

High Trypanosome Infection Rate and Blood-Feeding Patterns of Tsetse Flies in South Ethiopia

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Abstract

Background

Tsetse control has been implemented for decades using various methods. However, the impact of these interventions on tsetse infection rates remains unclear, highlighting the need for more comprehensive data on trypanosome infection rates and tsetse feeding patterns. This study aimed to assess trypanosome infection rates and feeding patterns in tsetse flies collected with different attractants in south Ethiopia.

Materials and Methods

The study was conducted in the Deme River Valley, Gamo Zone, South Ethiopia. Each month, sampling was conducted over two consecutive days at each site using 10 NGU traps, with trap locations rotated. Traps were baited with acetone, fermented cow urine, both, or left unbaited as controls. Anesthetized and recently deceased tsetse flies were examined for infections using microscopy, while the sources of their blood meals were analyzed using multiplex polymerase chain reaction (PCR). We used the Poisson regression model to analyse count data and the binary logistic regression model to assess binary outcomes (positive or negative) and infection rates.

Results

Of the 1208 tsetse flies captured, 751 (62.2%) were *Glossina pallidipes*, while 457 (37.8%) were *Glossina fuscipes fuscipes*. The overall trypanosome infection rate in the tsetse flies was 17.1% (103/603) (95% CI: 14.2–20.3). Out of the 103 flies that tested positive through microscopy, 66% were found to be infected with *Trypanosoma congolense*, 24.3% with *Trypanosoma vivax*, 1.9% with *Trypanosoma brucei*, and 7.8% with either *T. congolense* or *T. brucei*. The infection rate of trypanosomes in *G. pallidipes* was 19.6%, while it was 12.8% in *G. f. fuscipes*. Traps using a combination of cow urine and acetone captured more trypanosome-positive tsetse flies (21.2%), followed by acetone alone (18.9%), cow urine alone (12.2%), and traps without odour bait (6.1%). Of 107 freshly fed tsetse flies tested using multiplex polymerase chain reaction, 23.4% had fed on dogs, 8.4% on humans, 7.5% on cattle, and 4.7% on goats, including mixed blood meals.

Conclusions

The high trypanosome infection rate in tsetse flies suggests an increased risk of parasite transmission. The blood meal sources highlight the potential risk to humans. More research is needed to clarify the role of dogs in the transmission of parasites.

Introduction

Animal Trypanosomiasis (AT) is a major problem in animal production in sub-Saharan African countries, including Ethiopia [1]. The disease is caused by protozoan parasites called trypanosomes, and is transmitted cyclically by the tsetse fly (*Glossina* spp.) [2]. Several species of *Trypanosoma*, including *Trypanosoma congolense*, *Trypanosoma vivax*, and *Trypanosoma brucei brucei*, cause AT [3]. The parasites primarily affect vertebrate animals, particularly cattle [4, 5]. In Ethiopia, around 180,000 to 200,000km² of agricultural land, 14 million cattle and small ruminants, roughly 7 million equines, and 1.8 million camels are at risk of the disease [6]. Although there are five species of tsetse flies, namely *Glossina morsitans submorsitans*, *Glossina pallidipes*, *Glossina fuscipes fuscipes*, *Glossina tachinoides*, and *Glossina longipennis*, *Glossina pallidipes* is the most economically important species of AT in Ethiopia [6].

The World Health Organization reports that over 55 million people in Sub-Saharan Africa are at risk of human African trypanosomiasis (HAT) infection [4]. In Ethiopia, HAT was first identified in 1967 in the Baro and Akobo River valleys, and later in the southern Omo River basin [7, 8]. In 2022, an outbreak of HAT caused by *T. b. rhodesiense* occurred in the Deme River Valley, fifty-five years after the initial epidemic in western Ethiopia [9]. This suggests ongoing human exposure to tsetse flies and highlight the need to assess their blood meal sources to better understand human-vector interactions.

Tsetse flies usually feed on wild game animals instead of domestic animals. When wild game is not available, they often feed on domestic animals, especially cattle [10]. Knowing where tsetse flies get their blood meals helps identify which hosts might spread parasites and guides management plans. Understanding how tsetse flies choose their hosts and feed is important for assessing the risk of human infection and creating effective control strategies.

Controlling tsetse flies involves methods like insecticide spraying and the use of traps[11]. These traps, which can be enhanced with odour baits, attract flies visually. It is essential to identify the most effective baits considering the available resources and the specific species of the vector. This is crucial for the success of control programs [11].

Several programs were launched to control tsetse flies in the Deme River Valley [9]. In 1997, the Southern Tsetse Eradication Project (STEP) began deploying insecticide-impregnated targets, ground and aerial spraying, and cattle pour-on treatments using synthetic pyrethroids, applying 1% deltamethrin to cattle every two to three months [12]. From 2011 onward, sterile males of *G. pallidipes* and *G. f. fuscipes* were released weekly by aircraft for seven years. However, between 2011 and 2014, insecticide-based methods were suspended to avoid killing the released sterile males. This interruption caused a sharp rise in trypanosome infections in cattle, and farmers have since expressed dissatisfaction with renewed tsetse control efforts following the resurgence of tsetse flies. Additionally, the 2022 outbreak of HAT caused by *T. b. rhodesiense* was alarming [9].

The objective of this study was to assess trypanosome infection in tsetse flies and to analyze their blood-meal sources collected using various odour baits. The resulting data are intended to inform and enhance the efficiency of future trapping campaigns in the study area and adjacent regions.

Materials and methods

Study area description

The study was conducted in South Ethiopia in the Deme River Valley, Gamo Zone. The Deme River basin contains several small and large streams. The largest river is the Deme River, which feeds the Kulano River. At Dana *Kebele* (the smallest administrative unit in Ethiopia), the Gogara River meets the Deme River, and the combined rivers flow into the Omo River Valley (Fig. 1). Another minor tributary of the Deme River is the Kurtume River, which joins the Deme River in Sikole *Kebele*. The study area has a sub-humid climate with moderately high temperatures and an average annual rainfall of 900–1800 mm. The dry season stretches from December to March, while the wet season is from April to November. The highest recorded temperature was 32.8°C. The cooler months are June, July, and August, with the lowest temperature being 17.5°C.

The three *Kebeles* for our study are in rural areas near the savanna grasslands and riverine forests, which provide homes for wild animals. This ecological condition creates an optimal environment for tsetse fly reproduction.

Cattle, goats, and sheep are the primary domestic animals, while horses are rare. Dogs are also common in the areas. Surveys conducted in 1998 recorded *Glossina pallidipes* and *G. f. fuscipes* in the Deme River Valley [12]. Wild animals in the valley include gazelles, warthogs, bush pigs, monkeys, hyenas, and rabbits.

Study design and period

Randomized complete block design over time was employed to collect tsetse flies using odour-baited Nguruman (NGU) traps from September 2023 to February 2024. Sampling occurred over two consecutive days each month, with attractant locations rotated monthly. Ten NGU traps were placed in selected locations in each *Kebele* daily, resulting in 20 monthly trapping events. Of the ten traps, three were baited with acetone released at 500 mg/h from glass bottles with a 6 mm diameter. Another three traps were baited with fermented adult cow urine released at 1000 mg/h from plastic bottles with a 45 mm aperture [13]. Three traps were baited with a combination of cow urine and acetone. One trap was installed without any odour bait. A bait container was placed at the bottom of each trap. The feet of the traps' central and lateral poles were greased to deter predators. Fly sampling took place for six months, and 360 trapping events were conducted across the three *Kebeles*, with 120 in each.

We performed monthly randomization and rotation of odour baits to minimize variation due to trap location. This was achieved by creating ten lottery cards with codes representing odour baits. Upon

arriving at a trap location, a card was randomly drawn, and the corresponding odour was used. Each position had an equal chance of receiving any odour during the initial round. In the following rounds, the odour used in the previous month was excluded from the selection for the same position. This approach was implemented to avoid repeating the same odour at a location until all sites had been assigned a different odour. The diagram illustrating the rotation experiment is provided in the supplementary figure as an example for one *Kebele*.

We used GPS to measure the distance between the traps and Kebeles. In each *Kebele*, traps were spaced 200 to 250 meters. The distance between Dana and Gale was 6.8 kilometres, between Sikole and Dana was 3.2 kilometres, and between Sikole and Gale was 4.1 kilometres.

Tsetse fly sampling

Ten *Kebeles* near the Deme River Valley are infested with tsetse flies, and of these, three *Kebeles* (Gale, Sikole, and Dana) were randomly selected for the study. NGU traps were placed in the shade of a tree, 20 cm above the ground, to capture tsetse flies. The traps were set at 8:00 AM, collected at 6:00 PM the same day, and then redeployed the next morning at the same location. The trapping sites were kept open to allow the flies to see the traps. When there was no open location to deploy the traps, the vegetation obstructing trap visibility was cut within a 3–6-meter radius around the trap, depending on the height of the vegetation. The traps were set up in various locations, including riverbanks, forests, farmland, and village areas. The typical habitats include communal grazing areas with wooded grassland and riverine forest. Each trap in the field was labelled with the date, collection site, and bait type and then transported to the nearby animal health center. To quickly prepare live flies for dissection and parasite identification, they were placed in an icebox during transportation. Freshly fed flies were placed individually in a vial with silica gel for blood meal origin analysis. All the flies were identified at the species level.

Tsetse identification

The tsetse control personnel training manual key for *Glossina* species identification was used to distinguish between different species of tsetse flies[14]. The male fly has a hypopygium on the posterior end of the ventral abdomen, which is absent in a female tsetse fly.

Tsetse dissection and parasite identification

All live and recently deceased non-teneral flies, excluding those that were freshly fed, were selected for dissection. Trap cages were stored in an icebox during transportation to the Animal Health Centre. Upon arrival, flies were removed from the cages and euthanized by applying gentle pressure to the thorax. The location, bait type, cage label and coordinates were recorded. The species and sex of each fly were identified [14].

Only freshly euthanized or recently deceased flies were dissected. This was important because trypanosomes are less likely to be found in dead, desiccated flies. Initially, a drop of saline solution was placed in the Centre of a petri dish, and the fly was positioned on its back on top of it to prevent dehydration and maintain the isotonic state of the dissected organs. Using fine watchmaker forceps, each wing and leg was carefully removed from the fly, ensuring other organs were not damaged.

The process was conducted in a specific sequence to prevent contamination caused by trypanosomes found in the midgut during dissection [15]. First, the proboscis was dissected, followed by the salivary glands, and finally, the midgut. To start, the proboscis was firmly held on the thecal bulb and removed with the help of fine forceps. The labrum, hypopharynx, and labium were separated with the tip of tiny forceps and a hypodermic needle. The dissected organ was placed in one drop of saline solution on the glass slide and covered with a cover slip. The fly's thorax was held with one pair of fine forceps, the base of the abdomen was grasped with a second pair of fine forceps, and the abdomen was gently pulled back so that the "skin" slowly tears while the gut and other internal contents stretch out but do not break. Salivary glands on each lateral side of the abdomen were picked from the lateral sides of the first abdominal segments of the fly using a pair of fine forceps and then placed on a glass slide with a drop of 0.9% saline solution and covered with a cover slip. The abdominal contents were squeezed or pulled from the skin, and the mid-gut was picked up and placed on a glass slide with a drop of saline solution on one side, covered by a cover slip. To avoid contamination of the sample by dissecting needles and forceps, the needles and forceps were wiped with clean cloths, rinsed with sterile distilled water, and immersed in saline solution between dissections of each organ and fly.

The samples from all three dissected organs (mid-gut, salivary glands, and proboscis) were examined under a compound microscope at 400× total magnification, using a 10× eyepiece and a 40× objective. The presence of trypanosomes was determined by the motility in the places (organs) of the fly where trypanosome parasites were isolated due to their life cycle. Thus, infection in the labrum or hypopharynx alone (the proboscis) was classified as 'vivax-type.' Infections in the proboscis and mid-gut were classified as 'congolense-type,' while infections of the proboscis, mid-gut, and salivary glands were classified as 'brucei complex' infections[16]. Mixed trypanosome infections in tsetse flies may involve parasites at different developmental stages. For instance, immature *T. congolense* and mature *T. vivax* can co-occur when a tsetse fly acquires *T. vivax* during the extended developmental period of *T. congolense*. In this scenario, *T. vivax* reaches maturity in the proboscis before the immature *T. congolense* arrives. *Trypanosoma brucei* infections primarily localize in the mouthparts of tsetse flies, especially within the salivary glands.

Blood meal source identification

Freshly fed tsetse flies were carefully collected and stored in individual Eppendorf tubes. The tubes were placed in a plastic zip bag with silica gel beads and transported to the Advanced Medical Entomology and Vector Control laboratory at Arba Minch University. Upon arrival, the specimens were kept in a deep freeze at -80°C.

DNA extraction for blood meal origin detection was done following the Cetyltrimethylammonium bromide (CTAB) DNA isolation procedure [17]. The samples were analyzed to identify blood meal sources using a multiplex PCR method with primers targeting the mitochondrial cytochrome b gene in humans, dogs, cattle, goats, sheep, pigs, and horses [18]. The master mix was prepared with 1 µl nuclease-free distilled water, 8.5 µl DreamTaq master mix, 0.5 µl of each 25 pmol/µl UnRev for humans, goats, cows, dogs, horses, pigs and sheep (Invitrogen, USA). Then, 13 µl of master mix and 2 µl of DNA template were added, bringing the total volume to 15 µl per reaction. The PCRs were run on the Biometra T professional gradient Thermocycler with the thermocycler program set as: 95 °C/ 5 min x 1 cycle, [95 °C/ 60 sec; 56 °C/ 60 sec; 72 °C/ 60 sec] x 40 cycles, 72 °C/ 7 min x 1 cycle, 12 °C hold for amplification. The PCR products were visualized on a 2% agarose gel (Sisco Research Laboratories, India) using a 100 bp ladder (Promega, the Netherlands). Host species were inferred based on the DNA band size.

Statistical analysis

This study examined two main outcomes: the infection in tsetse flies with trypanosome parasites (a binary outcome), and the number of tsetse flies collected per trap per day. To analyse the infection status, we used a binary logistic regression model, while we employed a Poisson regression model to study the number of flies collected per trap per day. We estimated the parameters for both models using maximum likelihood estimation (MLE) and calculated standard errors to measure the statistical significance of the factors involved. Model fit was assessed using the Akaike Information Criterion (AIC). All analyses were conducted using R statistical software (version 4.1.1), and the ggplot2 package was employed for creating plots.

Results

Tsetse fly species in the study sites using various odour baits

The traps in the three *Kebeles* (120 trapping events in each) captured 1208 tsetse flies, averaging 3.4 per trap per day. The two identified species of tsetse flies were *G. pallidipes* and *G. f. fuscipes*. Out of the 1208 tsetse flies captured, 751 (62.2%) were *G. pallidipes*, with an average catch of 2.1 flies per trap per day. The remaining 457 (37.8%) were *G. f. fuscipes*, with an average catch of 1.3 flies per trap per day. Table 1 summarizes the number of tsetse flies captured in the three study *Kebeles* using various odour baits.

Table 1

The overall number of *G. pallidipes* and *G. f. fuscipes* collected using various odour baits in Deme River Valley in the Gamo zone, South Ethiopia (September 2023 to February 2024)

Kebeles	Number of traps	<i>G. pallidipes</i>		<i>G. f. fuscipes</i>	
		Number of flies	Mean	Number of flies	Mean
Gale	120	130	1.08	82	0.68
Sikole	120	294	2.45	164	1.37
Dana	120	327	2.73	211	1.76
Total	360	751	2.09	457	1.27
Acetone	108	145	1.34	119	1.10
Acetone and cow urine	108	368	3.41	140	1.30
Cow urine	108	223	2.06	153	1.42
Control	36	15	0.42	45	1.25
Total	360	751	2.09	457	1.27
Mean = catch/trap/day					

The trypanosome infection of tsetse flies

Out of a sample of 603 live and recently dead non-teneral tsetse flies dissected to determine the trypanosome infection, 103 tsetse flies were infected with at least one species of trypanosomes, giving an overall infection rate of 17.1% (95% Confidence Interval (CI): 14.2–20.3). Of the 377 dissected *G. pallidipes*, 73 (19.4%; 95% CI 15.6–23.8) had trypanosome infection in their proboscis, salivary glands, mid-gut, or all of these organs and higher than the infection rate among the *G. f. fuscipes* (30 of 226; 13.3%; 95% CI: 9.3–18.6) ($\chi^2 = 6.4$, $P = 0.01$). However, the infection rate of trypanosomes in the *G. pallidipes* group was similar to that of the *G. f. fuscipes* among *Kebeles* and species within the *Kebele* (Fig. 2).

The monthly variation in the number of tsetse flies collected in the study area was not statistically significant (supplementary table). Both acetone alone and a combination of acetone and cow urine attracted more tsetse flies infected with trypanosomes (Fig. 3).

Trypanosoma parasite species in tsetse flies

Out of the 103 positive tsetse flies, 68 flies (66%) were infected with *T. congolense*, 25 flies (24.3%) were infected with *T. vivax*, and two flies (1.9%) were infected with *T. brucei*. Eight flies (7.8%) were infected with either *T. congolense* or *T. brucei* (the immature stages identified in the mid-gut). *Trypanosoma congolense* and *T. vivax* were the most prevalent parasites, detected consistently throughout all six months. In contrast, the *T. brucei* complex was infrequently observed (Fig. 4).

Of the 377 *G. pallidipes* dissected, 18 tested positive for *T. vivax*, resulting in an infection rate of 4.8%. Seven of the 226 *G. f. fuscipes* dissected tested positive for *T. vivax*, resulting in an infection rate of 3.1%. The infection rate of *T. congolense* in *G. pallidipes* was 13.3% (50/377 dissected), while in *G. f. fuscipes*, the infection rate was 7.9% (18/226 dissected). Two *G. f. fuscipes* tested positive for *T. brucei* complex. Of the eight tsetse flies that tested positive for either *T. congolense* or the *T. brucei* complex, five were identified as *G. pallidipes* and three as *G. f. fuscipes*, with immature forms detected in the mid-gut. Among the three *Trypanosoma* species identified in the study area, *T. congolense* and *T. vivax* were present in all three *Kebeles* during the study period (Fig. 5).

Trypanosome infection rates in tsetse flies varied by bait type: cow urine and acetone (21.4%; 55/257), acetone alone (19.8%; 25/126), and cow urine alone (11.2%; 21/187). A statistically significant difference in infection likelihood was found between cow urine and acetone (P = 0.034), while tsetse fly species and geographical location (*Kebeles*) showed no significant effect. Traps deployed without any odour bait captured tsetse flies with the lowest infection rate (6.1%; 2/33) (Table 2).

Table 2
The estimated trypanosome infection rate of tsetse flies between odour baits, the three study *Kebeles* and tsetse species in the Deme River Valley in the Gamo zone, South Ethiopia (September 2023 to February 2024)

Predictors	Categories	Estimate	Std. error	Statistic	P. value
Intercept		-1.195	0.327	-3.66	< 0.001
Odour types	Cow urine	-0.682	0.323	-2.11	0.034
	Acetone and cow urine	0.04	0.273	0.146	0.883
	Control	-1.238	0.767	-1.61	0.106
	Acetone(ref)				
Species	<i>G. fuscipes</i>	-0.343	0.243	-1.42	0.156
	<i>G. pallidipes</i> (ref)				
Kebele	Sikole	-0.071	0.30	-0.23	0.814
	Dana	-0.079	0.293	-0.27	0.785
	Gale(ref)				

Blood meal sources of tsetse flies

Blood meal source identification was conducted on 107 freshly fed tsetse flies. The results showed that 31.8% (34/107) of the tsetse flies tested positive for the DNA of one or more vertebrate animals, while 68.2% (73/107) tested negative for the examined vertebrate animal DNA. None of the freshly fed tsetse flies were positive for sheep, horses, and pigs.

Overall, tsetse flies feed on dogs (*Canis lupus*) (14), cattle (6), humans (*Homo sapiens*) (2) and goats (*Caprines*) (1) (Table 3).

Table 3
The blood meal sources of tsetse flies collected using various odour baits in three *Kebeles* in the Deme River Valley of the Gamo Zone, South Ethiopia (September 2023 to February 2024)

Blood meal sources	Freshly fed tsetse species		Total
	<i>G. pallidipes</i>	<i>G. f. fuscipes</i>	
Unidentified	37	36	73
Human	2	0	2
Goat	0	1	1
Dog	10	4	14
Cattle	5	1	6
Cattle and goat	1	0	1
Goat and dog	2	0	2
Human and dog	5	1	6
Cattle and dog	0	1	1
Human, goat and dog	1	0	1
Total	63	44	107

Efficiency of odour baits in capturing tsetse flies

Using odour baits improved the effectiveness of NGU traps in catching tsetse flies. A combination of cow urine and acetone was the most effective for catching tsetse flies, followed by cow urine alone. The traps without odour bait caught fewer tsetse flies. The trapping effectiveness of cow urine was significantly higher than that of acetone but considerably lower than that of traps baited with a combination of acetone and cow urine. All traps baited with attractants were more efficient than the control in catching tsetse flies (Fig. 6).

The number of tsetse flies captured varied significantly across odour baits and study *Kebeles* (Table 4). Odour baited traps captured significantly more tsetse flies than the control, and both Dana and Sikole *Kebeles* exhibited higher tsetse fly counts compared to Gale.

Table 4

The estimated Poisson regression Log count variation of the number of tsetse flies between odour baits and the three study *Kebeles* in the Deme River Valley in the Gamo zone, south Ethiopia (September 2023 to February 2024)

Predictors	Categories	Estimate	Std. error	Statistic	P value
Intercept		0.26	0.08	3.0	0.002
Odour types	Cow urine	0.34	0.08	4.2	< 0.001
	Acetone and cow urine	0.64	0.07	8.5	< 0.001
	Control	-0.39	0.14	-2.8	0.034
	Acetone (ref)				
<i>Kebeles</i>	Sikole	0.77	0.08	9.2	< 0.001
	Dana	0.93	0.08	11.5	< 0.001
	Gale (ref)				

Discussion

The trypanosome infection rate in tsetse flies was highest for *Trypanosoma congolense*, followed by *T. vivax* and the *T. brucei* complex. Both *Glossina pallidipes* and *G. f. fuscipes* were found to carry these parasites. The higher prevalence is likely due to the flies' feeding habits, which commonly take mixed-blood meals from a variety of vertebrate hosts. The most effective baits for catching tsetse flies were a combination of cow urine and acetone, followed by traps with cow urine alone. Traps without baits had the lowest tsetse fly catch rate.

Two species of tsetse flies, *G. pallidipes* and *G. fuscipes*, were found in the study area. These two species are among the five *Glossina* species found in Ethiopia, particularly in the South Regional State[6]. Both species were documented in the Deme River Valley in 1998[12]. There is a high risk of tsetse fly invasion from the Omo River Valley into the Deme River Valley, and without adequate control, re-invasion is also likely [6]. To prevent tsetse fly invasion, insecticide-impregnated barrier targets were installed at entry points along the Omo River to the Deme River Valley [12]. However, from 2011 to 2014, insecticide-based tsetse control activities and maintenance of these barrier targets were halted, potentially allowing the reinvasion of tsetse flies. The currently high density of the two tsetse fly species observed in the study valley could be a consequence of this interruption in control efforts. The proportion of *G. pallidipes* was higher than *G. fuscipes*, consistent with previous findings in Nech Sar and Maze National Parks using NGU traps[19]. This difference may be attributed to the trap type [20]. *G. pallidipes* prefer a horizontal orientation facilitated by the NGU trap, while *G. fuscipes* prefer a vertical orientation. Another reason for the difference in species proportion may be *G. f. fuscipes*' poor response to the odour attractants used.

The study compared the infection rates of trypanosomes in two species of tsetse flies. Results indicated that *G. pallidipes* exhibited a higher rate of trypanosome infection (not statistically different) than *G. f. fuscipes*. This finding is consistent with previous research in the Akobo River system, western Ethiopia, which also employed the dissection technique [21]. However, the trypanosome infection rate in the current study site was lower than in previous research on the same species, *G. pallidipes*, at Nech Sar National Park [22]. This variation could be attributed to differences in the detection tools used to assess trypanosome infection in tsetse flies. Rodrigues et al.[22] employed a highly sensitive PCR assay, whereas we relied on dissection and examination under a compound microscope.

The finding also indicated that *T. congolense* was the most prevalent, followed by *T. vivax*, while the *T. brucei* complex was the least prevalent in both tsetse fly species. This finding aligns with Ethiopia's national atlas of tsetse flies and African animal trypanosomiasis, which also records the dominance of *T. congolense*, with *T. vivax* as the second most common species [6]. Although *T. vivax* exhibits a short developmental cycle in the tsetse fly proboscis and can be transmitted by various biting flies, *T. congolense* remains the primary causative agent of animal trypanosomiasis [6, 23]. Several studies have documented the low prevalence of the *T. brucei* complex [6, 23]. The risk of HAT associated with the *T. brucei* complex can be confirmed exclusively through molecular diagnostic methods. Nevertheless, insufficient surveillance and reporting systems, combined with limited resources for case management, may impede effective disease control in the event of case emergence.

The higher infection rate in tsetse flies caught in traps containing a mixture of cow urine and acetone may be due to the attraction of older tsetse flies to this odour combination. The age of tsetse flies is the most critical factor in determining the likelihood of trypanosome infection, as older flies are more likely to harbour mature trypanosome parasite [16]. Trypanosome infection alters tsetse fly behaviour by increasing hunger and enhancing olfactory sensitivity [24]. As a result, the infection prevents tsetse flies from successfully obtaining a blood meal and often interrupts blood feeding[25]. Consequently, infected flies may exhibit heightened responses to odours used for baiting. Additionally, the parasites compete for proline, a vital energy source for tsetse flies [26]. Therefore, infected tsetse flies, needing energy, are more likely to roam into odour-baited traps than uninfected ones, making them more susceptible to capture or kill by these devices. Consequently, using odour-baited traps can reduce the risk of infectious tsetse flies and help control them.

PCR-based detection of blood meal origins indicates that tsetse flies feed on a range of animals, including dogs, cattle, humans, and goats. The blood meal origins of tsetse flies vary, possibly due to the availability of host species and the ecology and species of tsetse fly vectors [27, 28]. Human activities such as hunting with dogs, farming, gathering firewood in the forest, and visiting rivers and streams can expose humans and dogs to tsetse fly bites. Dogs are common in the study area and frequently accompany domestic animals into forested areas. While accompanying livestock or hunting small wild animals, dogs may be exposed to tsetse flies. Unlike in wildlife-dominated regions, where tsetse flies tend to feed on a single preferred host species, the multiple vertebrate hosts' feeding patterns of tsetse

flies were observed[29]. The diverse feeding behaviours of tsetse flies may increase their exposure to various parasites.

As previously reported, odour-baited NGU traps were more effective at capturing *G. pallidipes*[19, 28] compared to traps without odour baits. When these baits are combined with killing agents, the odour-bait combinations can be employed as a "push-pull" strategy for comprehensive tsetse fly control across an area [27]. Large-scale deployment of odour-baited traps could enable effective tsetse control in a shorter period [1, 11]. In practical terms, using cow urine to catch tsetse flies is crucial as it emphasises its practicality and cost-effectiveness as an odour attractant in tsetse control strategies. Cow urine can be easily obtained and is effective after fermenting for up to three weeks. It is well-suited for community-based African trypanosomiasis control using traps, especially in resource-poor countries. Our research indicates that using cow urine alone or combined with baited traps could result in significant fly catches for controlling *G. pallidipes*.

This study offers important insights into tsetse fly infection and feeding behaviours; however, some limitations should be acknowledged. Although microscopic examination effectively detects *T. brucei* sensu lato, it does not distinguish among subspecies. Hence, conclusions apply only to *T. brucei* sensu lato, and molecular methods are recommended for confirmation. Additionally, identification of parasite species based on the developmental site may be prone to misclassification, as parasite development occurs across multiple organs within tsetse flies. Exclusive reliance on species-level identification of blood meals via PCR limits understanding of tsetse fly host preferences, as numerous blood meal sources remain unidentified. Further identification of unknown blood meal sources may reveal alternative host preferences. Additionally, the lower apparent density of *G. fuscipes* could be due to its reduced attraction to the specific bait odour used in the traps, which may be less effective for this species.

Conclusions

The high infection rate of *T. congolense* among tsetse flies in the area indicates a substantial risk of animal trypanosomiasis. The broad diversity of blood-meal sources may suggest a diverse set of potential reservoir hosts. The detection of human blood in tsetse meals further raises concern about possible human exposure to tsetse bites. Combining cow urine with acetone may boost the effectiveness of tsetse traps and be considered in tsetse suppression programs. The sources of dog blood meal identified in tsetse flies require attention, and further research is needed to understand its role in parasite transmission.

Declarations

Acknowledgments

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Author contributions

T.D., and F.M. contributed to the conceptualisation of the study: T.D., and K.K., collected the samples and conducted laboratory work: T.D., N.E., and G.T. conducted molecular analysis: Y.T., T.D., and F.M. conducted statistical analysis: F.M. supervised both the laboratory and fieldwork: F.M., and B.L. drafted the manuscript: T.D., K.K., N.E., G.T., Y.T., B.L., and FM. critically commented and approved the manuscript

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Data availability

All data generated and analyzed during this study are included in this article and its supplementary materials.

Ethics approval and consent to participate

Not applicable

Consent for publications

Not applicable

Competing interests

The authors have declared that no competing interests exist.

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Figures

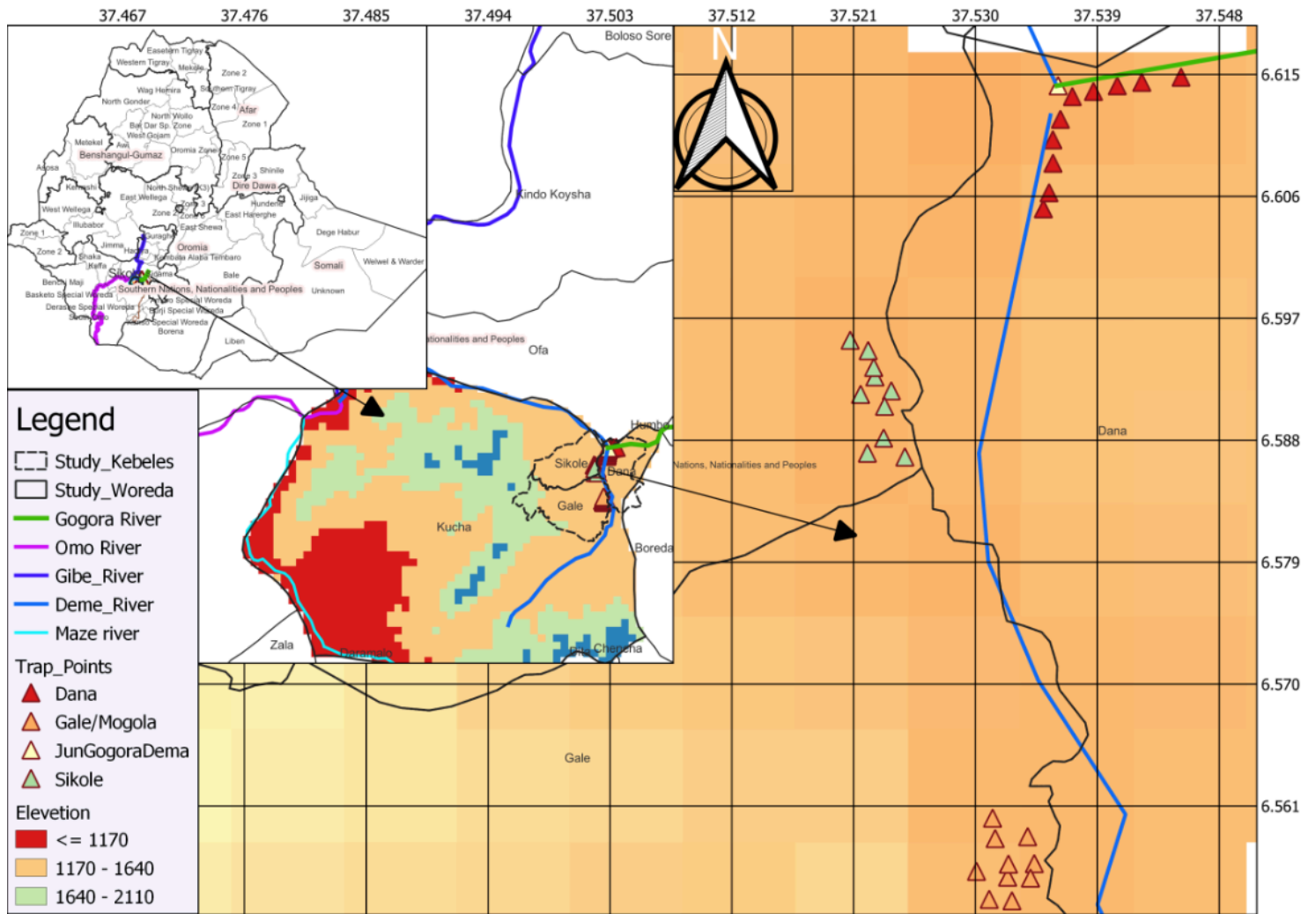


Figure 1

Map of Ethiopia, the study district, study *Kebeles* and trapping sites. Administrative boundaries, USGS Earth Explorer: (<https://earthexplorer.usgs.gov>), Roads, Water (River, lakes))–from DIVA-GIS (<https://www.diva-gis.org/gdata>)

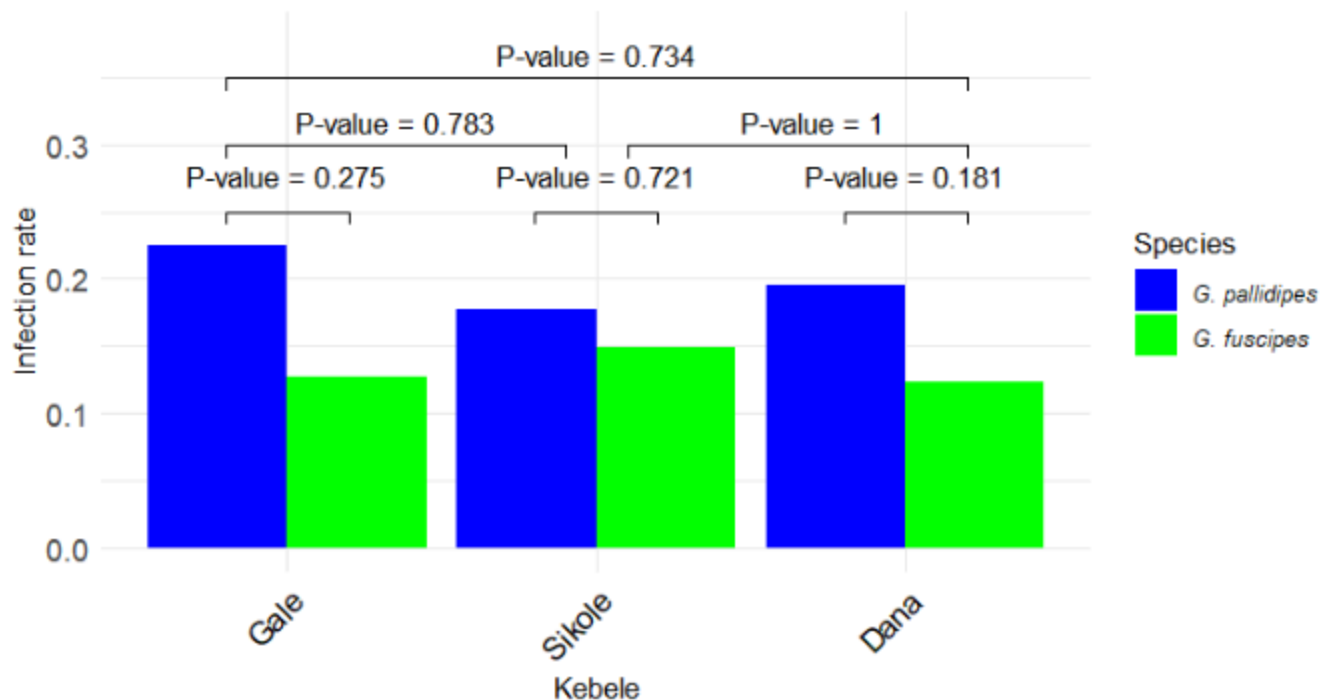


Figure 2

The *Trypanosoma* infection rate of tsetse flies collected using various odour baits during the study period across the three *Kebeles* of the Deme River Valley, Gamo Zone, South Ethiopia (September 2023 to February 2024).

The monthly variation in the number of tsetse flies collected in the study area was not statistically significant (supplementary table). Both acetone alone and a combination of acetone and cow urine attracted more tsetse flies infected with trypanosomes (Fig. 3).

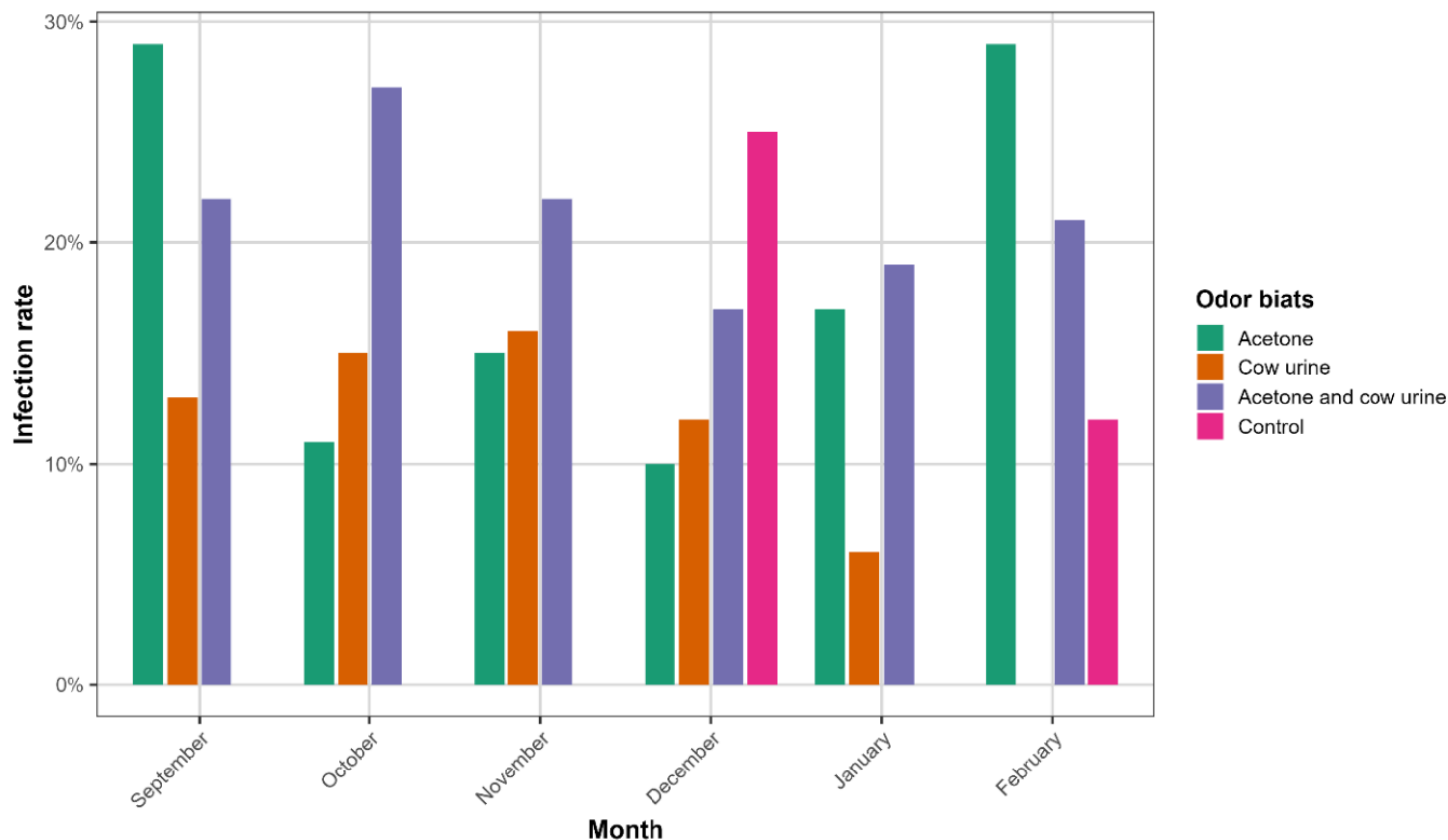


Figure 3

Monthly trypanosome infection rate of tsetse flies collected using various odour baits during the study period in the Deme River Valley of Gamo Zone, South Ethiopia (September 2023 to February 2024).

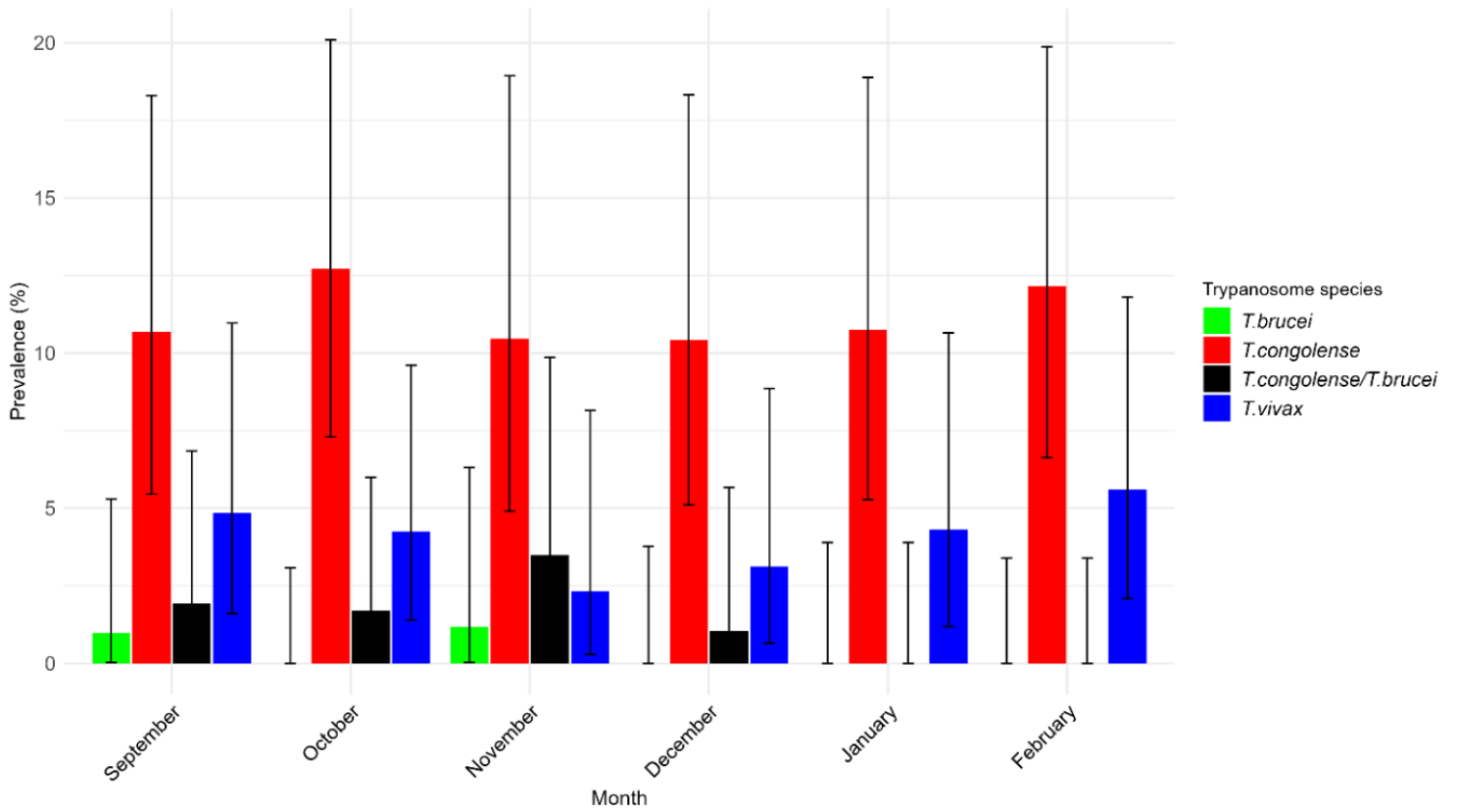


Figure 4

The proportion of tsetse flies that tested positive for various *Trypanosoma* species during the study period in the Deme River Valley in the Gamo Zone, South Ethiopia (September 2023 to February 2024).

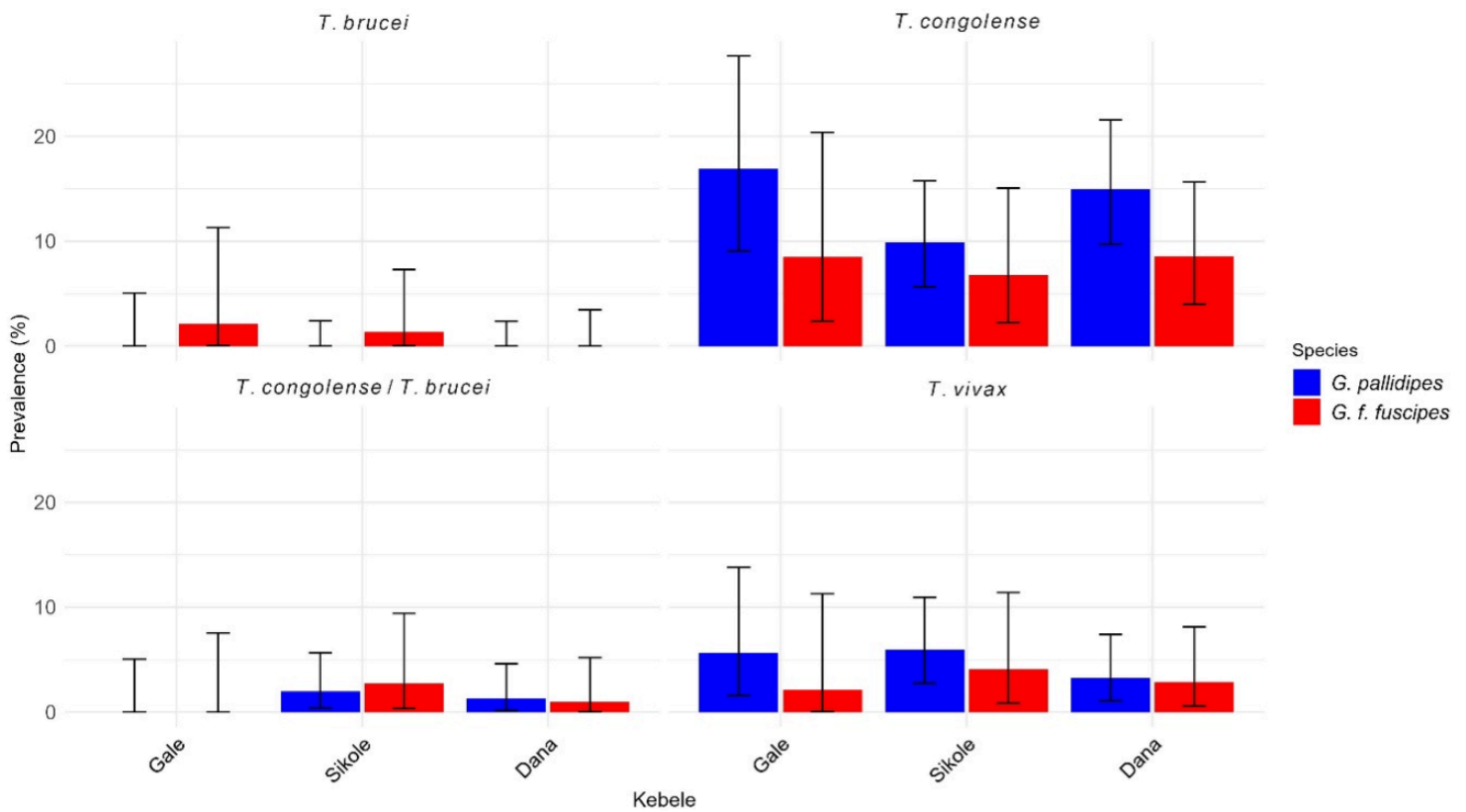


Figure 5

The number of *Trypanosoma*-positive tsetse flies in the three study Kebeles of the Deme River Valley in the Gamo Zone, South Ethiopia (September 2023 to February 2024).

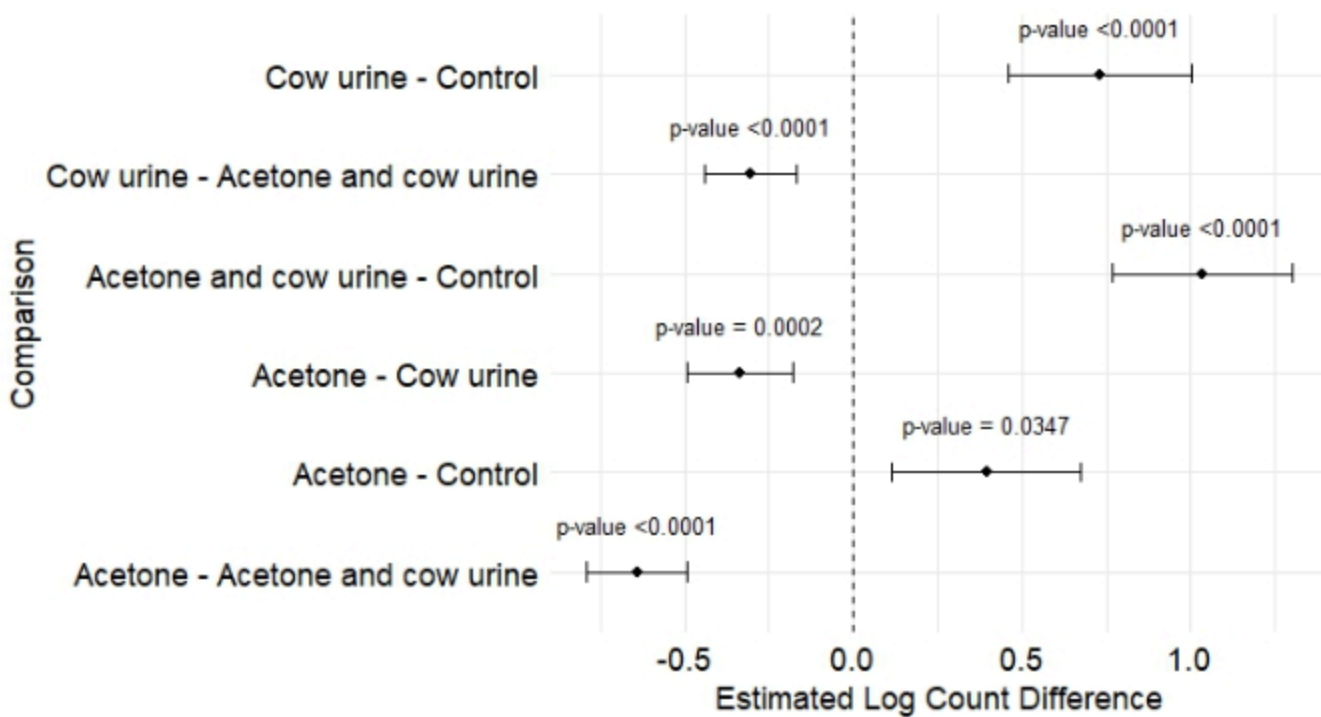


Figure 6

Pairwise comparison of the efficacy of odour baits using log counts of tsetse flies in Deme River Valley in Gamo Zone, south Ethiopia (September 2023 to February 2024).