*. The prevalence values in *G. p. palpalis* ($n = 28$) and *G. tachinoides* ($n = 36$) were 35.7% (95%CI: 20.7–54.2) and 27.8% (95%CI: 15.9–44.0) respectively, with no significant difference in infection rate. Although prevalence in females was higher in both tsetse species (*G. p. palpalis*, female = 41.2% (95%CI: 18.4–67.1), male = 27.3% (95%CI: 6.0–61.0), *G. tachinoides*, female = 31.8% (95%CI: 13.9–54.9, male = 21.4% (95%CI: 4.7–50.8) the difference was not significant.

In terms of location, the prevalence of *S. glossinidius* present in *Glossina* spp. trapped in Idiroko study site (40.0%, 95%CI: 22.7–59.4) was higher than Adebayo (23.5%, 10.8–41.2) but not significantly ($P = .156$). Prevalence of *S. glossinidius* found in *G. p. palpalis* showed 40.0% (95%CI: 16.3–67.7) and 30.8% (95%CI: 12.9–57.6), respectively for Idiroko and Adebayo, while *S. glossinidius* present in *G. tachinoides* according study sites revealed 40.0% (95%CI: 16.3–67.7) and 19.1% (95%CI: 7.7–40.0), for Idiroko and Adebayo, respectively.

3.3. Trypanosomes in tsetse flies

Of the 64-tsetse examined, 51.6% (95%CI: 39.6–63.4%) were positive of trypanosomes. The prevalence of trypanosomes identified from the *Glossina* species are shown in Table 1. *Trypanosoma vivax* was most prevalent (29.7% (95%CI: 18.9–42.4)) in both *G. p. palpalis* and *G. tachinoides* followed by *T. brucei* 17.2% (95%CI: 8.9–28.7). *T. congolense* DNA 4.7 (95%CI: 1.0–13.1) was only detected in *G. p. palpalis* and *T. simae* 3.1 (95%CI: 0.4–10.8) in *G. tachinoides*. There were dual infections of *T. vivax* and *T. brucei* at 3.1% (95%CI: 0.4–10.8) prevalence that reside in the midgut. Trypanosome prevalence was higher in *G. p. palpalis* 67.9% (95%CI: 47.7–84.1) compared to *G. tachinoides* 38.9% (95%CI: 23.1–56.5). Prevalence based on sex, species and study sites have been reported (Table 1).

3.4. Endosymbiont-trypanosome interaction

There was no significant interaction between the presence of *S. glossinidius* and trypanosomes that inhabit the midgut in either *G. p. palpalis* ($P = 1.00$) or *G. tachinoides* ($P = .370$).

4. Discussion

Two *Glossina* species of Palpalis group were captured in the study sites which are *G. tachinoides* and *G. p. palpalis*. The vegetational changes due to human activities in this rainforest habitat could provide

Table 1
Percentage detection (and 95% confidence intervals) of detection of *S. glossinidius* and trypanosomes in *G. palpalis* ($n = 28$) and *G. tachinoides* ($n = 36$) in southwest Nigeria. Midgut trypanosomes includes *T. brucei*, *T. congolense* and *T. simiae*. There were no double infections of these parasites.

Tsetse species	Prevalence (%) and confidence intervals (95%CI)					
	S. glossinidius	*T. brucei*	*T. congolense*	*T. simiae*	Midgut trypanosomes	*T. vivax*
G. palpalis	35.7 (20.7–54.2)	21.4 (10.2–39.5)	10.7 (3.7–27.2)	0.0	32.1 (17.9–50.7)	39.3 (23.6–57.6)
Sex						
Female ($n = 17$)	41.2 (21.6–64.0)	23.5 (9.6–47.3)	0.0	0.0	23.5 (9.6–47.3)	52.9 (31.0–73.8)
Male ($n = 11$)	27.3 (9.8–56.6)	18.2 (5.1–47.7)	27.3 (9.8–56.6)	0.0	45.5 (21.3–72.0)	18.2 (5.1–47.7)
G. tachinoides	27.8 (15.9–44)	13.9 (6.1–28.7)	0.0	5.6 (1.5–18.1)	19.4 (9.8–35.0)	22.2 (11.7–38.1)
Sex						
Female ($n = 22$)	31.8 (16.4–52.9)	18.2 (7.3–38.5)	0.0	4.6 (0.8–21.8)	22.7 (10.1–43.4)	22.7 (10.1–43.4)
Male ($n = 14$)	21.4 (7.6–47.6)	7.1 (1.3–31.5)	0.0	7.1 (1.3–31.5)	14.3 (4.0–39.9)	21.4 (7.6–47.6)
Locations						
Idiroko ($n = 30$)						
G. palpalis ($n = 15$)	40.0 (19.8–64.3)	13.3 (3.7–37.9)	6.7 (1.2–29.8)	0.0	20.0 (7.1–45.2)	40.0 (19.8–64.3)
G. tachinoides (n = 15)	40.0 (19.8–64.3)	26.7 (10.9–51.9)	0.0	13.3 (3.7–37.9)	40.0 (19.8–64.3)	26.7 (10.9–51.9)
Adebayo ($n = 34$)						
G. palpalis ($n = 13$)	30.8 (12.7–57.6)	30.8 (12.7–57.6)	15.4 (4.3–42.2)	0.0	46.2 (23.2–70.9)	38.5 (17.7–64.5)
G. tachinoides ($n = 21$)	19.1 (7.7–40.0)	4.8 (0.9–22.7)	0.0	0.0	4.8 (0.9–22.7)	19.1 (7.7–40.0)

suitable environment for these known savannah-dwelling species. Density of tsetse flies vary across Nigeria with low densities (< 2.0 flies/trap/day) of Palpalis group reported in northcentral [17,30–32]. Even though, high densities have also been reported in game reserves from northcentral such as 27.3 and 128.0 flies/trap/day for *G. palpalis* and *G. tachinoides*, respectively [18]. However, there seems to be dearth of information on tsetse abundance from southwest Nigeria. The low density from this study could be due to use of insecticides and improved management amongst livestock owners [33].

Tsetse infection rate varied across Nigeria due to the wide vegetational differences. Studies from northern Nigeria since year 2000 have reported infection rate of < 20% [18,32,34–36], while 58% [37], 67% [38] and 70% [39] have been reported in the southwest Nigeria. Recent tsetse infection rate from southeastern Nigeria reported 14.3% prevalence using PCR technique [40]. Meta-data on published work between 1960 and 2017 revealed mean trypanosome infection rate of 10.5 and 30.4% in the northern and southern regions of Nigeria, respectively [41]. The high prevalence of trypanosome in the flies from this study could be due to forest vegetation and animal husbandary practices in the area. Not until 2010, most studies have assessed trypanosomes in tsetse flies using microscopy rather than more sensitive PCR, reports range from very low to very high prevalence [41]. However, the use of PCR has improved detection.

Factors such as land pressure, transhumance, cattle management practices, vegetational changes, deforestation and urbanization could affect vector distribution and reproduction, vector capacity and competence, presence or absence of endosymbionts [42]. The study used Nzi traps, which are very effective, not biased for any tsetse species and recommended for tsetse catches [43]. Nzi trap is rarely used in Nigeria because only few studies on tsetse abundance are active and most experiments are designed based on existing methodologies.

None of the examined flies were shown to be infected with *Wolbachia*. Previous work has indicated the absence of *Wolbachia* in *G. p. palpalis* [15], however, more recent work using Illumina sequencing of the hypervariable V3-V4 region has identified *Wolbachia* in *G. palpalis* [15,44]. The advantage of the Illumina is to sequence millions of fragments simultaneously and easy detection of new or rare variants. Another study for Palpalis group, recently reported *Wolbachia* in 0.24% (2/834) of *G. tachinoides* trapped in Burkina Faso, 0.14% (1/731), 4.4% (16/364) and 4.14% (13/314) of *G. p. gambiensis* trapped from Burkina Faso, Mali and Guinea, respectively [45]. Therefore, it may be with more sensitive molecular tools and increased tsetse population analysed that *Wolbachia* may be found to be present within the tsetse population of southwestern Nigeria.

The detection of obligate *W. glossinidia* in the tsetse samples is not surprising has most field-trapped tsetse flies have reported similar prevalence [10]. This is because the symbiotic bacteria are vertically transmitted and readily colonise the developing larvae [10].

S. glossinidius, often referred to as commensal symbionts are considered non-essential and they are generally found at varying frequencies within host populations [46]. The presence of *S. glossinidius* suggests that more flies could be positive for trypanosomes [12]. *S. glossinidius* was detected in Nigerian *G. p. palpalis* and *G. tachinoides* at 35.7% ($n = 36$) and 27.8% ($n = 28$) prevalence respectively. This is lower than that found in Cameroon (54.9% ($n = 450$) in *G. p. palpalis* [21] and may be due to environmental variables such as precipitation, temperature, humidity and vegetation type, which have shown to affect prevalence under laboratory conditions [47]. The prevalence of *S. glossinidius* in *G. tachinoides* from this study (27.8%) is lower than 37% reported in Cameroon [48]. The differences could be due to vegetational changes and human activities. In this study, prevalence of *S. glossinidius* was observed to be non-significantly higher in flies captured around the coastline border area with Benin Republic. However, further studies will need to be undertaken in the field to investigate if this represents a real difference between the different areas that are separated by 150 km. *T. vivax* is the predominant trypanosome found in cattle in southwest Nigeria [42] and therefore it is not be surprising to find this trypanosome in the tsetse that we surveyed. The presence of trypanosomes in the tsetse flies implies active transmission in the study area [41], however that does not necessarily indicate the presence of

transmissible organism within tsetse [19]. Previous studies had found a link between the presence of *S. glossinidius* and trypanosome infection in *G. palpalis* in Cameroon [21] we did not find a similar association in southwest Nigeria. However, this might be due to the low numbers of flies sampled.

Apart from the primer sets used in this study, *S. glossinidius* can also be detected with *Hems* primers to target gene encoding haemolysin protein. *Wolbachia* can be also be assessed using *16S rRNA* or *ftsZ* genes, while *Wigglesworthia* can be assessed using *thic* gene. The differences in the detection level of these primer sets can be evaluated in future studies.

Overall the *Trypanosoma* species distribution was not influenced by the presence of endosymbionts in *G. palpalis* and *G. tachinoides* captured in southwest Nigeria. The complete absence of *Wolbachia* needs further investigations, while *S. glossinidius* prevalence could be important in future control of tsetse flies in this "fly-belt" region. In this study, we could not assess *Spiroplasma* which was recently reported in some *Glossina* spp. [22], due to financial constraints. The association of these endosymbionts would help in understanding on future approach of the functional role on tsetse and pathogen infections outcomes [10]. Due to the unique microbiota of tsetse flies, future studies will focus on several groups including *Spiroplasma*, *Rickettsia* and *Enterobacteriaceae* to understanding its many effects associated with trypanosomes in tsetse flies captured from West Africa. Further studies using genomic/complementary functional approaches are warranted to understand the implication of the endosymbionts in *Glossina* species captured in Nigeria.

Acknowledgements

This study was financially supported by the Commonwealth Scholarship Commission and the University of Edinburgh, United Kingdom. Paul Olalekan Odeniran is a Commonwealth Scholar funded by the UK government (reference no. NGCN-2016-196).

Declaration of interest

The authors declare there is no conflict of interest regarding the publication of this article.

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