

Article

Host Choice and Feeding Behaviours of *Glossina morsitans* Offspring Whose Parents Were Fed on Different Host Species

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Abstract: The success of any tsetse control program depends on the knowledge of their behaviour. This study assessed the host choice and feeding behaviours of *Glossina morsitans* siblings whose parents were bloodfed on rabbits, guinea pigs, rodents, and squirrels. Each individual host was placed in a screen cage, which allowed flies to enter through openings on each side. The groups of flies (20 per replicate), which were colour-marked differently based on their parents' blood meal hosts, were released from the centre of large semi-field cage. The released flies were aspirated after 24 h and then sorted based on their location, feeding status, and parents' blood meal. A total of 213 flies (72.95% of those recovered) were attracted to the hosts. The numbers of flies attracted to different hosts varied significantly ($\chi^2_4 = 33.685$, $p = 0.0001$): rodents ($n = 80$, $p = 0.006$), rabbits ($n = 59$, $p = 0.331$), guinea pigs ($n = 49$, $p = 0.057$), and squirrels ($n = 25$, $p = 0.005$). The numbers of flies attracted to their parent's blood meal source varied significantly ($\chi^2_{12} = 56.476$, $p < 0.001$): rabbits ($n = 35$, 59.32%, $p < 0.001$), rodents ($n = 25$, 31.25%, $p = 0.043$), and guinea pigs ($n = 19$, 38.78%, $p = 0.45$). But only 39 flies (18.31% of the total attracted) bloodfed on the hosts, including guinea pigs ($n = 10$, 25.64%), rodents ($n = 23$, 58.97%), rabbits ($n = 6$, 15.38%), and squirrels ($n = 0$, 0.0%). There was significant variation in the number of flies that fed successively across hosts ($\chi^2_4 = 49.478$, $p < 0.001$). The findings from this study confirm the presence of differential attractiveness of the hosts to flies and the so-called "Hopkins host selection principle" or "pre-imaginal conditioning". Therefore, the study attracts the need for detailed investigation on the influence of blood meal sources on tsetse fly siblings' behaviours across filial generations using small mammals or other large mammal species.

Keywords: *Glossina morsitans*; rabbits; guinea pigs; rodents; squirrels; bloodfed; attracted

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1. Introduction

Tsetse flies (Diptera, Glossinidae, *Glossina*) are blood-sucking, cyclical vectors of protozoan trypanosomes that cause sleeping sickness in humans (HAT) and nagana (AAT) in domestic animals [1,2]. Tsetse flies only inhabit sub-Saharan Africa [3] from the Kalahari to the Namibian deserts in the southern part and from the Sahara to the Somali desert in the northern part [1]. About 33 species and subspecies of these arthropods have been identified thus far [4] and subdivided into three subgenera, namely *Austenina* (Fusca group), *Nemorhina* (Palpalis group), and *Glossina* (Morsitans group) [5]. The savannah tsetse flies, *Glossina morsitans*, *G. pallidipes*, and *G. swynnertoni*, are the most dominant species in East African regions, including Tanzania [6]. There are also species that are

occurring with limited distribution, and these include *Glossina brevipalpis*, *Glossina longipennis*, *Glossina fuscipes martinii*, and *G. fuscipes fuscipes* [7–9]. Of all the species identified, only 6–10 species have public health and veterinary significance [1,4,9]. Examples of those species include *G. pallidipes*, *G. brevipalpis*, *G. m. morsitans*, and *G. swynnertoni* [10,11]. The major vectors of human African trypanosomiasis (HAT) in East Africa include *G. pallidipes* and *G. swynnertoni* [8,12].

Like other animals with learning ability, insects can also learn and adjust their intrinsic and extrinsic behaviours accordingly [13–16]. Such a learning ability helps insects to locate and assess the quality of resources such as food, breeding sites, and mates [15]. For instance, *Anopheles arabiensis* mosquitoes and *Lutzomyia whitmani* sandflies can return to the site and host where they were originally collected [14,17,18]. Similarly, *Glossina* species can return to the same host for a second blood meal whenever the feeding interval is within two days [15]. Such behaviours are sometimes referred to as host fidelity and site fidelity behaviours. Another study has proposed the so-called “Hopkins host selection principle” or pre-imaginal conditioning [18]. This concept explains that the larvae of the flies become attracted to their parents’ food sources, possibly due to olfactory cues experienced during development.

The feeding behaviours of tsetse flies are genetically determined [19,20]. They are mostly opportunistic feeders; however, in the absence of a preferred host, they adapt to feeding on available hosts [20]. Their choice to feed on a specific host is influenced by multiple factors, such as the shape of the host, the colour of the host, odour emanation, the size of the host, and host availability [20,21]. Understanding these and other behaviours is critical in designing and implementing surveillance and control strategies [15,16,22]. In-depth knowledge of host choice and feeding behaviours of tsetse flies could be important, as it may influence parasite transmissions in different vectors [15], thus facilitating the formation of new strategies to minimize vector–host contact, especially in settings where humans, wildlife, and livestock interact. Similarly, such knowledge may be useful during the monitoring and evaluation of tsetse surveillance and control programs [21]. Despite their urgent and adverse impacts on public health, the behaviours of most hematophagous insects, including tsetse flies, are understudied. Therefore, in this study, we investigated the host choice and feeding behaviours of *Glossina morsitans*, one of the most predominant tsetse vector species in Tanzania and elsewhere in Africa.

2. Materials and Methods

2.1. Rearing the Tsetse Flies

A colony of *Glossina morsitans* was established using pupae from the Tsetse and Vector Control Centre, Tanga, Tanzania. The pupae were transferred into large, fine-meshed emergence cages and rearing cages and therein maintained under ambient conditions at 25 ± 2 °C with $70 \pm 2\%$ RH and a 12 h photo phase within an insectary at the Institute of Pest Management (IPM), Sokoine University of Agriculture, Tanzania. Emerging flies were sorted by sex and transferred to separate cages with either 25 flies ($7.5 \times 5 \times 4$ cm) or 40 flies ($13.5 \times 8 \times 4.5$ cm). Virgin female flies were mated with 6- to 8-day-old virgin males in separate cages at 1:3 male-to-female ratios [23,24]. While in the insectarium, different cohorts of adult tsetse, consisting of at least 80–100 tsetse flies, were respectively blood-fed on a guinea pig (*Cavia porcellus*), rodent (*Cricetomys gambianus*), rabbit (*Oryctolagus cuniculus*), and squirrel (*Paraxerus ochraceus*). Prior to feeding the flies, hosts were shaved on one side of the abdomen then cleaned using warm water. During blood-feeding, hosts were humanely restrained, then one cage (which contained 10–15 flies) was attached on the abdomen of the hosts to allow the flies to blood-feed for 10 min. The same process of blood-feeding was conducted for 3 h on Monday, Wednesday, and Friday every week from 11:00 am to 2:00 pm for five weeks.

2.2. Experimental Setup

A large semi-field cage made of inert mosquito nets (size: 245 cm × 185 cm × 203 cm) was constructed and placed inside a room (size: 336 cm × 195 cm × 308 cm) with ambient conditions similar to those of the rearing insectary. Then, four small screen cages of the same size (size: 62 cm × 42 cm × 62 cm) were fixed one in each of the four sides of the semi-field cage (Figure 1). The host screen cages were made of metal bars and improvised with four openings, one on each of their four sides, through which the host-seeking tsetse would access the hosts. The openings were tapered such that tsetse flies visiting the large cages were unable to leave. The large semi-field and screen cages were regularly checked for intactness to prevent experimental tsetse flies from escaping to the outside.

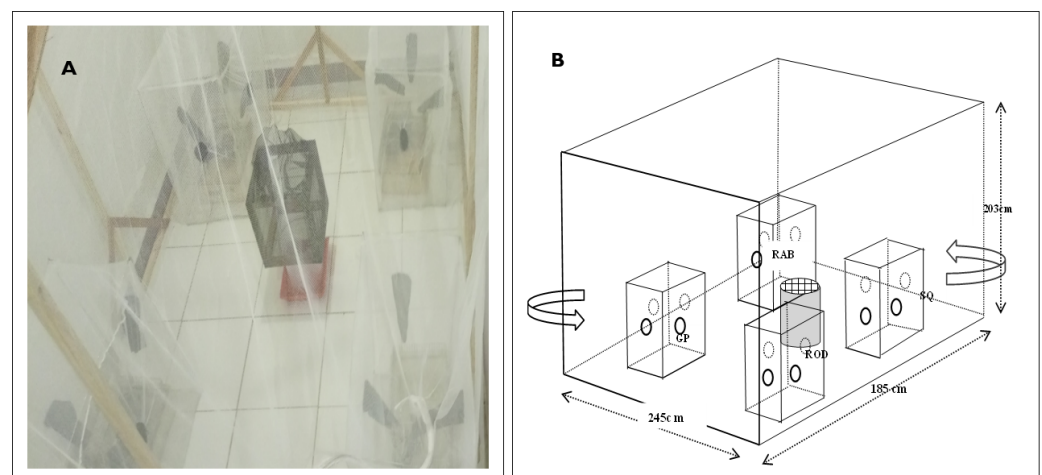


Figure 1. Host-choice experimental setup with four small screen cages containing different host species. During the experiment, the position of the four cages were alternated such that each host occupied all four different positions within the large semi-field cage. (A) Shows four screen cages and releasing cage positioned within large semi-field cage. (B) Shows the experimental layout.

2.3. Assessing the Host Choice and Feeding Success of Offspring Whose Mothers Were Fed on Different Host Species

Using a 4 by 4 Latin Square design, 4 host species (1 guinea pig, 1 rodent, 1 rabbit, and 1 squirrel) were placed in each of the four screen cages inside the large semi-field cage (Figure 1). In each replicate, four cohorts of offspring (20 tsetse flies each) obtained from mothers, for *Glossina morsitans* species, blood-fed on the different hosts and labelled with different colours of fluorescent powder, were released simultaneously at the centre of the large semi-field cage. Before releasing, the tsetse flies were starved for 72 hours to maximize their physiological demand for blood and urge for host-seeking. After release, the tsetse flies were left to forage for 24 hours and recaptured independently from all the four screen cages and elsewhere in the semi-field system using aspirators. Recaptured tsetse flies were identified and categorized as fed, unfed, live, or dead, as well as the location where released flies were collected (hosts' screen cage). This experiment was repeated four times, and each time hosts were randomly rotated across the four cages such that all hosts occupied all positions in the large semi-field cages. To assess the feeding success of released flies, the proportion of live or dead engorged flies obtained in host-choice experiment were compared. By observing the abdomen, engorged recaptured flies were sorted out, counted, and recorded.

2.4. Determination of Haemoglobin (Hb) Concentrations and Total Plasma Protein of Blood Samples from the Experimental Host Species

Haemoglobin concentration and total plasma protein were assessed in all hosts before they were deployed in the experiment. Prior to blood samples' collection, hosts were anaesthetized shortly using diethyl ether for about 2 min. Using micro-capillary tubes,

blood samples (2 mL per host) were drawn from the retro-orbital sinus and transferred into two well-labelled EDTA K3 2.5 mL tubes. These samples were shipped to the laboratory for analysis inside a cool box with recyclable ice.

Haemoglobin concentration (Hb) was determined using the Cyanomethemoglobin method. The blood samples were gently mixed before taking 0.02 mL of the sample using a pipette. Excess blood on the pipette surface was wiped using clean tissue paper. The individual samples were then transferred into test tubes containing 5 mL Drabkin's reagents. The tubes containing samples were stoppered then gently mixed, and then left for 10 min for maximum colour development. The samples were then poured into the cuvette, where absorbance at 540 nm versus a reagent blank was compared [25]. Haemoglobin concentration (mg/mL) and percentage (%) were obtained and recorded accordingly.

The Biuret method was used to determine total blood protein for all collected blood samples following the Erba Total Protein protocol. This method involves the formation of a blue-violet ion complex resulting from the reaction between the peptide bonds of protein and copper II ions in the alkaline solution.

2.5. Data Analysis

The data were entered, cleaned, and organized in Microsoft Excel 2010 prior to statistical analysis. The variation in the total number of flies that entered screen cages and the proportion of flies which returned to the same host were analysed using a generalized linear mixed model (GLMM) in R statistical software version 4.2.2. The hetero-scedasticity and non-normal distribution of count data were confirmed using Bartlett's test and the Shapiro–Wilk test, respectively. Hence, a negative binomial distribution (glmer.nb function of the lme4 package) was used to account for the over-dispersion of the data. An initial model fixed the number of flies entering screen cages as a dependent factor predicted by fixed factors: Hb concentration, total blood protein, screen cage, blood meal sources used to maintain parents, average temperature, and relative humidity. The cage position was set as a random effect in all models. Insignificant fixed predictors such as Hb concentration, total plasma protein, average temperature, and relative humidity were removed from the model until the lower Akaike information criterion (AIC) was achieved.

2.6. Ethical Approval

Ethical clearance for conducting this particular study was obtained from the Sokoine University of Agriculture Research and Publication Committee (reference number SUA/DRRTC/R/186/18), and the Tanzanian Commission for Science and Technology (reference number: 2022-735-NA-2022-082).

3. Results

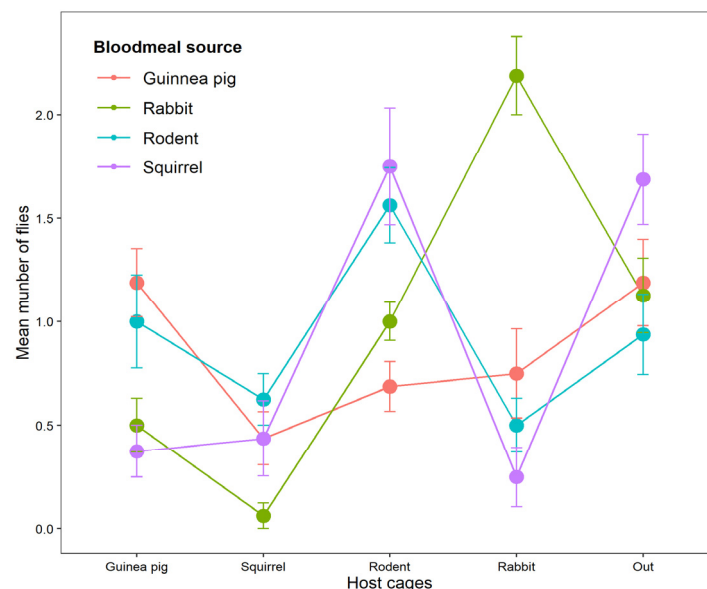
3.1. Choice of Adult Tsetse Fly, *Glossina Morsitans*, on Different Host Species

A total of 320 adult *G. morsitans* were released for the host choice experiment, of which 292 (91.25% of the released) were recovered and 28 (8.75% of released flies) unrecovered. Out of the recovered adult tsetse, 213 (72.95%) were attracted to different hosts: rodent (n = 80, 27.4%), rabbit (n = 59, 20.21%), guinea pig (n = 49, 16.78%), and squirrel (n = 25, 8.56%). The remaining flies (n = 51, 17.47% of recovered flies) were collected in the large semi-field cage (Table 1). The number of flies attracted to individual hosts varied significantly regardless of position ($\chi^2_4 = 33.685$, $p = 0.0001$). Rodents attracted the highest number of flies ($p = 0.006$), followed by rabbits ($p = 0.331$), guinea pigs ($p = 0.057$), and squirrels ($p = 0.005$) (Table 1). Nevertheless, the difference in the number of flies attracted to guinea pigs and rodents ($p = 0.001$), rabbits and rodents ($p = 0.003$), guinea pigs and squirrels ($p = 0.002$), rabbits and squirrels ($p < 0.001$), and rodents and squirrels ($p < 0.001$) was statistically significant.

Table 1. The distribution of offspring flies attracted to their parent's bloodmeal sources (guinea pig, rabbit, and squirrel).

Bloodmeal Source	Host Cages									
	Guinea Pig		Rabbit		Rodent		Squirrel		Out	
	n	%	n	%	n	%	n	%	n	%
Guinea pig	19	38.78	12	20.34	11	13.75	7	28.00	19	24.05
Rabbit	8	16.33	35	59.32	16	20.00	1	4.00	18	22.78
Rodent	16	32.65	8	13.56	25	31.25	10	40.00	15	18.99
Squirrel	6	12.24	4	6.78	28	35.00	7	28.00	27	34.18
TOTAL	49	100.00	59	100.00	80	100.00	25	100.00	79	100.00

The number of offspring attracted to their parent's blood meal source varied significantly regardless of position ($\chi^2_{12} = 56.476$, $p < 0.001$). Furthermore, the highest number of offspring flies attracted to their parent's bloodmeal source was observed in rabbits ($n = 35$, 59.32%, $p < 0.001$), rodents ($n = 25$, 31.25%, $p = 0.043$), and guinea pigs ($n = 19$, 38.78%, $p = 0.45$). Considering the contribution of offspring from different bloodmeal sources attracted to individual hosts in the host-choice experiment, rodents attracted more flies from squirrel ($n = 28$, 35%) and rodent blood ($n = 25$, 31.25%); guinea pigs attracted more flies from guinea pig blood ($n = 19$, 38.78%) and rodent blood ($n = 16$, 32.65%); rabbits attracted more flies from rabbit blood ($n = 35$, 59.32%) and guinea pigs ($n = 12$, 20.34%); finally, squirrels attracted more flies from rodent blood ($n = 10$, 40%) (Table 1). The distribution of the mean number of flies that were attracted to the parent's blood meal source is shown in Figure 2 and Table 1. Interestingly, 34.18% ($n = 27$) of the offspring flies that remained outside of the host's cages or screen cages were those from parents which bloodfed on squirrels (Figure 2). Unlike the significant variation in the number of offspring flies attracted to rabbits, guinea pigs, and rodents ($p < 0.001$), no significant variation was observed in the number of flies attracted to squirrels ($\chi^2_3 = 4.9624$, $p = 0.1746$).

**Figure 2.** The average number of offspring flies that were attracted to their parent's bloodmeal source (guinea pig, squirrel, rodent, and rabbit).

3.2. Feeding Success of Tsetse Flies Attracted to Different Hosts

Of the flies attracted to different hosts, only 39 flies (18.31%, alive = 6, dead = 33) successively bloodfed on hosts. The number of flies that were attracted and successfully fed varied across the different hosts ($\chi^2_4 = 49.478$, $p < 0.001$): guinea pigs ($n = 10$, 25.64%), rodents ($n = 23$, 58.97%), and rabbits ($n = 6$, 15.38%). None of the flies attracted to squirrels

were bloodfed. Most of the flies that were attracted and successively fed on rodents ($n = 13$, 56.52%) originate from parents maintained on blood from squirrels (Table 2).

Table 2. The distribution of the number flies bloodfed successively on different hosts (guinea pig, squirrel, rodent, and rabbit).

Host Cages	Blood Meal Sources				Total
	Guinea Pig	Rabbit	Rodent	Squirrel	
Guinea pig	1	0	7	2	10
Rabbit	1	3	1	1	6
Rodent	0	1	9	13	23
Squirrel	0	0	0	0	0
Total	2	4	17	16	39

3.3. Haemoglobin Concentration (Hb) and Total Plasma Protein in Different Hosts

Of all hosts used in the choice experiment, squirrels had the highest Hb concentration (mean: 19.32 ± 0.51 g/dL), while rabbits had the least (mean: 14.515 ± 0.05 g/dL). Furthermore, rodents had the highest total plasma protein (mean: 75.17 ± 0.497 g/dL), and squirrels had the least among all (mean: 7.756 ± 0.028 g/dL) (Table 3). There was a statistically significant difference in both mean Hb concentration ($\chi^2_3 = 155.24$, $p < 0.001$) and total blood protein ($\chi^2_3 = 302.91$, $p < 0.001$) between hosts. The number of flies attracted to specific hosts is not significantly correlated with either the host's haemoglobin concentration ($r(1) = -0.03$, $p = 0.5368$) or the total plasma protein ($r(1) = 0.05$, $p = 0.3431$). Furthermore, the number of bloodfed flies positively correlated with the host's haemoglobin concentration (Hb) ($r(1) = 0.17$, $p = 0.002$) and was insignificantly correlated with the total plasma protein ($r(1) = 0.04$, $p = 0.478$).

Table 3. The average haemoglobin (Hb) and total plasma protein concentration of different attracted and successfully bloodfed flies.

Host Type	Hb Concentration (g/dL)		Total Plasma Protein (g/dL)		Number of Flies (n, %)	
	Mean	Std. Error	Mean	Std. Error	Attracted	Bloodfed
Guinea pig	14.730	0.071	52.695	0.033	49 (23.00%)	10 (25.64%)
Rabbit	14.515	0.050	65.225	0.003	59 (27.7%)	6 (15.38%)
Rodent	17.335	0.244	75.170	0.497	80 (37.56%)	23 (58.97%)
Squirrel	19.320	0.505	7.756	0.028	25 (11.74%)	0 (0.000%)

4. Discussion

This study assessed the variation in the host choice and feeding success behaviours of *Glossina morsitans* siblings whose parents were maintained from guinea pigs, rabbits, rodents, and squirrels.

The results show the variation in the proportion of attracted tsetse flies across individual hosts. Rodents attracted the highest proportion of released flies (27.4%), followed by rabbits (20.21%), guinea pigs (16.78%), and squirrels (8.56%). This can be explained by the variation in the level of hosts' attractiveness to the flies. Hosts' cues, such as the odour emanating from hosts' bodies, trigger fly-searching behaviours, while host shape, colour, and size determine their choice of specific host [20,26]. It is likely that rodents and rabbits had relatively larger bodies than squirrels and guinea pigs, which influenced their level of attractiveness to flies. A similar finding was also reported in other studies [27,28], where hosts with a larger body size attracted a relatively larger proportion of flies than hosts with a smaller body size. For example, most tsetse flies were attracted to cattle and donkeys compared to monitor lizards, goats, and sheep. Moreover, rodents had buff-grey pelage, and rabbits had black pelage, squirrels had dull yellowish-brown pelage, and guinea pigs had yellow-white pelage. Considering tsetse flies' preference for black or dark colours [29–32], it is possible that the deployed host colours influenced the flies' host choice. For example, one of these studies reported a higher proportion of flies being attracted to hosts with a relatively dark pelage than those with yellow pelage/colour [30].

Likewise, rabbits attracted the highest proportion (59.32%) of flies originating from parents that were maintained on rabbits, followed by rodents, which attracted more of the flies (31.25%) whose parents bloodfed on rodents. This can be attributed to hosts' differential attractiveness to released flies. Similar studies conducted elsewhere, which deployed teneral flies, reported similar results where hosts with larger body sizes (cows) attracted more flies than those with smaller bodies (lizards) [28]. The study conducted on mosquitoes reported evidence that host choice for mosquitoes can be explained by physiological or behavioural conditioning rather than genetic variability [33]. This may be true in the case of deployed tsetse flies, where feeding of the flies on experimental hosts was conducted in only one generation. Hence, there could be less chance for parents' behaviour to be inherited by their siblings if this process existed at all.

Interestingly, about 34% of the flies whose parents bloodfed on squirrels remained in the large semi-field cage (they did not visit any screen cage that contained hosts). This can be explained by the variation in the flies' physical fitness and their ability to detect hosts. Several studies have reported the influence of blood quality on the physiology and biology of flies [34,35]. These studies reported the impact of bloodmeal sources on mosquitoes' feeding rates, survivorship, and fecundity [34], as well as the variation in feeding activities and reproductive capacity and efficiency [36]. Since hosts' haematological properties influence blood nutrition [35] and are known to vary across species [37], this could have influenced flies' physical fitness. Squirrels had the least total plasma protein and the largest haemoglobin (Hb) concentration among all of the hosts (Table 3). Most of these offspring that remained in the large cage were originating from parents that were maintained on squirrels. Future studies may perhaps focus on assessing the influence of hosts' haematological parameters on tsetse siblings' behaviours.

Despite the higher proportion of attracted flies on rabbits and rodents, only 28.75% (23 flies) and 10.17% (6 flies) of attracted flies fed successfully on these hosts, respectively. This finding can be explained by the variation in the level of hosts' defensive behaviour, which affects flies' feeding success [38–41]. It is possible that the hosts deployed in this study varied in the level of their defensive behaviours, thereby affecting flies' feeding success. This finding agrees with the study which reported reduced feeding success for tsetse flies due to hosts' defensive behaviour [38]. And the study which reported a relatively higher feeding rate of *G.pallidipes* on adult cattle compared to young cattle due to variation in the level of their defensive behaviours [39]. In addition to the above factors, the nature of the hosts' furs could have also affected flies' feeding success. The rabbits' bodies, unlike those of rodents, were covered with long, dense furs, which could have minimized the surface area available for the attracted flies to feed on. Finally, the taste system determines attracted flies' biting decisions [22]. The difference in chemical signatures that results from the host's dermal secretions or metabolism of microbiota could have influenced flies' biting decisions. But, since these factors were not assessed in this study, we lack evidence to confirm their influence on observed behaviours. Future studies can be performed to assess the influence of host chemical signatures on the behaviours of tsetse fly siblings using similar host species in Tanzania.

5. Conclusions

This study reports the varied proportion of tsetse flies' siblings that were attracted to and successfully bloodfed on different hosts that were used to maintain their parents. It was hard to confirm the presence of the inherited behaviours of the parents in their siblings; however, only host-related factors explained the observed variations in the deployed tsetse flies' siblings. Future studies need to be conducted to assess the same behaviours using more species of tsetse flies and small mammals, which, on the other hand, could be alternative blood hosts for these flies in the absence of their preferred hosts. The resulting findings will inform tsetse control programs on the possible ways of altering offspring choice and feeding behaviours as a way of controlling African trypanosomiasis.

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Institutional Review Board Statement: The study was conducted in accordance with the Sokoine University of Agriculture and the Tanzanian Commission for Science and Technology research ethics. The study ethical clearances were approved by the Institutional Ethics Committees of Sokoine University of Agriculture (reference numbers: SUA/DRRTC/R/186/18) and the Tanzanian Commission for Science and Technology for studies involving animals (reference numbers: 2022-735-NA-2022-082).

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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