

***Sodalis Glossinidius* and *Wolbachia* Infections in Wild Population of *Glossina Morsitans Submorsitans* Caught in the Area of Lake Iro in the South of Chad**

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Abstract

Background: To improve vector control of tsetse transmitted trypanosomiases through a better understanding of the vectorial competence of tsetse flies, investigations have been undertaken on the bacterial fauna of different tsetse species. It is in this light that *Wolbachia* and *Sodalis glossinidius* were detected in wild populations of *Glossina morsistans submorsistans* caught in the area of lake Iro in the south of Chad with the aim of generating data that may help to understand the influence of these symbiotic microorganisms on the vectorial competence of *G. m. submorsistans*.

Methods: Tsetse flies were captured using biconical traps. DNA was extracted from tsetse body using the resin chelex. *Sodalis glossinidius* and *Wolbachia* were investigated by PCR using specific primers for pSG 2 and wsp genes. Comparisons between *S. glossinidius* and *Wolbachia* infections were performed according to sex and sampling periods and association studies between trypanosome infections and *S. glossinidius* or *Wolbachia* were performed.

Results: From 345 *G. m. submorsistans* analyzed, 9.0% and 14.5% were respectively infected with *S. glossinidius* and *Wolbachia*. Only 2.31% of all tsetse flies hosted the 2 bacteria. Of all trypanosome-infected flies, 7.1% and 9.8% hosted respectively *S. glossinidius* and *Wolbachia*. No association was observed between *Wolbachia* and trypanosomes while significant association ($r = 4.992$; $P = 0.025$) was found between *S. glossinidius* and trypanosome infections. Although significant association ($r = 3.147$; $P = 0.043$) was observed between *S. glossinidius* and *T. simiae*, none was found with *T. congolense* or *T. godfreyi*.

Conclusion: This study revealed *S. glossinidius* and *Wolbachia* in wild population of *G. m. submorsistans* of lake Iro. It showed that few tsetse flies are co-infected by *Wolbachia* and *S. glossinidius*. The presence of *S. glossinidius* seems to favor trypanosome infections while *Wolbachia* has probably no effect. Deciphering the tripartite association requires to investigate the relationship between haplotypes or genotypes of *Wolbachia* and/or *S. glossinidius* and trypanosome infections.

Introduction

Tsetse flies are biological vectors of African trypanosomes that cause human and animal African trypanosomiases respectively in humans and animals. Human African trypanosomiasis (HAT) is caused by two subspecies of trypanosomes: *Trypanosoma brucei rhodesiense* which is responsible of the acute form of HAT in eastern and southern Africa, and *T. b. gambiense* that causes the chronic form of HAT in western and central Africa [1]. About 65 million people are at risk of HAT and currently, the number of reported cases is, for the first time below 2000 new cases [2]. Control efforts undertaken in the last three decades have brought HAT under control and led to its inclusion into the WHO "roadmap for the interruption of transmission to human by 2030 [3].

Animal African trypanosomiases (AAT) are caused by several trypanosomes species and subspecies including for instance *T. b. brucei*, *T. congolense*, *T. vivax* and *T. simiae*. These diseases remain a

constraint for animal production and agriculture development. In absence of vaccine for African trypanosomiasis, the control strategies deployed against these infectious diseases rely mainly on the diagnosis and treatment of infected mammals and vector control. The development of drug-resistant trypanosomes could jeopardize the control measures aiming to eliminate the protozoan parasites through treatment of infected hosts. Moreover, the strategy relying on treatment of infected mammals cannot be applied on wild animals that could serve as reservoirs of different trypanosome species and subspecies. In such context, vector control remains a very important component for the management of African trypanosomiasis [4]. Integrating vector control as key component of new control strategies is becoming crucial to achieve the complete interruption of HAT transmission and boost AAT control. In this light, several approaches including the setup of tsetse traps, screens or “tiny targets”, the use of insecticide and the modification tsetse biotopes have been developed to fight tsetse flies [5]. Although the implementation of these approaches enabled to reduce tsetse populations in most settings, their sustainability remains challenging and some of these approaches have environmental impacts [6]. There is a need to develop innovative vector control methods that may not have such impacts. In recent decades, growing interests have been focused on factors enable to interfere with the vectorial competence of tsetse flies [7, 8]. It is in this light that interactions between trypanosomes, tsetse and symbiotic microorganisms have been investigated [9, 10]. Three symbiotic microorganisms including *Wigglesworthia glossinidia*, *Sodalis glossinidius* and *Wolbachia* have been associated with tsetse.

Wigglesworthia glossinidia is an obligate primary symbiont on which tsetse depend for their vital physiological functions such as host fertility and immune maturation [11, 12, 9]. *Sodalis glossinidius* is a secondary and non-essential symbiont. Although its biological function remains unknown [13, 14], *S. glossinidius* has been suspected to play a role in the susceptibility of tsetse to trypanosome infections by favoring midgut establishment of trypanosomes through a complex biochemical mechanism [13, 15, 11]. *Wolbachia* are also non-essential symbionts found in a wide range of arthropods and nematodes [16, 17, 18]. They are transmitted from mother to offspring and can protect their hosts against viral pathogens [19, 20]. Abundant in both male and female germ-cells as well as somatic tissues, *Wolbachia* are able to induce cytoplasmic incompatibility which leads to embryonic death in tsetse flies [21]. With such ability, investigations on *Wolbachia* could improve vector control through the development of transgenic tsetse that have the ability of releasing specific molecules that can interfere with the establishment of trypanosomes.

In wild tsetse populations from several tsetse infested regions, *S. glossinidius* and *Wolbachia* have been reported in *G. m. morsitans*, *G. m. centralis*, *G. f. fuscipes*, *G. austeni*, *G. pallidipes*, *G. p. palpalis*, *G. f. quanzensis* and *G. brevipalpis* [7, 22, 23, 24, 20; 25, 26, 27]. Previous investigations on the tripartite association reported contrasting results in different ecosystems. A negative association has been reported between *Wolbachia* and trypanosomes in *G. f. fuscipes*, suggesting that the presence of *Wolbachia* could prevent trypanosome infections [27]. In tsetse of *palpalis* group, no association has been reported between *Wolbachia* and trypanosome infections [28]. For *S. glossinidius*, some authors reported a positive association between *S. glossinidius* and trypanosome infections [29] while others found that the presence of *S. glossinidius* does not seem to favor trypanosome infections in *G. p. palpalis*

[25]. The tripartite association between tsetse, symbiotic microorganisms and trypanosome infections seems to vary according to tsetse species as well as ecological settings. Deciphering the role that each symbiotic microorganisms could play in the establishment and the development of trypanosomes in tsetse species of each ecological setting is importance for the understanding of vectorial competence of tsetse flies.

In the present study, *Wolbachia* and *S. glossinidius* were screened in wild populations of *G. m. submorsistans* caught in the area of Lake Iro in the south of Chad with the aim of generating data that may help to understand the influence of these symbiotic microorganisms on the vectorial competence of *G. m. submorsistans*.

Results

Entomological survey

During the three entomological surveys, 54 tsetse traps were set up and 617 tsetse flies were caught: 12 tsetse caught in November 2018, 323 in February 2019 and 282 in February 2020. The entomological data have been previously described in Djoukzoumka et al. (accepted in Parasites and Vectors). From the 617 caught flies, 359 non teneral flies were randomly selected and their identification revealed 345 (96.1%) *G. m. submorsitans*, 11 (3.06%) *G. f. fuscipes* and 3 (0.84%) *G. tachinoides*. For the present study, only the 345 *G. m. submorsitans* were subjected to the molecular identification of *S. glossinidius* and *Wolbachia*. These 345 flies included 105 (30.4%) females and 240 (69.6%) males.

Molecular identification of *S. glossinidius*

A tsetse fly was considered as harboring *S. glossinudius* infections if, after electrophoresis on agarose gel (Fig. 2) and sequencing, a DNA fragment of 120 bp was obtained. In addition, the obtained sequences must also show at least 98% of similarity with those of *S. glossinidius* available in the data base.

Of the 345 *G. m. submorsitans* analysed, 31 (9.0%) harboured *S. glossinidius*. The infection rates of *S. glossinidius* were 5.71% (6/105) in females and 10.41% (25/240) in males. Comparing the *S. glossinidius* infection rates between males and females, no significant difference ($\chi^2 = 1,975; P = 0.16$) was observed. According to sampling period, the infection rate of *S. glossinidius* was 18.8% (2/11) in November 2018, 10.8% (22/216) in February 2019 and 5.9% (7/118) in February 2020. Between the sampling periods, no significant difference ($\chi^2 = 3.213; P = 0.2201$) was observed in the *S. glossinidius* infection rates (Table 1).

Table 1

Infection rates of *S. glossinidius* and *Wolbachia* in *G. m. submorsitans* caught in the Lake Iro area according to the sampling period

Sampling period	Number of tsetse flies analyzed	NTW (%)	NTSG (%)	NTWSG (%)
November 2018	11	2 (18.8)	2 (18.8)	0(0.0)
February 2019	216	31 (14.3)	22(10.8)	7 (3.2)
February 2020	118	17(14.0)	7(5.9)	1(0.8)
X2		0.154	3.213	2.376
P-value		0.926	0.201	0.305
Total	345	50(14.5)	31(9,0)	8(2.3)

NTW: number of tsetse flies with *Wolbachia* infections; NTSG: number of tsetse flies found with *Sodalis glossinidius* infections; NTWSG: number of tsetse flies with coinfections of *Wolbachia* and *Sodalis glossinidius*; (%): infection rate

Molecular identification of *Wolbachia*

A sample was considered as having *Wolbachia* infection if after electrophoresis on agarose gel (Fig. 3) and sequencing, a DNA fragment of 513 bp was obtained. The obtained sequences must also have at least 98% of similarity with those of *Wolbachia* available in the database.

From the 345 *G. m. submorsitans* analyzed in the present study, 50 (14.5%) were found with *Wolbachia* infections. The *Wolbachia* infection rates were 19.0 % (20/105) and 12.5% (30/240) in females and males respectively. Comparing the *Wolbachia* infection rates, no significant difference ($\chi^2 = 2,527$; $P = 0.11$) was observed between males and females. The *Wolbachia* infection rate was 18.8% (2/11) in November 2018, 14.3% (31/216) in February 2019 and 14.0% (17/118) in February 2020. Between the sampling periods, no significant difference ($\chi^2 = 0.154$; $P = 0.926$) was observed in the *Wolbachia* infection rates (Table 1).

Of the 345 tsetse flies that were simultaneous analyzed for the presence of *Wolbachia* and *S. glossinidius*, 8 (2.31%) were co-infected with these two symbiotic microorganisms.

Trypanosome infections in *G. m. submorsitans*

To evaluate the tripartite association between trypanosome infections, *G. m. submorsitans* and symbiotic microorganisms (*S. glossinidius* and *Wolbachia*), results of trypanosome infections in *G. m. submorsitans* were retrieved from the paper of Djoukzoumka *et al.* (accepted in *Parasites and Vectors*). From the 345 *G. m. submorsitans* analysed in this paper, 212 (61.4%) were infected by different trypanosome species including 196 (56.7%) single infections and 16 (4.6%) mixed infections. The single infections were constituted of 68 (19.7%) *T. simiae*, 55 (15.9%) *T. congolense* savannah, 39 (11.3%) *T. vivax* and 34 (9.8%) *T. goffreyi*. Considering the fact that the life cycle of *T. vivax* occurred mainly in the

mouthparts of tsetse flies, the single infections involving this parasite were excluded in the tripartite association. Thus, from 345 tsetse flies that were simultaneously analyzed for the presence of trypanosomes, *Wolbachia* and *S. glossinidius*, the 39 tsetse flies with single infections of *T. vivax* were excluded. The 16 flies with mixed infections involving *T. vivax* were considered in the tripartite analysis. For association studies, only 306 tsetse flies were considered.

Coinfection of *S. glossinidius* and trypanosome

Of the 306 tsetse flies that considered for association studies, coinfections between *S. glossinidius* and trypanosomes (S + T+) were found in 7.1% (22/306) of them (Table 2). Nine (3.0%; 9/306) tsetse flies (S + T-) without trypanosome infections were infected with *S. Glossinidius*. In the contrary, 155 (50.6%; 155/306) tsetse flies (S-T+) harbored trypanosome infections without *S. glossinidius*. Taking together all trypanosome infections, a significant association ($r = 4.992$; $P = 0.025$; 95% CI= [0.178–5.012]) was observed between the presence of *S. glossinidius* and trypanosome infections in *G. m. submorsitans*. When each trypanosome species was considered for association studies, a significant association ($r = 3.147$; $P = 0.043$; 95% CI= [0.178–5.012]) was observed between *S. glossinidius* and *T. simiae* while no significant association was observed for *T. congolense* ($r = 1.066$; $P = 0.901$; 95% CI= [0.390–2.912]) and *T. godfreyi* ($r = 0.608$; $P = 0.509$; 95% CI= [0.139–2.677]) (Table 2).

Table 2
Combined results of *S. glossinidius* and trypanosome infections

Trypanosome infections	No	S+ T-	S+ T+	S- T+	S-T-	S+ T+	r	P value	95% CI	
<i>Tsimiae</i>	306	21	10	57	357	31	67	3.147	0.043*	0.959–4.806
TCS	306	26	5	48	266	31	53	1.066	0.901	0.390–2.912
<i>T. godfreyi</i>	306	29	2	32	282	31	34	0.608	0.509	0.139–2.677
All trypanosome species	306	9	22	155	159	31	177	4.992	0.025*	0.178–5.012

No = Number of tsetse flies analyzed; **TCS** = *Trypanosoma congolense* savannah; **S +** = tsetse flies infected by *S. glossinidius*; **T +** = tsetse flies infected by at least one trypanosome species. **S + T +** = tsetse flies co-infected by *S. glossinidius* and at least one trypanosome species. **S + T -** = tsetse flies infected by *S. glossinidius* but without any trypanosome infection; **S - T +** = tsetse flies without *S. glossinidius* but harboring trypanosome infections; **S - T -** = tsetse flies without trypanosome and *S. glossinidius* infections. **CI** = Confidence Interval; TCS: *Trypanosoma congolense* savannah; *T. simiae*: *Trypanosoma simiae*; *T. godfreyi*: *Trypanosoma godfreyi*; **r** = coefficient of the generalised linear modeling.

Coinfection of *Wolbachia* and trypanosomes

Of the 306 tsetse flies that were considered for tripartite association, 30 (9.8%) (W + T+) were coinfecting with *Wolbachia* and at least one trypanosome species. Amongst these 306 tsetse flies, 20 (6.6%) (W + T-) harbored *Wolbachia* infections without any trypanosome while 147 (48.0%) (W-T+) were infected only with trypanosomes. When all trypanosome infections were considered for association studies, no significant association ($r = 1.754$; $P = 0.185$; 95% CI = [0.360–1.219]) was observed between the presence of *Wolbachia* and trypanosome infections in *G. m. submorsitans* (Table 3). Between the presence of *Wolbachia* and each trypanosome species, no significant association was also observed (Table 3).

Table 3
Combined results of *Wolbachia* and trypanosome infections

Trypanosome species	No	W+ T-	W+ T+	W- T+	W- T-	W+ T+	r	P-value	95% CI	
<i>T. Simiae</i>	306	39	11	56	239	50	67	1.204	0.618	0.580–2.497
TCS	306	42	8	45	250	50	53	1.058	0.892	0.466–2.403
<i>T. godfreyi</i>	306	45	5	29	266	50	34	1.019	0.970	0.375–2.771
All trypanosome species	306	20	30	147	148	50	177	1.754	0.185	0.360–1.219

No = Number of tsetse flies analyzed; **TCS** = *Trypanosoma congolense* savannah; **W+** = tsetse flies infected by *Wolbachia*; **T+** = tsetse flies infected by at least one trypanosome species. **W+T+** = tsetse flies coinfecting by *Wolbachia* and at least one trypanosome species. **W+T-** = tsetse flies with *Wolbachia* infection without any trypanosome species; **W-T+** = tsetse flies without *Wolbachia* but infected by at least one trypanosome species; **W-T-** = tsetse flies without *Wolbachia* and trypanosome infection; TCS: *Trypanosoma congolense* savannah; *T. simiae*: *Trypanosoma simiae*; *T. godfreyi*: *Trypanosoma godfreyi*; **CI** = confidence interval; **r** = coefficient of the generalised linear model.

Discussion

Challenges limiting the appraisal of tsetse microbiome include the difficulties to identify bacterial species in these flies. To fill these gaps in understudied ecological settings, PCR-based method was used to identify *S. glossinidius* and *Wolbachia* in wild population of *G. m. submorsitans* caught in the area of lake Iro in the south of Chad. The identification of *S. glossinidius* and *Wolbachia* in wild population of *G. m. submorsitans* of Lake Iro is in line with previous studies reporting these two symbiotic microorganisms in wild populations of *G. m. morsitans*, *G. tachinoides*, *G. p. palpalis*, *G. pallidipes*, *G. f. quanzensis* and *G. brevipalpis* [2010, 30, 31, 25, 26, 22, 23].

The *S. glossinidius* infection rate of 9.0% obtained in the present study is similar to 9.3% reported in Liberia for *G. p. palpalis* [29]. This rate is higher than 1.4% reported in Zambia for wild populations of *G. pallidipes* [31], but lower than 15.65%, 17.5%, 54.9% and 93.7% previously reported respectively in the

Democratic Republic of Congo for *G. f. quanzensis*, in Zambia for *G. m. morsitans*, in Cameroon for *G. p. palpalis* and in Zambia for *G. brevipalpis* [7, 31, 23]. These results confirm the high heterogeneity of *S. glossinidius* infection rates according to tsetse species [31, 7]. Nevertheless, reliable comparisons between data from by different studies require to understand the study designs. In the present study, *S. glossinidius* was identified in tsetse body while in other studies, whole tsetse or parts of the insect such as the abdomen, the thorax and the legs were used. The heterogeneity observed in the *S. glossinidius* infection rates could be explained by some variations in methodological approaches, the intrinsic characters of each tsetse species and environmental factors (vegetation, humidity, temperature) encountered in different ecological settings. In natural conditions where environmental factors vary and have impacts on the biology of tsetse flies, the relationship between tsetse and its symbiotic micro-organisms is affected. As the survival and the transmission of these symbiotic micro-organisms are linked to tsetse biology because of their limited metabolic capacities, each environmental factor affecting the biology of tsetse could change its interactions with symbiotic micro-organisms. In such a scenario, *S. glossinidius* could not undergo horizontal transmission with the same efficiency and in consequence, its infection rates could vary with environmental factors. When environmental variations are removed like in experimental studies or in insectarium [33], the symbiotic association between tsetse and its symbionts is not affected. As already reported in *G. p. gambiense* and *G. m. morsitans*, high vertical transmission and high infection rates of symbiotic micro-organisms are observed in tsetse flies [11].

The significant association ($r = 4.992$; $P = 0.025$; 95% CI= [0.178–5.012]) observed between *S. glossinidius* and trypanosome infections indicates that the presence of *S. glossinidius* seems to favor trypanosome infections in *G. m. submorsitans* of the area of lake Iro in the south of Chad. Although these results are not in agreement with those reporting no significant association between the presence of *S. glossinidius* and trypanosome infections in *G. austeni* [20], *G. brevipalpis*, *G. m. morsitans* and *G. pallidipes* [31], our findings are in line with those reported in *G. p. palpalis* of some sleeping sickness foci of Cameroon [7] and other tsetse species [20, 8, 11]. The discrepancies observed in the tripartite association between tsetse, *S. glossinidius* and trypanosomes may result from differences in the biology of different tsetse species as well as the bioclimatic conditions impacting the relationship between tsetse and its symbiotic micro-organisms. Moreover, our results showing significant association ($r = 3.147$; $P = 0.043$; 95% CI= [0.178–5.012]) between *S. glossinidius* and *T. simiae*, but no association for other trypanosome species identified in this study suggest that the tripartite association between tsetse, *S. glossinidius* and trypanosomes could vary according to trypanosome species. Better understanding these tripartite associations requires more in-depth investigations on wild populations of different tsetse species of various tsetse infested areas.

The identification of *Wolbachia* in wild populations of *G. m. submorsitans* may have some implications in the development of new vector control strategies. On the basis of its capacity of inducing cytoplasmic incompatibility and to be transmitted from mother to offspring, *Wolbachia* can be genetically modified with the objective of producing biomolecules able to interfere with the establishment and/or the development of trypanosomes in tsetse flies. If that occurs, the vectorial competence of tsetse will be

affected and disease transmission could be blocked through genetically modified *Wolbachia* strains that conferred resistance to tsetse fly [26].

The overall *Wolbachia* infection rate of 14.5% obtained in the present study is lower than 25.32%, 44.3%, 88.8%, 98% and 100% reported respectively in *G. p. palpalis* [26], *G. f. fuscipes* [27], *G. f. Quanzensis* [23], *G. austeni* [33, 20] and *G. m. morsitans* [34]. These results show a certain heterogeneity in the *Wolbachia* infection rates according to tsetse species. As already reported by Kante et al. [26], this heterogeneity could be related to specific biological characteristics of each tsetse subspecies. For identical stimulus, it has been reported that interactions between tsetse fly and its symbiotic micro-organisms vary according to specific biological response of each tsetse species or subspecies [26]. Such variations affect not only the interactions between tsetse and its symbiotic micro-organism, but also the *Wolbachia* infection rates. Some discrepancies observed in the *Wolbachia* infection rates could be explained by some differences in the study design as well as the analytical methods. In the present study, *Wolbachia* was searched in tsetse body (whole tsetse without legs, wings and proboscis) while in other studies, investigations were undertaken on isolated tissues or whole tsetse fly. In addition, the fact that one molecular marker was used to detect *Wolbachia* infections has probably underestimated its infections rates. Indeed, in tsetse flies from the same ecological setting, Kante *et al.* [26] reported significant differences in the *Wolbachia* infection rates when different molecular markers were used. In Cameroon for instance, the detection of *wsp* gene was two-fold more sensitive in tsetse from Campo while 16S rDNA showed higher sensitivity in flies from Fontem [26]. In addition to differences in the sensitivity of molecular markers, the technical approaches could also have impacts on the *Wolbachia* infection rates. If the density of *Wolbachia* in some *G. m. submorsitans* is below the detection threshold of standard PCR-based method, some infections could pass undetected. Wamwiri *et al.* [20] highlight a high density of *Wolbachia* in *G. austeni* populations from Kenya and a low density in the same tsetse species of South Africa. In addition, a low density of *Wolbachia* has been reported in *Rhagoletis cerasi* [35] and *Drosophila paulistorum* [36]. While searching for sensitive and reliable markers or tools for *Wolbachia* identification remains a goal to achieve, the use of one marker or standard PCR-based method may lead to an underestimation of the real *Wolbachia* infection rates.

The 9.8% of *G. m. submorsitans* harboring co-infections of *Wolbachia* and trypanosomes is low compared to 29.84% and 26% reported respectively in *G. p. palpalis* [26] and *G. tachinoides* in Cameroun [22]. Although the technical approach and the study design could partially explain this low co-infection rate, such co-infections are probably not common in *G. m. submorsitans* of lake Iro. The absence of significant association ($r = 1.754$; $P = 0.185$; 95% CI = [0.360–1.219]) between *Wolbachia* and trypanosome infections suggests that the presence of this bacterium does not seem to be an obstacle for the establishment of trypanosomes. These results are in agreement with those of Kante et al. [26] reporting no association in *G. p. palpalis* from in sleeping sickness foci of Cameroon. They contrast data of Alam et al. [27] showing a negative correlation between *Wolbachia* and trypanosome infections and suggesting that the presence of this bacterium prevent trypanosome infections in *G. f. fuscipes*. The tripartite association between tsetse, *Wolbachia* and trypanosomes seems to vary according to tsetse species or subspecies.

Results of the present study showing that only 2.31% of tsetse flies were co-infected by *Wolbachia* and *S. glossinidius* are in agreement with the 5.43% previously reported in *G. f. quanzensis* [23]. They indicate that co-infections between *Wolbachia* and *S. glossinidius* are rare in wild populations of *G. m. submorsitans*. The co-infection rate between *S. glossinidius* and *Wolbachia* is probably underestimated in the present study because the molecular markers used have been reported to be of low sensitivity, especially when only one marker was used to detect symbiotic microorganisms. The low co-infection rate revealed between *S. glossinidius* and *Wolbachia* can be also explained by the biological effects of each of these bacteria. Indeed, association studies revealed that the presence of *S. glossinidius* seems to favor trypanosome infections while no association was reported between *Wolbachia* and trypanosome infections. In other studies, the negative correlation reported between trypanosomes and *Wolbachia* infections suggested that the presence of *Wolbachia* seems to prevent trypanosome infections [27, 30]. These observations suggest that some antagonistic actions, resulting from different biological actions of *Wolbachia* and *S. glossinidius* could occur in tsetse fly during trypanosome infections.

Our investigations on tripartite associations were based on presence/absence of trypanosome or *S. glossinidius* or *Wolbachia*. Instead of focusing on this presence/absence, the genetic characterization of *S. glossinidius* or *Wolbachia* strains could provide additional values to decrypt these associations. In previous investigations, it has been reported that the tripartite association could be affected by specific genotypes of *S. glossinidius* and some trypanosome species such as *T. b. gambiense* and *T. b. brucei* [8]. For some trypanosome species, specific *S. glossinidius* genotypes have been reported to affect the vectorial competence of *G. p. gambiensis* and *G. m. morsitans* [11]. Genetic characterization of bacteria populations could provide additional data to improve knowledge on this tripartite association, and also to better understand the real contribution of symbiotic microorganisms (*S. glossinidius* or *Wolbachia*) in the vectorial competence of tsetse flies. To obtain the real overview of the vector competence of tsetse flies, it is also important to take into consideration other factors such as the level of lectin in the tsetse gut at the time of parasite uptake, the fly species, the age, the general status of tsetse and its first blood meal on a non-infected host because these factors affect its ability to be infected and could mitigate the influence of symbiotic micro-organisms. Such factors could play a significant role in the success or failure of parasite establishment because the processes leading to this establishment involve complex interactions between these factors [13].

Conclusion

This study revealed *S. glossinidius* and *Wolbachia* in wild population of *G. m. submorsitans* of Lake Iro in the south of Chad. It showed that few tsetse flies harbor co-infections of *Wolbachia* and *S. glossinidius*. Co-infections of *Wolbachia* and trypanosomes or *S. glossinidius* and trypanosomes are not common in *G. m. submorsitans*. No association was revealed between *Wolbachia* and trypanosomes while significant association was observed between the presence of *S. glossinidius* and trypanosome infections. Decrypting the tripartite association involving tsetse, symbionts and trypanosomes requires additional studies aiming to understand the relationship between haplotypes or genotypes of *Wolbachia* and/or *S. glossinidius* and trypanosome infections.

Methodology

Study area

Tsetse flies were caught in the area of Lake Iro, along the Salamat River in the Middle Chari region of the south of Chad (Fig. 1). This area is considered as a buffer zone of the Zakouma national park where domestic and wild animals can meet. It is located between latitude 09°59'N and longitude 019°26'E and has a climate of Sudano-Sahelian type with one dry season (November to April) and one rainy season (May to October). This locality has an average annual temperature and relative humidity of respectively 27°C and 50% [37]. The rainfall varies from 800mm to 1200mm per year [38] and the vegetation is dominated by floodplains and dense forests containing shrubs. The hydrographic network is mainly dominated by the Lake Iro and the Salamat River, which flows into the Chari River that feeds Lake Chad in the north. The population is estimated at 174,195 inhabitants who are mainly herdermen, farmers and fishermen [39].

Entomological survey

Three entomological surveys were performed in November 2018, February 2019 and February 2020. During these surveys, biconical traps [40] were set up along the Salamat River, especially where the bioclimatic conditions were considered favourable for the development of tsetse flies. The geographical coordinates of each tsetse trap were recorded using a global positioning system (GPSMAP® 60CSx Garmin). The temperature and the relative humidity were recorded using a thermohygrometer (EasyLog TH, Lascar, Whiteparish, UK). Tsetse flies were collected each day at 9 am. The collected flies were morphologically identified and their sex and species determined.

Collection of tsetse fly organs

From each tsetse fly, the wing pairs (for morphometric analyses) as well as the legs (for genetic studies) were removed and introduced separately into dry microtubes. Thereafter, the proboscis (for the identification of trypanosome species) and the remaining body (for the identification of trypanosome species and symbiotic micro-organisms) of each tsetse were collected and each of them placed in a 1.5mL cryotube containing 200µl of nucleic acid preservative solution (25 mM sodium citrate, 10 mM EDTA and 70% ammonium sulphate). The dissecting tweezers were decontaminated in 5% solution of sodium chloride and then rinsed with distilled water after dissection of each tsetse fly. In the field, the samples were preserved at 4 degrees and once in the Laboratory, they were stored at -80 degree.

DNA extraction from the tsetse body

DNA was extracted from the body of each tsetse fly using 5% chelex-resin (Chelex 100, Bio-rad). Briefly, each tsetse body was removed and put into a new 1.5 mL microtube. This tsetse body was crushed using the tip of the Pasteur pipette. Thereafter, 100 µl of chelex 5% solution were added. Each microtube was vortexed and incubated at 56°C for 30 minutes in a Thermomixer. After this incubation, the microtubes were vortexed and reincubated at 95°C for 5 minutes. These microtubes were subsequently centrifuged at

10,000 rpm for 1 minute. The concentration of DNA extracts was determined using a Nanodrop 1000 (Thermo Scientific-Germany). DNA extracts were stored at -20°C for molecular analysis.

Tsetse species identification

The confirmation of each tsetse fly was performed by amplifying and sequencing the Cytochrome oxidase I (COI) gene. This amplification was done using COI-sense (5' TTG ATTTTT TGG TCA TCC AGA AGT-3') and COI-non-sense (5'-TGA AGC TTA AAT TCA TTG CAC TAA TC-3') primers designed by Dyer *et al.* [41]. Briefly, PCR reaction was performed in a final volume of 25 µL containing 2.5 U of dream taq polymerase, 2.5µL of the dream taq buffer (10X), 0.2 mM of dNTPs (all provided by Thermo Scientific, Dreieich, Germany), 2 µM of each primer and 1µL of DNA extract. The amplification program consisted of an initial denaturation step of 95°C for 5 min, followed by 35 cycles. Each of these cycles was made up of a denaturation step at 94°C for 1 min, an annealing step at 55°C for 1 min and an elongation step at 72°C for 1 min. This was followed by a final elongation at 72°C for 5 min.

After each PCR reaction, 20 µL of PCR products were checked by electrophoresis on 1.5% agarose gel containing 3 µL G-stain (Serva, Heidelberg, Germany). The agarose gel was stained, visualized under ultraviolet light (UV) and photographed.

Each sample for which a DNA fragment of 930 bp was revealed by electrophoresis was selected and the remaining amplicons purified using GeneJet DNA purification kit (Thermo Scientific, Dreieich, Germany). This purification was performed following the manufacturer's instruction. Each purified COI DNA fragment was sequenced by a commercial company (SeqLab, Göttingen, Germany). To identify each tsetse species or subspecies, each sequenced fragment of COI was compared to those available in the database (Genbank) of the National Center for Biotechnology Information (NCBI). This was done through a BLAST search.

Identification of *S. glossinidius*.

Sodalis glossinidius was identified by PCR using the pSG2 primers (pSG2-sense: 5'-TGAAGTTGGGAATGTCG-3': pSG2-non-sense: 5'-AGTTGTAGCACAGCGTGTA-3') as previously described by Darby *et al.* [42]. The PCR reaction was performed in a final volume of 15 µL containing 1.5 µL of Dream taq buffer, 0.5 µL of Dream taq polymerase (5U/µL), 0.3 µL of dNTPs (10 mM/µL), 0.5 µL of each primer (100mg/µL) and 0.5 µL of DNA extract. The amplification program consisted of an initial denaturation step at 94°C for 3 minutes followed by 30 cycles. Each of these cycles was made up of a denaturation step at 94°C for 30 sec, an annealing step at 51°C for 45 sec and an elongation step at 72°C for 1 min. The final elongation was done at 72°C for 5 min. For each PCR reaction, positive and negative controls were used. In the positive control, purified genomic DNA of *S. glossinidius* was used while in the negative control, the DNA solution was replaced by nuclease free water.

After PCR reactions, amplicons were separated by electrophoresis on 2% agarose gel containing 3 µL of G-stain (Serva, Heidelberg, Germany). The electrophoresis was performed at 100 volt for 60 min. At the

end of each electrophoresis, the gel was visualized under UV light. Samples were considered positive for *S. glossinidius* if a DNA fragment of 120 bp was observed.

The presence of *S. glossinidius* was confirmed by sequencing 5 positive randomly selected samples. For these 5 selected samples, the same PCR was performed in a final volume of 50 µL. After electrophoretic separation of PCR products of these 5 samples, amplicons were purified from agarose gel using the GenJet purification kit following the manufacturer's instructions. The purified PCR products were cloned into pJET 1.2 cloning vector (Thermo Scientific) following the manufacturer's instructions. Recombinant clones or clones containing pSG2 sequences were identified by PCR using pSG2 primers. PCR reactions were carried out in the same conditions as described above.

Recombinant clones were picked up from petri dish and cultured overnight at 37°C into LB medium supplemented with ampicillin (100 µg/ml). The bacteria cultures were centrifuged at 4500 xg for 15 min at 4°C and the pellet was recovered. Plasmid DNA was purified from each pellet using the GeneJET Plasmid MiniPrep Kit (Thermo Fischer Scientific). The Plasmidic DNA was sent for sequencing that was performed by a commercial company (institute SeqLab, Göttingen, Germany).

Molecular identification of *Wolbachia*

Wolbachia was identified by amplifying the *wsp* (*Wolbachia* surface protein) gene as described by Baldo *et al.* [43]. The amplification was done using *wsp*-sense (5'-GTCCAATARSTGATGARGAAAC-3') and *wsp*-non-sense (CYGCACCAAYAGYRCTRTRTAAA-3') primers designed by Baldo *et al.* [43]. PCR reaction was performed in a total volume of 15 µL containing 1.5 µL of Dream taq buffer, 0.1 µL of Dream taq polymerase (5U/µL), 0.3 µL of dNTPs (10mM/µL) (all from Thermo Fischer Scientific) and 0.5 µL of each primer (100mg/µL) (provided by Sigma-Aldrich, Darmstadt, Germany) and 0.5 µL of DNA extract. The amplification program consisted of an initial denaturation step for 5 minutes at 95°C followed by 35 cycles. Each cycle was made up of a denaturation step at 94°C for 30 sec, an annealing step at 53°C for 30 sec and an elongation step at 72°C. The final elongation was done at 72°C for 10 minutes. During each PCR, positive and negative controls were used. The positive control was a purified genomic DNA of *Wolbachia* while in the negative control, the DNA solution was replaced by nuclease free water.

Amplicons of each PCR reaction were subjected to an electrophoresis that was carried out as described above. Samples were considered as having *Wolbachia* infections if a DNA fragment of 513 bp was identified after electrophoresis. As for *S. glossinidius*, the presence of *Wolbachia* was confirmed by sequencing amplicons of 5 positive randomly selected samples. For each of these samples, another PCR was performed in a volume of 50 µL. The amplicons were purified by the GenJET purification kit (Thermo Fischer Scientific). The purified PCR product was directly sent to a commercial company (SeqLab, Göttingen, Germany) for sequencing.

Geneious Pro version 5.5.9 software was used to store, organize and analyze the sequences obtained from PSG2 and *wsp* genes of *S. glossinidius* and *Wolbachia*, respectively. The presence of *S. glossinidius*

and *Wolbachia* was confirmed by BLAST searching respectively of pSG2 and wsp sequences at the data base (Genbank) of the National Center for Biotechnology Information (NCBI). After BLAST search, a sequence was considered as belonging to *S. glossinidius* if the sequenced DNA fragment of 120 bp had at least 98% of similarity with those of *S. glossinidius* available in the data base. For *Wolbachia*, the nucleotide sequence of the sequenced DNA fragment of 513 bp must also have at least 98% of similarity with the sequence of *Wolbachia* available in the database.

Statistical analysis

The R software was used for statistical analysis [44]. The Chi-square test was used to compare the infection rates of *S. glossinidius* and *Wolbachia* according to sex and sampling periods. The test was considered significant when the P value was below 0.05. Generalised linear modelling (glm) with 95% confidence intervals (CIs) was used to evaluate the association between symbiotic microorganisms and trypanosome infections. For association studies, data on trypanosome infections were retrieved for results of Djoukzoumka *et al.* (submitted). To carry out these association studies, *T. vivax* was excluded because its lifecycle is restricted to the mouthparts of tsetse flies.

Abbreviations

ITS-1: Inner Transcribed Spacer 1; pSG 2: Plasmid Sodalis glossinidius 2, wsp: Wolbachia surface protein; AAT: Animal African Trypanosomiasis; HAT: Human African Trypanosomiasis.

Declarations

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Author's contributions

GS, DS, VKP, SK and HMH contributed to the design of the Project. DS, IMAM, SK, PB and YMM contributed in sample collection and laboratory analysis. DS, STK and GS analyzed the data. DS and GS

and wrote the Manuscript.

Availability of data and materials

All data generated and/or analyzed during this study are included in this article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing Interests

The authors declare that they have no competing of interests

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Figures

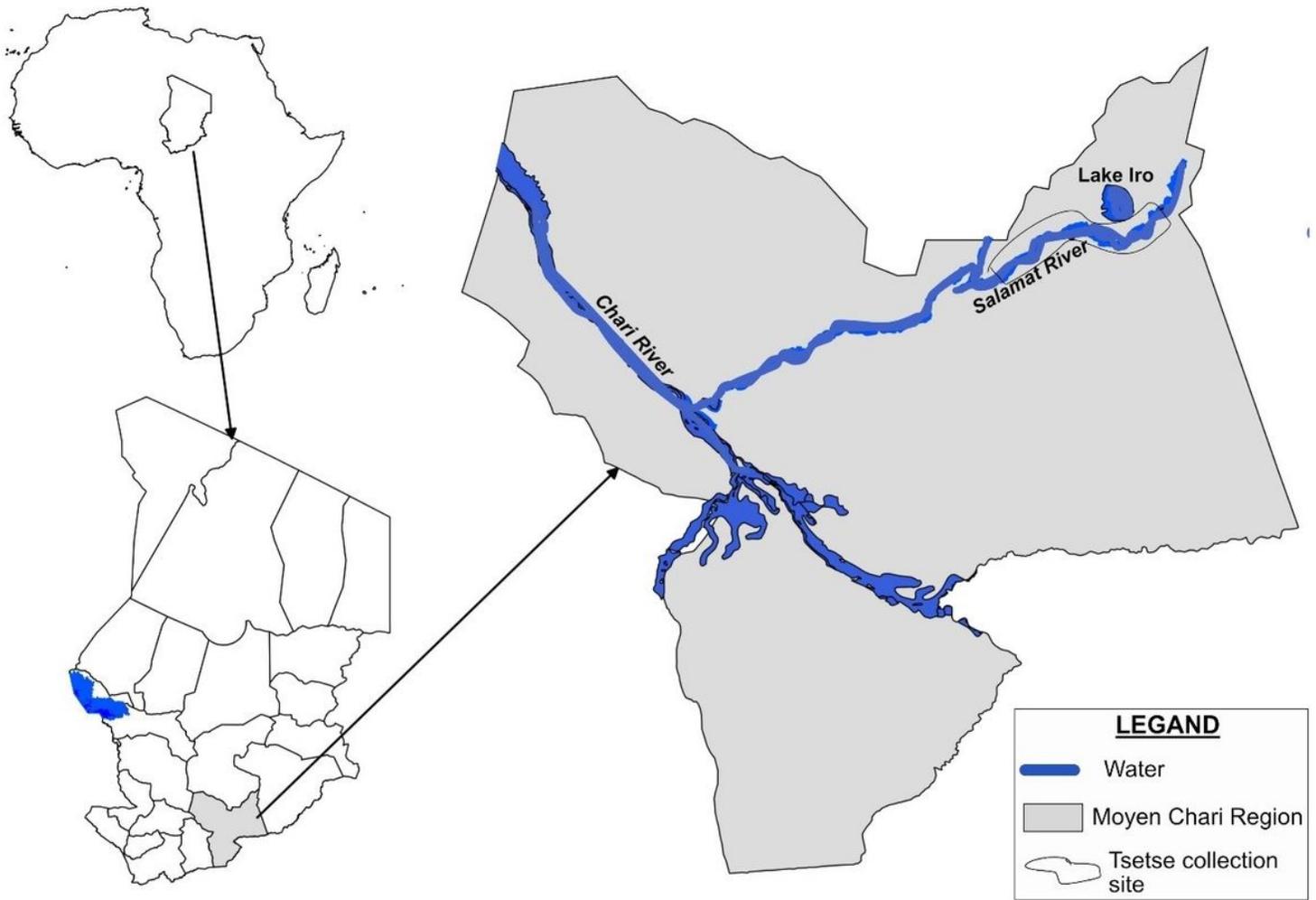


Figure 1

Tsetse flies were caught in the area of Lake Iro, along the Salamat River in the Middle Chari region of the south of Chad. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

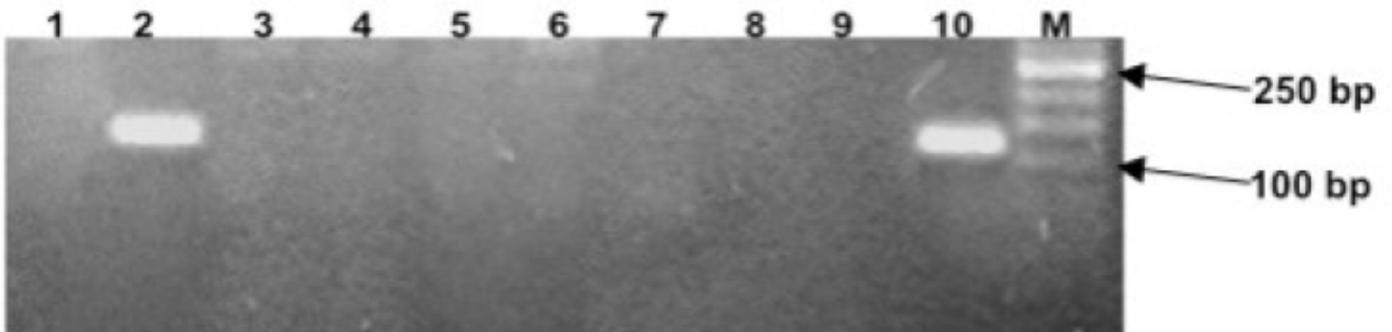


Figure 2

Electrophoretic profile illustrating the separation of amplified products of PSG2 gene of *S. glossinidius*. 1, 3, 4, 5, 6, 7 and 8: samples without *S. glossinidius* infection; 2: sample with *S. glossinidius* infection; 9: negative control; 10: positif control (genomic DNA of *S. glossinidius*); M: GeneRuler 50 bp Ladder (Thermo Fischer Scientific).

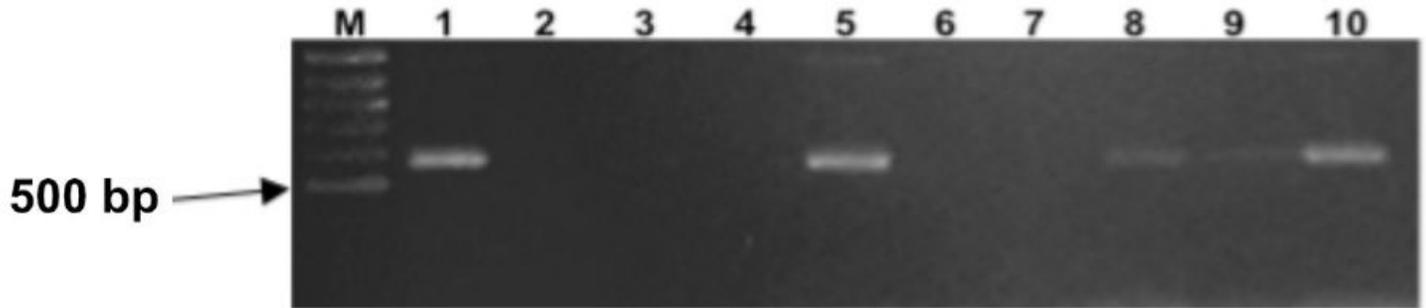


Figure 3

Electrophoretic profile showing the amplified DNA fragment of WSP gene of *Wolbachia*. M: Massruler DNA ladder mix (thermo Fischer Scientific); 1: positif control (genomic DNA of *Wolbachia* sp.); 2: negative control; 5, 8, 9 and 10: samples with *Wolbachia* infections; 3, 4, 6 and 7: samples without infection due to *Wolbachia*.