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RESEARCH ARTICLE

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Ecodistribution, infection rates and host preference of tsetse flies in the sleeping sickness focus of Bonon, west-central Côte d'Ivoire

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The sleeping sickness focus of Bonon was the last one still active at a low endemic level in Côte d'Ivoire. An entomological survey carried out in June 2015 during the rainy season using "Vavoua" traps guided subsequent control activities. Indeed, it improved knowledge of tsetse fly ecology. All the tsetse flies caught (i.e. 1909) belonged to the subspecies *Glossina palpalis palpalis* (Robineau-Desvoidy, 1830), the major vector of Human African Trypanosomiasis (HAT) in Côte d'Ivoire. In this paper, we looked at the relationship between the apparent density (AD, flies/trap/day) and biotopes. The AD significantly varied according to biotopes, with high density around villages. The trypanosomes overall infection rate (mature and immature) according to microscopic observation was 23.2%. When considering mature infections, the infection rate was 5.5 %. Polymerase chain reaction (PCR) analyses confirmed the presence of *Trypanosoma brucei* s.l. and *Trypanosoma congolense* "forest type". Blood meals analysis using cytochrome *b* gene sequences revealed that tsetse flies fed on pigs. The edges of the villages seem to constitute preferred habitats for tsetse flies where they are protected from insecticide pressure in the fields, and where they can easily take bloodmeals from free-ranging pigs. The findings of this study provided a baseline in decision-making for subsequent vector control activities.

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INTRODUCTION

Tsetse flies (*Glossina* Wiedemann, 1830 species) are important cyclical vectors of protozoan parasites, trypanosomes, which cause animal and human African trypanosomiasis. Human African Trypanosomiasis (HAT) is a parasitic, neglected tropical disease that mostly affects remote rural and poor communities in Africa (WHO 2013; Büscher et al. 2017) thus maintaining the cycle of poverty. In West Africa, the aetiological agent, *Trypanosoma brucei gambiense* Dutton, 1902, is transmitted to humans by tsetse flies belonging to the genus *Glossina*.

In Côte d'Ivoire, the development of cash crops (coffee and cocoa) replacing the original forest in the western part of the country has led to profound changes in biotopes, which have become more favourable to the main vector of HAT, *Glossina palpalis palpalis* (Robineau-Desvoidy, 1830) (Laveissière and Hervouët 1988, Djohan et al. 2015). According to the World Health Organisation (WHO), the country was the second most affected by HAT in West Africa in the 2000s (Simarro et al. 2010). In more recent years, the focus of Bonon, located in the western-central part of the country, has reported the most cases (Djè et al. 2002, Koffi et al. 2016) with 13 of the 23 cases reported in the country from 2013 to 2023 (Franco et al. 2024). The last case reported in this focus dates to 2022. Studies carried out in this focus since the 2000s have identified only *G. palpalis* (Courtin et al. 2005; Ravel et al. 2007; Kaba et al. 2021). However, previous studies around this area had revealed a diversity of species (Challier and Gouteux 1980).

The geographical distribution of sleeping sickness results from a complex interaction between pathogenic trypanosomes, the tsetse, wildlife, and behavioural patterns of humans and livestock that expose these hosts to infective flies. Therefore, elimination of sleeping sickness relies on understanding this ecology to protect humans from infective tsetse, to break the disease cycle. Indeed, vector control is considered by WHO as an important tool contributing to the elimination of gambiense HAT (FAO and WHO 2022).

An entomological survey was undertaken in Bonon focus in 2015, with summarised results on tsetse distribution and abundance already published (Kaba et al. 2021). In this study we provide more detailed information and data analysis from this survey, including biotope preferences, infection rates and blood meal source, to improve the understanding of the relationship between trypanosomes, tsetse, and their human and animal hosts. These data provided an important starting point to help define the follow-up tsetse fly control strategy (Kaba et al. 2023).

MATERIALS AND METHODS

Study area

The study was carried out in June 2015 in the Bonon focus located in the Western-central part of Côte d'Ivoire (Figure 1). This period corresponds to the rainy season, and the annual rainfall is around 1200 mm. Bonon focus is in a mesophyll forest area, although nowadays the forest has almost completely disappeared, replaced by coffee and cocoa plantations. In the town and its surroundings, many livestock farms occur, harbouring pigs, goats, sheep and cattle (Courtin et al. 2005; N'Djetchi et al. 2017).

Entomological survey

Tsetse flies were collected with Vavoua traps (Laveissière and Grébaut 1990), deployed for two days in favourable locations for tsetse flies and human-tsetse contact such as water supply points, intersection runways and watercourses, bathing areas and edge of villages' plantation. Trap sites were selected to include all vegetation types/habitats that could be favourable to fly reproduction, feeding and resting (Gouteux et al., 1983). Trapping sites were georeferenced and biotopes were described. Biotopes were divided into 7 categories according to our knowledge about tsetse fly bio-ecology and human activities in this area. Coffee and cocoa plantations which have replaced mesophilic forest represent a large part of Bonon focus's landscape. Rice cultivation in the lowlands is one of Bonon's main activities. Lowland rice cultivation are therefore potential areas of contact between humans and vectors. Village's edge and Bonon town have been considered to assess the risk of suburban transmission of trypanosomiasis. Water courses and riparian forest have been considered because of their ecological importance for certain tsetse fly species, of the *palpalis* group. Finally, we considered fallows to assess the behaviour of tsetse flies in areas where humans are not present in high numbers.

Tsetse flies were collected daily and identified by species and sex using morphological characteristics as described by FAO identification key (Pollock 1982). A random subset of non-teneral flies were then dissected in a drop of sterile saline solution using binoculars to determine their ovarian age and to look for trypanosomal infections. The age of female tsetse was assessed by observing the content of the uterus and the relative size of the follicles in each of the two ovarioles and in each of the two ovules that constitute each ovary. The sub-division of each of the age category was carried out as described in Sané et al. (1999).

Blood meals analysis

Blood meals were collected during tsetse dissection in case of presence of blood in the midgut. The contents were smeared onto numbered sector on filter paper (Whatman No. 4) (Späth

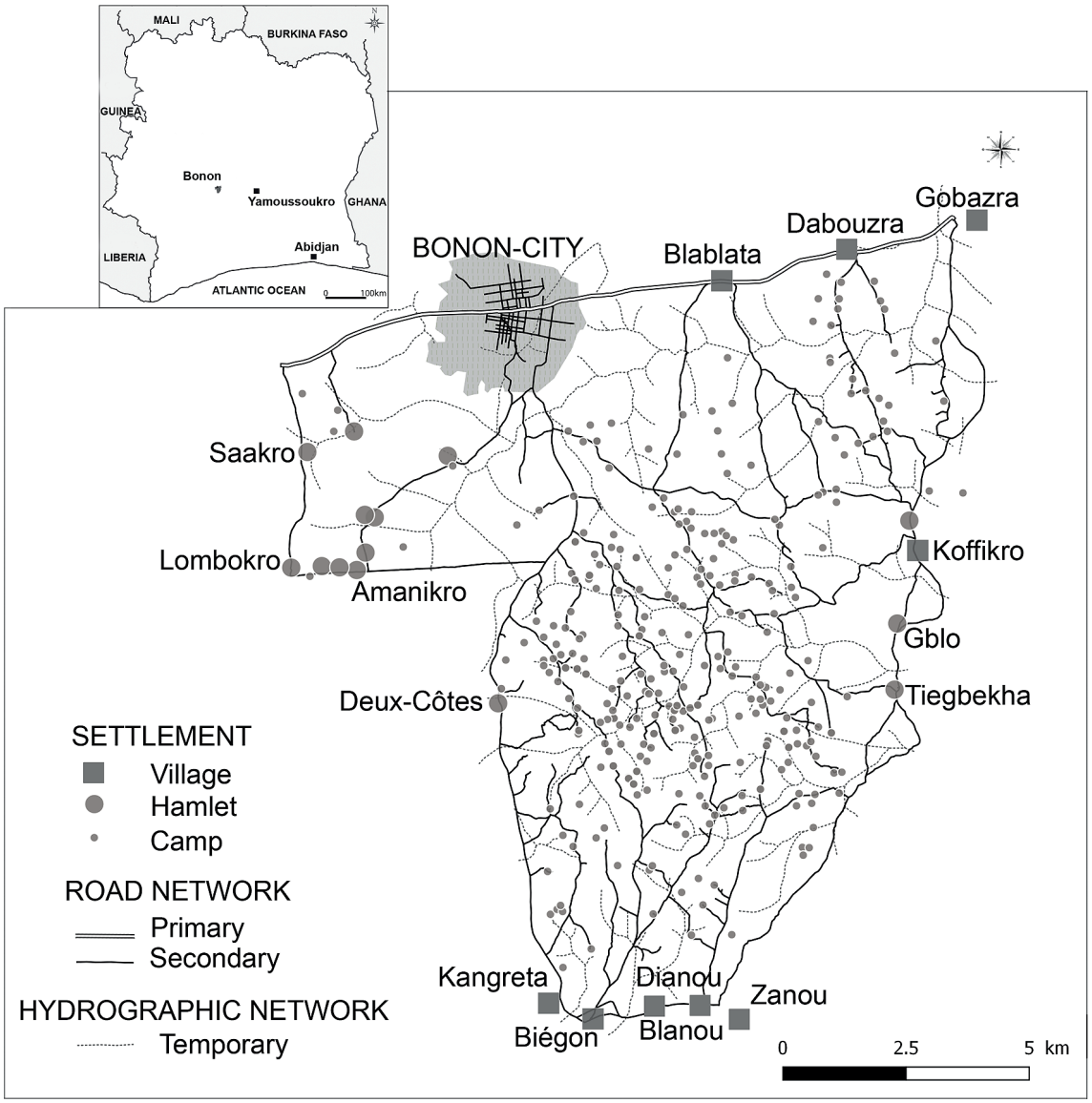

Figure 1: Location map of study area

2000), dried and packed in aluminium foil. The packed blood meals were placed in a sterilised jar containing silicagel until they were analysed.

Blood meal DNAs were extracted using chelating resins as describe by Walsh et al. (1991). 200 µl of 5% Chelex 100® (Chelating Ion Exchange Resin, Biorad, CA, USA) were added for each tube containing blood meal. These were heated at 56 °C for an hour and 95 °C for 30 min. Tubes were centrifuged at 14 000 rounds per minute for 3 minutes at room temperature then supernatants were used for subsequent PCR assay (Njiokou et al., 2004).

PCR was performed using universal primers complementary to the conserved region of the cytochrome *b* gene of the mitochondrion DNA of vertebrates to amplify 358 bp segment of the vertebrate host mitochondrial cytochrome *B* (Farias et al. 2001; Hebert et al. 2003; Steuber et al. 2005). Primers sequences were:
CybBF: 5' CCCCTCAGAATGATATTTGTCCTCA 3'
CytBR: 5'CCATCCAACATCTCAGCATGATGAAA 3'

A negative control containing distilled water was run in parallel. The PCR reaction was carried out in a Thermal Cycler PTC 100 (Biorad, CA, U.S.A.) with the following parameters: DNA initial denaturation and polymerase activation at 95°C for 3 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 50 s, primer extension at 72°C for 40 s, and final extension at 72°C for 5 min. An amount of 7 µl of each PCR product was checked by electrophoresis in 2% agarose gel and visualised by ethidium bromide staining under UV light. PCR products were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA, U.S.A.) using the manufacturer's instructions, then sent for sequencing to GATC biotech.

For each PCR product sequenced, forward and reverse sequences were aligned and traces examined using CodonCode Aligner (CodonCodeCorporation). The sequences obtained (358 bp) were compared to reference cytochrome *B* gene sequences from the GenBank/EMBL/DDBJ database, using blast searching (Lah et al. 2012). The origin of blood meal sample was assigned to the reference species which showed the highest percentage of homology with our sample.

Identification of trypanosomes

Mouth parts, salivary glands and midguts were isolated and examined for trypanosome infection with microscopy (Pollock, 1982). When at least one of these organs was found to be infected, all three organs were collected separately in microfuge tubes containing 25 µl of sterile water. Samples were stored at –20°C and sent back to the laboratory until use. DNAs were extracted using Chelex method. 25 µl of 5%. Chelex 100 were added for each tube containing organ. These were heated at 56°C for an hour and 95°C for 30 min. Tubes were centrifuged at 14 000 rounds per minute for 3 minutes at room temperature and 5µl of supernatant were used for PCR assays.

Primers specific for *Trypanozoon* (i.e. *T. brucei s.l.* (Moser et al. 1989), *T. vivax* (Masiga et al. 1992) and riverine forest type of *T. congolense* (Masiga et al. 1992) were used. We did not use primers specific to the savannah type of *T. congolense* because previous studies have shown the absence of this type of parasite in this area (N'Djetchi et al., 2017). DNA samples were amplified in a final volume of 25 µl. Tubes were incubated at 95°C for 3 min as an initial denaturation step, followed by 40 cycles of 30 sec at 95°C, 30 sec at 55°C, and 1 min at 72°C. A final extension was processed at 72°C. A 7 µl aliquot of each sample was analysed on an agarose gel, stained with ethidium bromide and photographed under UV illumination.

Data analysis

The average Apparent Density (AD) of tsetse flies was determined by summing daily catch and dividing by the number of days and the number of traps. Infection rates were calculated by dividing the number of infected tsetse flies by the total number of tsetse flies we dissected and expressed as percentage. Sex-ratio was estimated by dividing the number of males by the number of females (M/F). We tested the average number of flies caught per trap per day between landscapes using the Kruskall-Wallis test. The statistical significance of differences in catch was assessed using Dunn's nonparametric pairwise multiple-comparison test with Benjamini. A two-tailed Fisher's exact test was used to compare rates of infection All statistical analyses were carried out using R 4.0.2 (https://www.r-project.org/).

RESULTS

Entomological parameters

All the tsetse flies captured were identified as *Glossina palpalis*. They were assumed to belong to the subspecies *G. palpalis palpalis* based on the criterion of geographical distribution of the two subspecies of *G. palpalis* established by previous studies. (Challier et al., 1983; Nekpeni et al. 1989) A total of 1909 tsetse flies were captured with 277 traps for two days, giving an AD of 3.4 ± 14.6 ranging from 0 to 129.5 flies depending on the traps as published in Kaba et al (2021). The highest AD was observed at the edges of the villages (13.8 flies/day/trap), followed by Bonon town (2.0 flies/day/trap) (Table 1). The Kruskal-Wallis test revealed significant difference of ADTs according to the landscapes (p = 0.02). Although the density at the villages' edges was very high compared with the other sites, Dunn's post hoc test only revealed significant differences of densities with riparian forest and fallow (Table 2).

The 1909 flies consisted of 430 males and 1479 females, for a sex-ratio (M/F) of 0.3. A total of 436 flies (328 females and 108 males) were dissected and observed under the microscope. Among them, 101 were found infected hence a parasitological overall trypanosomes infection rate of 23.17% ±25.4 (Table 1). When considering separately the different organs, 76.2% of the infections were found in the midgut only, and are therefore considered as immature infections, 10.5% in the mouth parts only (*T. vivax*-type) and 13.3% were mixed infection of midgut and mouth parts (*T. congolense*-type). No salivary gland infection

Table 1: Tsetse flies' abundance and infection rate according to landscapes, with standard deviation (SD)

Landscape	Number of traps	Number of trapped flies	ADT ± SD	Dissected	Infected	Infection rate (%)
Bonon town	16	64	2.0 ± 3.3	47	10	21.3
Fallow	36	13	0.2 ± 0.4	9	1	11.1
Lowland rice cultivation	58	54	0.5 ± 0.9	31	8	25.8
Cocoa and coffee orchard	68	119	0.9 ± 2.3	81	11	13.6
Riparian forest	25	11	0.2 ± 0.8	10	1	10.0
Village's edges	59	1624	**13.8 ± 29.5**	246	67	27.2
Water courses	15	24	0.8 ± 1.7	12	3	25.0
Total	**277**	**1909**	**3.44 ± 14.6**	**436**	**101**	**23.2**

Table 2: Results of post-hoc comparisons of tsetse densities by landscape using Benjamini–Hochberg correction

	Bonon Town	Lowland rice cultivation	Ochard	Riparian forest	Water course	Fallow
Lowland rice cultivation	0.1114					
Ochard	0.1615	0.4155				
Riparian forest	0.0571	0.2166	0.1698			
Water course	0.1684	0.4751	0.4413	0.2896		
Fallow	0.058	0.32	0.2255	0.397	0.3722	
Village	0.4116	0.0676	0.1021	**0.0477***	0.1815	**0.0369***

(*T. brucei*-type) was found. The infection rate was significantly higher in female than in male (p = 0.03). When considering mature infections, i.e. mouth parts' infection and mixed infection of midgut and mouth part, the infection rate is 5.5 %.

The 328 female flies dissected were divided into 45.7% of old parous, 48.7% of young parous and 2.6% of nulliparous while 3% of females dissected could not be aged. The proportion of young flies (young parous + nulliparous) was 51.3% and there was no significant difference in numbers between young and old flies.

Host preferences

Out of the eight (8) blood meals collected from dissected tsetse flies, seven could be sequenced. Sequencing of cytochrome *B* genes showed that for the 7 blood meals, BLAST exhibited 99% similarity with the pig sequence (*Sus scrofa domesticus*).

Identification of trypanosomes

PCR could be performed on 36 samples of the 101 tsetse flies positive with microscopy. In 19.4% (7/36) of these flies, the species of trypanosome was identified: six were *T. brucei* and one was *T. congolense* forest type.

DISCUSSION

Tsetse flies' division in different biotopes is related to their survival conditions (WHO 1986). Several studies have been carried out in the focus of sleeping sickness of Bonon in the early 2000s (Solano et al. 2003; Jamonneau et al. 2003, 2004; Courtin et al. 2005). Population movements during political crisis contributed to a doubling of Bonon's population between 2002 and 2015 (Krouba et al. 2018). The rate of net forest decreased from 7% to less than 1% from 2002 to 2015, leaving only the sacred forest (Coulibaly et al. 2018). Outbreaks of Cacao Swollen Shoot Disease have been confirmed in this area in 2003 (Kébé and N'Guessan 2003). It has contributed to reduction of the surface of cocoa plantations from 63% to 26% in the same period (Coulibaly et al. 2018), which has been replaced by food crops, rubber and cashew. These factors have certainly significantly modified the distribution of tsetse flies. Only one tsetse subspecies was caught, *Glossina palpalis palpalis* which is known as the major vector of HAT in Côte d'Ivoire (Allou et al. 2009, Fauret et al. 2015). These observations are like those of studies carried out in recent years (Courtin et al. 2005; Ravel et al. 2007). However, Challier and Gouteux identified five tsetse species in this region, divided between the three groups of *Glossina*, some forty years ago (1980). This reflects the global changes underway in the area. Formerly, the western part of Côte d'Ivoire was mesophilic forest in which were found tsetse belonging to the *fusca* group and to a lesser extent, tsetse from the *palpalis* group. The destruction of forest for cash crops has led to the decline of tsetse of the *fusca* group, which are zoophilic (Gouteux 1991). On the opposite, those of the *palpalis* group especially *G. p. palpalis,* can subsist in this recently anthropised area and it was noted in the past that coffee and cocoa plantations were areas with high trypanosomiasis risk (Roubaud 1913; Laveissière and Hervouët 1981; Franco et al. 2014; Djohan et al. 2015). The fact that densities are currently low in these areas may be attributed to the massive use of pesticides and insecticides, combined with forest destruction (Tano 2012). Indeed, no resistance of tsetse flies to insecticides has yet been reported. The edges of the villages seem to constitute new habitats for tsetse flies where they are protected from insecticide pressure of the fields, and where they can easily take bloodmeals from free-ranging pigs. We also observed a strong presence of tsetse flies in Bonon town. Indeed, the town of Bonon is made up of five villages whose inhabitants have preserved their rural way of life. The activities therefore do not differ from those practised in the villages. Agriculture and traditional breeding are the main activities in these areas. The existence of an urban transmission of HAT was reported in Bonon (Courtin et al. 2005). Several studies also suggested a suburban sleeping sickness transmission in Libreville, Gabon (Kohagne et al. 2013) and in Kinshasa, Democratic Republic of the Congo (Simo et al. 2006; Lumbala et al. 2015) among others. This presence of tsetse within and around villages also reflects the resilience of this species (*G. p. palpalis*) and its adaptations to an anthropised environment, at least to a certain extent.

Difference in flies' sex-ratio was largely in favour of females (1 male for 3 females) and this is in agreement with Laveissière et al. (2000). It has been proved that some types of traps catch a higher proportion of females than other sampling methods (Leak et al. 2008). The gestation period, which increases appetite in tsetse, also results in the increased capture of females (Bouyer et al. 2015). However, Leak (1998) reported that females could represent 70% to 80% in an unbiased sampling. The predominance of females could constitute an additional risk factor because of their higher feeding frequency and long-life span, when compared to males (Kazadi et al. 2000).

Out of the total number of tsetse caught, more than half were young (51.3%). This agrees with Kaba et al. who found in 2006, 53% of young flies near Abidjan, Côte d'Ivoire (Kaba 2006). Young flies will become more dangerous as they mature. The infection rate of 21.5% in our study, that includes all trypanosomes found in the tsetse, is comparable to the rates of 28.7% reported by Jamonneau et al. (2004) and the 25% reported by Courtin et al. (2005) and Ravel et al. (2007) in the same area. More females were infected (24.2%) than males (14.42%) as reported in earlier studies in Côte d'Ivoire (Yao et al. 1997; Bosson-Vanga et al. 2012). Females are more likely to be infected because of their greater number of bloodmeals (Laveissière et al. 2000). We observed trypanosomes only in the proboscis and in both the proboscis and midgut. This suggests the presence of *T. vivax* and *T. congolense* respectively, parasites responsible for animal trypanosomiasis. Trypanosomes were in flies' midgut and/or mouthparts. However, the absence of salivary gland infection does not necessarily mean absence of *T. b. gambiense*, the etiological parasite of sleeping sickness, because even in areas of trypanosomiasis transmission, the prevalence of this parasite in tsetse is very low, when examined parasitologically (Jamonneau et al. 2004). In addition, it has been demonstrated in this same area that pigs are heavily infected with trypanosomes belonging to *T. brucei* s.l. (Jamonneau et al. 2004; N'Djetchi et al. 2017). It should be noted that the identification

of trypanosome species according to their location in tsetse flies is not very reliable due to the low specificity microscopic examination. Infection of an organ can also go unnoticed or be temporary (Yoni et al., 2005). Identification of the species of trypanosomes present in the tsetse flies indicated the presence of *T. brucei* s.l. (which may include *T. b. gambiense*) and *T. congolense* forest type. Technical problems prevented the identification of trypanosomes in a large proportion of infected organs and a more specific testing for *T. brucei gambiense* infections, which are limitations of the study.

Only 8 blood meals could be collected out of 488 tsetse flies dissected (1.63%). Although a bit disappointing, this result was expected since tsetse caught in traps are mainly the ones that are looking for bloodmeals (Leak 1998), together with the ones flying for mating. Nevertheless, all the blood meals analysed were identified as coming from pigs. Most studies on the feeding behaviour of peridomestic population of tsetse flies in Côte d'Ivoire have highlighted their preference for pigs. Indeed, an analysis of 84 blood meals from peridomestic populations of *Glossina palpalis palpalis* and *G. longipalpis* in central Côte d'Ivoire showed that 94% of them came from pigs (Späth 2000). Dagnogo et al. (1985) showed that 98% of the blood meals examined from *G. palpalis* were from pigs in savanna-forest mosaic area, this rate was 72% in the pre-forest area (Gouteux et al. 1982). Tsetse flies of *palpalis* group are reported to be opportunistic regarding their bloodmeal sources in natural conditions, and it has been reported that on the edge of villages in the western central Cote d'Ivoire they feed almost exclusively on pigs (Laveissière et al. 1985). Indeed, villages where pig breeding is practised are adjacent to wetlands Climatic conditions prevailing in these basins favour the gathering of free-ranging pigs. These biotopes are also place of refuge for tsetse flies (Gouteux 1982). These results confirm *G. p. palpalis* food opportunism, pigs wandering in villages constituting their preferred host as showed by several studies (Laveissière et al. 1985; Sané et al. 2000). Based on these observations, feeding on humans in such areas appears more accidental than frequent, but each time it happens, it represents a risk for humans to be infected by *T. b. gambiense*.

CONCLUSION

This study brings further knowledge on the peridomestic behaviour of *Glossina palpalis palpalis* in a HAT focus in Côte d'Ivoire. Its findings helped design vector control activities that were subsequently initiated in 2016 (Kaba et al, 2021 & 2023) and sustained until 2023. The data collected in this type of survey will also help improve the mapping of tsetse distribution at the national and continental level in the framework of the atlas initiatives (Boulangé et al., 2022; Cecchi et al, 2024). Our results highlighted the importance of baseline data collection to characterize the targeted ecosystem before any control measure is implemented, and the findings also suggest that it would be useful to improve our understanding of the role of pigs in HAT epidemiology.

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