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1 **Tsetse blood-meal sources, endosymbionts, and trypanosome infections**
2 **provide insight into African trypanosomiasis transmission in the Maasai Mara**
3 **National Reserve, a wildlife-human-livestock interface**

4 **Short title: Tsetse blood-meals/symbionts/trypanosomes in a human-wildlife**
5 **interface**

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35 ## Abstract

36 **Background**: African trypanosomiasis (AT) is a neglected disease of both humans and animals
37 caused by *Trypanosoma* parasites, which are transmitted by obligate hematophagous tsetse flies
38 (*Glossina* spp.). Understanding of AT transmission is hampered by limited knowledge on
39 interactions of tsetse flies with their vertebrate hosts and the influence of endosymbionts on
40 vector competence, especially in wildlife-human-livestock interfaces. We identified the tsetse
41 species, their blood-meal sources, and the correlation between endosymbiont and trypanosome
42 infection status in the trypanosome-endemic Maasai Mara National Reserve (MMNR) of Kenya.

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43 **Methodology/Principal Findings**: Among 1167 tsetse flies (1136 *Glossina pallidipes*, 31
44 *Glossina swynnertoni*) collected from 10 sampling sites, 28 (2.4%) were positive by PCR for
45 trypanosomes, majority (17/28) being *Trypanosoma vivax*. Blood-meal analyses based on high-
46 resolution melting analysis of mitochondrial cytochrome c oxidase 1 and cytochrome b gene
47 PCR products (n = 345) identified humans as the most common vertebrate host (37%), followed
48 by hippopotamus (29.1%), African buffalo (26.3%), elephant (3.39%) , and giraffe (0.84%).
49 Trypanosome-infected flies had fed on hippopotamus and buffalo. Additionally, PCR analysis
50 revealed that tsetse flies were more likely to be infected with trypanosomes if they were infected
51 with the *Sodalis glossinidius* endosymbiont (P = 0.0022 Fisher's exact test).

52 **Conclusions/Significance**: Diverse species of wildlife hosts may contribute to the maintenance
53 of tsetse populations and/or persistent circulation of African trypanosomes in the MMNR.
54 Although the African buffalo is known to be a key reservoir of AT, the higher proportion of
55 hippopotamus blood-meals in trypanosomes-infected flies identified here indicates that other
56 wildlife species may also be important to transmission cycles. No trypanosomes associated with
57 human disease were identified, but the high proportion of human blood-meals identified are
58 indicative of human African trypanosomiasis transmission risk. Furthermore, this work provides
59 data showing that *Sodalis* endosymbionts can is associated with increased trypanosome infection
60 rates in endemic ecologies.

61 Author summary

62 Human and animal African trypanosomiasis are neglected tropical diseases with potential to
63 spread to new areas. Wild animals are important reservoirs for African trypanosomes and crucial
64 in the emergence and re-emergence of AT. Vertebrate host-vector-parasite interactions are
65 integral to trypanosome transmission. We investigated the vertebrate blood-meals and

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66 trypanosomes-endosymbionts co-infections in tsetse flies, which have been associated with
67 reservoirs and vector competence, respectively, on AT transmission in Kenya's Maasai Mara
68 National Reserve. We identified tsetse fly diversity, trypanosome and endosymbiont infection
69 status, and vertebrate blood-meal hosts to infer potential transmission dynamics. We found
70 that *Glossina pallidipes* was the major tsetse fly vector and that *Trypanosoma vivax* was the
71 main trypanosome species circulating in the region. Humans, hippopotamus, and buffalo were
72 the most frequented for blood-meals. Buffalo and hippopotamus blood-meals were identified in
73 trypanosome infected flies. Feeding of the flies on both humans and wildlife may potentiate the
74 risk of the human trypanosomiasis in this ecology. Additionally, we found that the
75 endosymbiont *Sodalis glossinidius* is associated with higher trypanosome infection rates in wild
76 tsetse flies. These findings emphasize the importance of understanding the interaction of tsetse
77 flies with vertebrate blood-meal sources and their endosymbionts in the transmission and control
78 of AT.

79 **Introduction**

80 African trypanosomes (genus *Trypanosoma*), cyclically transmitted by the tsetse fly vector
81 (genus *Glossina*), cause a group of diseases known as African trypanosomiasis (AT). In man, the
82 disease is called sleeping sickness (human African trypanosomiasis, HAT), while in animals it is
83 called nagana (African animal trypanosomiasis, AAT). African trypanosomiasis is endemic in 37
84 countries in Africa, in regions inhabited by the insect vector. Approximately 70 million people
85 and 60 million cattle in AT endemic regions are at risk of infection [1,2]. Consequently, reduced
86 productivity due to chronic disease in humans animal and loss of livestock through death,
87 particularly in regions where pastoralism is the main economic activity threatens food security,

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88 quality of living, and economic stability [3,4]. Therefore, more effective control and
89 management strategies of AT are required.

90 Control of AT has involved combinations of active surveillance, vector control strategies,
91 and mass chemotherapy [5]. Notably, chemotherapy has been limited by increasing levels of
92 resistance to the available trypanocides, chemotoxicity, and unavailability of new drugs [5,6]. To
93 address limitations associated with chemotherapy, transmission disruption through vector control
94 is crucial. Vector control is largely applied in areas where livestock are kept [7,8]. However,
95 wild animals sustain the life cycles of tsetse flies [9,10] and the parasites they transmit [11,12],
96 and are thus an important factor of transmission dynamics of AT, particularly in wildlife
97 ecologies. Tsetse fly blood-meal sources are highly variable, especially in wildlife areas. Hence,
98 one sampling area cannot be used to make a generalized conclusion of tsetse feeding behavior
99 [12]. Consequently, identification of tsetse fly host blood-meal sources in specific regions can
100 help elucidate potential wild animals involved in AT transmission and provide baseline for
101 research towards improving vector-control strategies, particularly in wildlife-human-livestock
102 interfaces that serve as hotspots for the emergence and re-emergence of AT.

103 Moreover, transmission of trypanosomes from an infected animal to another by tsetse
104 flies is highly influenced by vector competence – the vector's ability to successfully acquire and
105 transmit a pathogen. Competence is influenced by various factors including genotype, sex,
106 species, immune status, and endosymbionts, [13–17]. Endosymbionts have been shown to
107 influence the susceptibility of tsetse flies to trypanosomes [18]. *Wigglesworthia glossinidia*,
108 *Sodalis glossinidius*, and *Wolbachia pipentis* are well-defined tsetse fly endosymbionts with
109 direct and indirect effects on the tsetse fly vectorial capacity [19]. Despite numerous studies on

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110 the influence of endosymbionts on vectorial competence [13,14,20–22], studies on the presence
111 and influence of tsetse fly endosymbionts in wildlife-livestock-human interfaces are scant.

112 The Maasai Mara National Reserve (MMNR) is a prime tourist destination in Kenya that
113 is surrounded by a number of ranches and is thus characterized by constant interactions between
114 wildlife and humans and their livestock. With endemic tsetse fly populations, there have been
115 recent cases of tourists contracting HAT in the MMNR [23,24]. Therefore, the MMNR is an
116 ideal study site for investigating the contribution of tsetse fly blood-meal sources and the major
117 endosymbionts of tsetse flies in relation to transmission of African trypanosomes in a human-
118 livestock-wildlife interface. We sought to understand the interactions occurring among natural
119 tsetse fly populations, trypanosome species, endosymbionts, and vertebrate hosts in the MMNR.
120 Specifically, we investigated the diversity of tsetse fly species and their trypanosome species,
121 vertebrate blood-meal sources, and *Sodalis*, *Wolbachia*, and salivary gland hypertrophy virus
122 (SVGH) endosymbionts.

123 Materials and methods

124 Study area

125 Field sampling was carried out within the MMNR (1°29′24″S 35°8′38″E, 1500 m above sea
126 level) located in southwest region of Kenya, which is contiguous with the Serengeti National
127 Park (SNP) in Tanzania (Figure 1). This sampling site is located approximately 150 km south
128 from the equator and covers an area of 1500 km^2. The MMNR is home to a diverse variety of
129 flora and fauna, and is famously known for its wild animals and 'Great Migration' of
130 wildebeests, zebras, and antelopes across the Mara River. Grassland forms the major vegetation
131 cover in this ecosystem, with swampy grounds found around the riverbanks. The sampling sites
132 were selected along the rivers due to their high populations of animals (Figure 1). Ethical

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133 clearance for this research in protected areas was sought from and approved by the Kenya
134 Wildlife Service (KWS) Research Authorization committee.

135 **Fig 1. Map showing tsetse fly sampling sites from Maasai Mara National Reserve and**
136 **number of tsetse species sampled.** Each sampling site has its total sampled flies indicated in the
137 pie chart.

138 **Tsetse collection and identification**

139 Tsetse flies were trapped at the start of the annual wildebeest migration between June and July
140 2016 using Nguruman (Ngu) traps baited with acetone and cow urine. Traps were set in the
141 morning (10 - 11 am) at different sampling sites in the various regions demarcated by Mara,
142 Talek, and Sand Rivers, and at the wildlife crossing points across the Mara River at the border of
143 Kenya and Tanzania's SNP (Figure 1). The traps were emptied after 24 hours, and trapped flies
144 were transferred into 50-mL falcon tubes and stored in dry ice before transportation in liquid
145 nitrogen to the laboratory at the International Centre of Insect Physiology and Ecology (*icipe*),
146 Nairobi where they were sorted. The flies were identified to species level under a light
147 microscope (Stemi 2000-C, Zeiss, Oberkochen, Germany) based on standard published
148 taxonomic keys [25].

149 **Nucleic acid extraction**

150 Individual flies (after removal of legs and wings) were homogenized using six 2-mm zirconium
151 beads in 1.5-ml microcentrifuge tubes using Mini-beadbeater-16 (BioSpecs Inc., Bartlesville,
152 OK, USA) for 20 seconds. DNA was extracted from the homogenate of each sample using the
153 ammonium acetate protein precipitation method described by Adams *et al.* [26], with slight
154 modifications. Briefly, 300 µl of cell lysate buffer (10 mM Tris-HCl, pH 8.0, 0.5% SDS and 5
155 mM EDTA) was added into homogenized samples and incubated for 90 minutes at 65°C.

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156 Thereafter, 100 µl of protein precipitate solution (8M ammonium acetate and 1M EDTA) was
157 added to each mixture, which were vortexed for 30 seconds, incubated in ice for 30 minutes, and
158 centrifuged at 14,000 x g for 15 minutes at 4°C. The supernatants were transferred into new 1.5-
159 ml microcentrifuge tubes containing 300 µl of isopropanol, and mixed gently by inverting 100
160 times and centrifuging at 14,000 x g for 30 minutes. The supernatants were pipetted off and
161 subsequently, 300 µl of ice-cold 70% molecular grade ethanol was added to each pellet, gently
162 mixed by inversion and centrifuged at 14,000 x g for 30 minutes. Ethanol was pipetted off and
163 the pellets were air-dried overnight. The DNA pellets were solubilized by adding 100 µl of PCR
164 grade water and quantified using a NanoDrop™ 2000 Spectrophotometer (Thermo Scientific,
165 NJ, USA). Concentrations were adjusted to 50 ng/µl using PCR grade water.

166 **PCR identification of African trypanosomes**

167 Trypanosome infections in flies were detected using trypanosome-specific ITS1 CF and BR
168 primers (S1 Table) as described by Njiru *et al.* [27]. *Trypanozoon* species were further resolved
169 using species-specific primers (S1 Table). Briefly, glycosylphosphatidylinositol-phospholipase C
170 polypeptide (GPI-PLC) and serum resistant-associated (SRA) species-specific primers were used
171 to identify *T. brucei brucei* and *T. brucei rhodesiense*, respectively, by PCR [28]. *Trypanosoma*
172 *congolense savannah* was identified according to Masiga *et al.* [29].

173 PCR amplification was carried out in final reaction volumes of 20 µl containing 10.4 µl of PCR
174 grade water, 1× GeneScript PCR reaction buffer and 1.6 units of Green Taq DNA polymerase
175 enzyme (GeneScript, New Jersey, USA), 1 µl (final concentration 0.5 µM) of each primer and
176 200 ng DNA template. The PCR reactions were performed in a SimpliAmp™ Thermal Cycler
177 (Applied Biosystems, California, USA) programmed as follows; initial denaturation step at 94°C
178 for 3 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at a

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179 temperature specific for each primer (S1 Table) for 30 seconds and extension at 72°C for 45
180 seconds and a final extension at 72°C for 7 minutes. PCR grade water was used as a negative
181 control, in place of DNA template. DNA obtained from characterized and archived stocks of
182 African trypanosome species were used as positive controls. The PCR products were size
183 separated by ethidium stained agarose gel electrophoresis and viewed under UV light. Gel
184 images were captured using Genoplex (VWR International GmbH, Darmstadt, Germany) and
185 images processed using Adobe Photoshop (Adobe Photoshop CC).

186 **Host blood-meal identification**

187 Blood-meal sources were determined by PCR coupled with high-resolution melting (HRM)
188 analysis of vertebrate cytochrome c oxidase subunit I (COI) and cytochrome b (cyt b)
189 mitochondrial genes as previously described [30–32]. We analyzed 760 flies, representing 65%
190 of the sampled population, which included all engorged flies (n = 39) and 721 randomly selected
191 non-engorged flies. Final concentrations of the PCR reactions were performed in 20 µl PCR
192 reaction volumes, which included 4 ul of 5× Hot FIREPol EvaGreen HRM Mix (Solis BioDyne,
193 Teaduspargi, Estonia), 0.5 µM of each primer, 250 ng of DNA template, and 10 µl of PCR grade
194 water. The PCR cycling conditions included an initial denaturation at 95°C for 15 minutes
195 followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at specific temperatures
196 for COI and cyt b primers (S1 Table) for 30 seconds and elongation at 72°C for 30 seconds. This
197 was followed by a final extension at 72°C for 7 minutes. Thereafter, HRM analysis of PCR
198 products was conducted as described by [30–32]. HRM profiles were analysed using the Rotor-
199 Gene Q software v2.1 with normalized regions between 76.0-78.0°C and 89.50-90.0°C.
200 Amplicons representative of each unique HRM profile were purified using ExoSAP-IT kit (USB
201 Corporation, Cleveland, Ohio, USA) according to the manufacturer's instructions and sequenced

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202 at Macrogen (South Korea). The sequences were analysed and aligned using the MAFFT plugin
203 in Geneious software version 11.1.4 [33]. Vertebrate species were confirmed by sequence
204 alignments with Basic Local Alignment search tool (BLAST) [34] hits obtained from the
205 GenBank database.

206 **PCR identification of *Sodalis glossinidius*, *Wolbachia*, and salivary gland hypertrophy virus**

207 We screened 760 tsetse flies for their endosymbionts, *S. glossinidius*, *Wolbachia*, and salivary
208 gland hypertrophy virus (SGHV). The PCR reactions were done in 20 µl reaction volumes using
209 endosymbiont-specific primers (SI table) and similar concentrations of PCR components as those
210 described above for host blood-meal identification. The PCR cycles steps were the same as those
211 used in host blood-meal analysis with changes made only in the annealing temperatures, which
212 were specific for the different primer pairs [35–37]. Positive controls for *Wolbachia* and *Sodalis*
213 were isolated from *Aedes aegypti* and *Glossina pallidipes*, respectively, while a plasmid standard
214 from a synthetic construct of the *P74* gene of SGHV from GenScript was used as a positive
215 control. PCR-grade water used to reconstitute the extracted genomic DNA was used as a
216 negative control. The amplified products were size separated in 2% (W/V) agarose gels. The
217 amplicons were visualized, images captured, and processed as previously described.

218 **Statistical analysis**

219 Data were processed and analyzed through descriptive statistical tools using RStudio at 95%
220 confidence levels. Two-tailed t-tests were used to compare variations of tsetse fly species
221 proportions between the sampling blocks and the mean differences of host blood-meals between
222 the tsetse fly species. Fisher’s exact test was used to determine whether endosymbiont infections
223 were correlated with trypanosome co-infections.

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224 # Results

225 ## Tsetse fly species identified

226 A total of 1167 tsetse flies were collected from the ten sampling sites, of which 1136 were *G.*
227 *pallidipes* and 31 were *G. swynnertoni* (Table 1). The highest number of *G. swynnertoni* flies
228 (87.1%; n = 27) were sampled from sites closer to the border between the MMNR and the SNP
229 (i.e. Sand River and Mara Bridge sampling sites) as depicted in Fig 1. Female tsetse flies
230 constituted 61% of the collected samples, whereas 39% were male. There was no statistical
231 difference between the mean sex proportions of *G. pallidipes* (0.963±0.029) and *G. swynnertoni*
232 (0.037±0.029) ($t(4) = 31.54$, $p < 0.001$).

233 **Table 1. Numbers and proportions of trypanosome species identified in tsetse fly species.**
234 *The percentage of trypanosomes infecting each tsetse fly species is indicated in brackets.

235

Tsetse fly species	Number of tsetse flies screened	Number of tsetse infected with African trypanosomes*			Percent (%) trypanosome infection
		T. b. brucei	*T. c. savannah*	*T. vivax*	
G. pallidipes	1136	3 (0.3%)	7 (0.6%)	11 (1%)	2.1
G. swynnertoni	31	1 (3.2%)	0 (0%)	6 (19.4%)	12.9
Totals	1167	4 (0.4%)	7 (0.6%)	17 (1.5%)	2.4

236

237 ## Trypanosome species identified in sampled tsetse flies

238 Trypanosome DNA was amplified in 28 (2.40%) out of the 1167 tsetse flies sampled (Table 1).
239 The African trypanosome species identified were *T. vivax* (17/28), whereas 25% were *T.*
240 *congolense savannah* (7/28), and 14.3% were *T. brucei brucei* (4/28) (S3 Table, GenBank
241 accessions MK684364-MK684366). A representation of the samples positive for trypanosomes

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242 by PCR is shown in S1 Fig. Trypanosome infection rate was higher in *G. swynnertoni* (12.9%, n
243 = 4/31) than in *G. pallidipes* (2.1%; n=24/1136). There were no mixed trypanosome infections in
244 either of the fly species.

245 **Tsetse blood-meal sources identified**

246 Vertebrate blood-meals were detected and identified in 46.6% (354/760) of the tsetse flies
247 analyzed which comprised of 328 *G. pallidipes* and 26 *G. swynnertoni* (Fig. 2; S2 Table). The
248 most common source of blood-meal was from humans (*Homo sapiens*) (n = 131) (S3 Table,
249 GenBank accession MK684355, MK684357), followed by hippopotamus (*Hippopotamus*
250 *amphibious*) (S3 Table, GenBank accession MK684356) (n = 103), African buffalo (*Syncerus*
251 *caffer*) (S3 Table, GenBank accessions MK684354, MK684358) (n = 93), African savannah
252 elephant (*Loxodonta africana*) (S3 Table GenBank accession MK684359) (n = 12), and giraffe
253 (*Giraffa camelopardis*) (S3 Table, GenBank accession MK684360) (n = 3). There were 406
254 samples that had HRM peaks lower than 0.5 rate in fluorescence (dF/dT), thus qualified as
255 having a non-detectable blood-meal.

256 **Fig 2. Blood-meal melt curves and proportions of vertebrate species identified.** A. High
257 resolution melt curves of single species and mixed species blood-meals. Mixed blood-meals
258 were determined by matching melt profile peaks to those of more than one blood-meal control.
259 B. Overall blood-meal proportions and proportions per tsetse species.

260 Humans were the most frequently identified blood-meal sources in *G. pallidipes*, whereas
261 African buffalo was the major blood-meal sources for *G. swynnertoni* (Fig. 2B; S2 Table).
262 However, there was no significant difference in the mean blood-meal source proportions
263 between the two tsetse fly species ($t(4) = 2.47$, $p = 0.069$). We further observed that of the 28
264 trypanosome-infected tsetse, 14 (10 *G. pallidipes* and four *G. swynnertoni*) had blood-meals

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265 from African buffalo, while eight *G. pallidipes* had blood-meals from hippopotamus. The blood-
266 meal sources for six trypanosome-infected flies could not be identified. The vertebrate blood-
267 meal detection rates were 94.87% and 43.69% in engorged and non-engorged flies, respectively.

268 Twelve mixed blood-meals were detected (Fig 2), accounting for 3.4% of the positively
269 identified samples. These samples had distinct melt curves that mapped onto those of known
270 reference samples. Of these, human and buffalo mixed blood-meals was the most frequent
271 combination (6/12), followed by human and elephant (3/12), elephant and giraffe (2/12), and
272 human and hippopotamus (1/12) (Fig 2; S2 Table). Mixed blood-meals were further confirmed
273 by sequencing of representative PCR-HRM amplicons (S2 Fig).

274 **Co-infection of tsetse with endosymbionts and African trypanosomes**

275 A total of 69 (n = 760, 9.08%) flies (66 *G. pallidipes,* three *G. swynnertoni*) were infected with
276 the endosymbiont *S. glossinidius* (S3 Table, 16S-23S rRNA GenBank accessions MK684361-
277 MK684363) (S3 Fig.). Notably, a greater proportion of *S. glossinidius*-positive *G. pallidipes* flies
278 were co-infected with trypanosomes (7/68, 10.3%) than *G. pallidipes* without *Sodalis* infections
279 (16/661, 2.42%) (P = 0.0033 Fisher's exact test) (Table 2). Five of the co-infections were with *T.*
280 *congolense* and two were with *T. vivax*. As only one *G. swynnertoni* fly, which was also
281 trypanosome infected (*T. vivax*), was positive for *S. glossinidius*, the sample size was too small
282 for meaningful association analysis in this tsetse species. Nonetheless, across both species, the
283 proportion of *S. glossinidius* infected flies that were co-infected with trypanosomes (8/69,
284 11.6%) was similarly significantly higher than in tsetse without the *S. glossinidius* endosymbiont
285 (20/691, 2.89%) (P = 0.0022, Fisher's exact test).

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286 Eighteen flies (n = 760; 2.37%), 16 females and two males (all *G. pallidipes*), were
287 infected with *Wolbachia* (S3 Table, GenBank accessions MK680053-MK680056) (S3 Fig).
288 However, there was no statistical significance between *Wolbachia* and trypanosome co-
289 infections in *G. pallidipes* (P = 0.555, Fisher's exact test). No SGHV was detected in this study
290 and therefore there were no co-infection studies.

291

Table 2. Statistical analysis of *Sodalis glossinidus* and *Wolbachia* co-infection with trypanosomes in *G. pallidipes* and *G. swynnertoni*. Abbreviations: **S+/S-** *S. glossinidus* positive/negative, **W+/W-** *Wolbachia* positive/negative, **T+/T-** trypanosome positive/negative.

	G. pallidipes			*G. swynnertoni*			*G. pallidipes*	
	T+	T-		T+	T-		T+	T-
S+	7 (0.96%)	61 (8.37%)	S+	1 (3.23%)	0 (0.00%)	W+	1 (0.14%)	17 (2.33%)
S-	16 (2.19 %)	645 (88.47%)	S-	4 (12.90%)	26 (83.87%)	W-	22 (3.02%)	689 (94.51%)
P = 0.0033*; α < 0.05			P = 0.1613; α > 0.05			P = 0.555; α > 0.05		

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292 **Discussion**

293 Transmission of vector-borne diseases is dependent on the vector competence and the
294 interactions between their vectors and vertebrate hosts that are reservoirs of the parasites
295 [12,38,39]. This cross-sectional study focused on the transmission of AAT in the MMNR, a
296 wildlife ecology in Kenya, with respect to the sources of tsetse fly blood-meals and the potential
297 impact of three endosymbionts on their infection with animal trypanosomes. Our findings
298 revealed that humans, hippopotamus, and African buffaloes were the most frequent blood-meal
299 sources of tsetse flies in the MMNR. We also found that the endosymbiont, *S. glossinidius*, was
300 positively correlated with trypanosome infection in wild-caught *G. pallidipes* tsetse flies in the
301 MMNR, supporting the hypothesis that *Sodalis* potentiates AAT transmission in tsetse flies [40–
302 42]. However, we found no correlation between *Wolbachia* and trypanosome infections, and no
303 evidence of SGHV endosymbionts in the tsetse populations analyzed. These findings emphasize
304 the importance of understanding the complete spectrum of interactions amongst vertebrates,
305 tsetse fly vectors, endosymbionts, and trypanosome parasites, particularly in the context of
306 wildlife-livestock-human interfaces where emergence and reemergence of AAT and other
307 vector-borne diseases are reported.

308 *Glossina pallidipes* was the most abundant tsetse species sampled in the MMNR in this
309 study, while *G. swynnertoni* was less abundant. This finding corroborates previous studies in
310 which these two savannah tsetse species were found to be predominant in the Maasai Mara-
311 Serengeti ecosystem of Kenya and Tanzania [43–45]. As both species are competent vectors of
312 human and animal trypanosomes [46,47], their presence highlights the persistent risk of AAT
313 and HAT in the MMNR. Despite their lower abundance relative to *G. pallidipes, G. swynnertoni*
314 had higher rates of infection with trypanosomes, underpinning its critical role in transmission of

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315 African trypanosomes as demonstrated by previous studies [48]. However, unlike *G. pallidipes*,
316 which is widely distributed across many habitats in Kenya, the geographical range of *G.*
317 *swynnertoni* is limited to a narrow belt within the Maasai Mara-Serengeti ecosystem, which has
318 resulted in the prioritization of this tsetse species as a target for elimination in East Africa [49].
319 Extensive efforts have been employed over the last four decades to reduce *G. swynnertoni*
320 populations using various techniques as comprehensively reviewed by Nagagi and co-workers
321 [49]. These have included spraying with both residual and non-residual insecticides, use of
322 mechanical traps and baits with insecticide-impregnated traps or cloth targets, and insecticide-
323 treated animals as live mobile targets. Coordinated studies are needed to evaluate their effect on
324 tsetse populations and quantify their impact in East Africa.

325 Despite recent cases of HAT (caused by *T. b. rhodensiense*) being reported in East Africa
326 [50], the trypanosome species identified in this study are only those responsible for causing
327 trypanosomiasis in animals. Kenya is currently classified by the WHO as a country with
328 diminished incidence of HAT (<10 cases in the last decade), with the recent cases being reported
329 in 2012 in tourists returning from the MNNR [23,24]. Nevertheless, the persistent presence of *G.*
330 *pallidipes* and *G. swynnertoni*, which are competent vectors of *T. b. rhodensiense*, coupled with
331 the relatively higher incidences of HAT in neighboring Tanzania and Uganda and increased
332 tourism, reinforces the need for coordinated surveillance and diagnosis in the MMNR and other
333 HAT foci in eastern Africa. With reference to animal trypanosomes, this study identified *T. vivax*
334 as the most prevalent species, followed by *T. congolense* and *T. brucei brucei*. Our findings are
335 congruent with previous findings within the East African savannah [51,52]. The higher numbers
336 of flies infected with *T. vivax* may be due to differences in development cycles in tsetse flies; *T.*
337 *vivax* has all its development stages in the fly's proboscis unlike *T. congolense* and *T. brucei*,

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338 which establish in the fly mid-gut where they are affected by low pH, proteases, and lectins
339 [53,54]. Moreover, *T. vivax* usually achieves higher parasitemia in hosts than do *T. congolense*
340 and *T. brucei*, further increasing its chances of being transmitted to tsetse flies during blood-
341 feeding on infected hosts [54]. Infection rates were similar for both female and male tsetse flies,
342 as found in another recent study conducted in Mtito Andei, Kenya [47].

343 The greater abundance of *G. pallidipes*, but higher trypanosome infection rate observed
344 in *G. swynnertoni*, in the MMNR highlights the need for understanding the difference in
345 susceptibility between the two tsetse species. Vector susceptibility of *G. pallidipes* to mid-gut
346 trypanosomes has been shown to be lower compared to *G. morsitans morsitans* and *G. morsitans*
347 *centralis* [55,56]. Further still, it has been shown that tsetse protection against trypanosome
348 invasion is different for *G. pallidipes* and *G. morsitans morsitans* [56]. Similarly, field studies
349 have shown *G. swynnertoni* to be more susceptible than *G. pallidipes* [46,49]. Given that *G.*
350 *swynnertoni* is an important species in the Mara-Serengeti ecosystem, its potentially greater
351 susceptibility to trypanosome infection needs further investigation to elucidate its relative role in
352 trypanosome transmission relative to the more abundant sympatric *G. pallidipes*.

353 Blood feeding of tsetse fly populations in the wild is influenced by the composition of
354 vertebrate host species in an area and how these species attract tsetse flies [12]. Our
355 identification of animal trypanosome DNA in flies with hippopotamus and African buffalo
356 blood-meals was not surprising as these vertebrates are known to be reservoirs for *T. vivax*, *T.*
357 *congolense*, and *T. brucei* [12,55]. Nevertheless, these results suggest that active transmission
358 cycle of animal trypanosomiasis in this wildlife-livestock interface may be maintained by
359 multiple potential vertebrate hosts. Despite the abundance of wildebeest, zebra, and other
360 antelopes when the study was conducted during the Great Migration season, no blood-meals

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361 from these hosts were detected in the tsetse flies. These findings are congruent with previous
362 reports that *G. pallidipes* and *G. swynnertoni* exhibit significant specificity in host selection
363 whereby wildebeest are not preferred blood-meal sources [57,58], and that zebra skin odors are
364 repellant to *G. pallidipes* [59]. The influx of people in the MMNR due to high tourism activities
365 in the Great Migration season, may partially explain why humans were frequent blood-meal
366 sources. Identification of mixed blood-meals from humans and wildlife is indicative of the
367 inherent risk of HAT transmission in the MMNR [11,12], even though *T. b. rhodensiense* was
368 not detected in this study.

369 Visual cues and odors released by vertebrate hosts influence tsetse fly host choice and
370 have been pivotal to the development of baited traps and targets for the control and management
371 of tsetse fly populations, HAT, and AAT. A tsetse repellant formulation mimicking the odor of
372 waterbuck (*Kobus ellipsiprymnus defassa*), a non-host animal, was recently developed and used
373 as an innovative collar device to protect cattle from tsetse bites and AAT [60]. Visual cues have
374 been extensively exploited in the development of improved traps – stationery and mobile targets
375 impregnated with insecticides for riverine/"palpalis" [61–63] and savannah/"morsitans" [49,64]
376 groups of tsetse. However, it has been noted that for the morsitans group of tsetse flies, including
377 *G. pallidipes* and *G. swynnertoni*, host odors play a more significant role than visual cues as they
378 strongly attract the tsetse flies across long ranges of up to 100 m [65]. Acetone and butanone
379 odors obtained from cattle have long been used as attractants of choice in tsetse fly control [66].
380 However, other better tsetse fly attractants, such as 2-propanol, have been identified [67]. Our
381 observed high rates of buffalo, hippopotamus, and human blood-meals imply that
382 semiochemicals from these vertebrates may be possible candidates to advance research for novel
383 host-derived cues in control of *G. pallidipes* and *G. swynnertoni*. Better understanding of the

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384 molecular mechanisms of tsetse fly olfaction associated with host selection can help to evaluate
385 candidate host semiochemicals as potential attractants or repellants [67–69]. Knowledge of
386 emergent repellant odors (such as those described from zebra and waterbuck) [59,60], coupled
387 with new host attractants, present unique opportunities to further improve tsetse bait technology
388 using "Push-Pull" and/or "Attract-and-Kill" approaches.

389 This study further highlights the sensitivity of HRM technique to accurately, reliably,
390 rapidly, and reproducibly identify arthropod blood-meal hosts. We were able to identify blood-
391 meals from wild-caught non-engorged flies. Unlike serological and other PCR-based techniques
392 for blood-meal identification [57,70,71], the use of HRM to detect sequence variants is fast, cost-
393 effective, accurate, easy-to-use, and sensitive, making it a more economical tool for blood-meal
394 analysis [30,72] and pathogen detection/identification [71,73,74]. Sequencing of representative
395 samples with combined human-hippopotamus and human-African buffalo peaks confirmed their
396 mixed blood-meal status (S2 Fig.).

397 Our finding that higher proportions of tsetse flies infected with the endosymbiont *S.*
398 *glossinidus* were infected with trypanosomes than those without *S. glossinidius* corroborates
399 previous studies that suggest that *S. glossinidius* infection may have the potential to increase the
400 ability of both wild caught [40,41] and lab reared [42,75] tsetse flies to acquire trypanosomes.
401 Further, this finding implies that *S. glossinidius* infection in tsetse populations may be used as a
402 positive indicator of trypanosomiasis risk. Nonetheless, the functional role of *S. glossinidius* in
403 tsetse flies was not explored in this study and knowledge on the same remains limited [76].
404 However, inhibition of tsetse mid-gut and mouthpart lectins by Nacetyl-d-glucosamine
405 (GlcNAc), a product of chitin catabolism by *S. glossinidius*, has been proposed as the main factor
406 associated with *S. glossinidius* and increased tsetse-vector competence [18,77,78]. Nevertheless,

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407 this association is complex and a number of other factors, including geographic location, tsetse
408 fly species, sex, and age also affect the capacity of *S. glossinidius* to increase vector competence
409 in wild-caught tsetse flies [77]. While more studies are needed to elucidate the role of *Sodalis*
410 endosymbionts on tsetse competence to vector trypanosomes, our findings support that *S.*
411 *glossinidius* infection increases probability of savannah tsetse flies to acquire animal
412 trypanosome infection in this wildlife-livestock interface.

413 **Conclusions**

414 Emergence and/or reemergence of AT, especially in human-wildlife-interfaces like the MMNR,
415 where AT has been recently reported, happens occasionally. With limitations on current methods
416 of control and management of AT and its tsetse fly vectors, more research on the factors
417 influencing trypanosome transmission is required. Identification of trypanosome-infected tsetse
418 flies that had fed on hippopotamus and African buffalo highlights these two vertebrate species as
419 possible reservoirs of trypanosomes in the MMNR, providing a basis for investigating their
420 contributions to AT in other human-wildlife-interfaces. Further understanding of the
421 attractiveness of hippopotamus and expounding on existing knowledge on African buffalo to
422 tsetse flies based on the volatiles they release may help improve tsetse baits and repellants. In
423 addition, our findings demonstrate that the endosymbiont *S. glossinidius* increases tsetse fly
424 susceptibility to trypanosome infection in this endemic ecology. These findings support the idea
425 that *S. glossinidius* can be a potential target for vector control [79]. Despite *T. b. rhodensiense*
426 not being detected, it has previously been reported in the MMNR. Hence, there is need for
427 intense prevalence studies of *T. b. rhodensiense* in tsetse flies from the MMNR.

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445

446 ## Author's contributions

447 Conceived and designed the experiments: LW, JV, MJ, VOA. Performed the experiments: EEM,
448 EM, JWO, AAM. Analyzed the data: EEM, JV, LW, VOA, EMK. Tsetse collection: LW, JV,
449 MJ. Tsetse fly identification: EEM. Wrote the paper: EEM, LW, JV, VOA, MJ, EMK.
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451

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680 ## Supporting Information

681 **S1 Fig. PCR detection of trypanosome species in tsetse flies**. **A**. Agarose gel electrophoresis
682 images of representative tsetse fly samples positive for Trypanozoon, *T. vivax*, and *T. congolense*
683 PCR amplicons with ITS BR/CR primers specific for African trypanosomes species. The
684 trypanozoon group were further resolved using primer pairs specific for *T. b. rhodesiense* and *T.*
685 *b. brucei*. **B**. PCR amplification results for detection of *T. b. rhodesiense* using primers targeting
686 the SRA gene. **C**. PCR amplification results for detection of *T. b. brucei* using primers targeting
687 the TbbGPI-PLC gene. M represents the molecular ladder – represents negative control and +
688 represents positive control.

689 **S2 Fig. DNA sequence analysis of mixed blood-meals. A**. Hippopotamus and human mixed
690 blood-meal cytochrome b sequences aligned and edited using Geneious v8.0.1. **B**. Buffalo and
691 human mixed blood-meal cyt b sequences aligned and edited using Geneious v8.0.1. Scientific
692 names and the GenBank accession numbers highlighted in red represent sequences obtained from
693 this study.
694 **S3 Fig. PCR detection of endosymbionts in tsetse flies. A.** Agarose gel electrophoresis image
695 of a representative PCR amplicons of *S. glossinidus* DNA. **B.** Agarose gel electrophoresis image
696 of a representative PCR amplicons for *Wolbachia*.

697 **S1 Table. Primer list with annealing temperatures.** Details of primer sequences and PCR
698 conditions used in this study.

699 **S2 Table. Trypanosome species and host blood-meals among the tsetse fly species in this**
700 **study.** Distribution of trypanosome infections and blood-meals sources in *Glossina pallidipes*
701 and *Glossina swynnertoni* in Maasai Mara, Kenya.

702 **S3 Table. Data URL repository associated with this study**. Details of the nucleotide
703 sequences generated in this study and URLs for obtaining the respective accessions in GenBank.

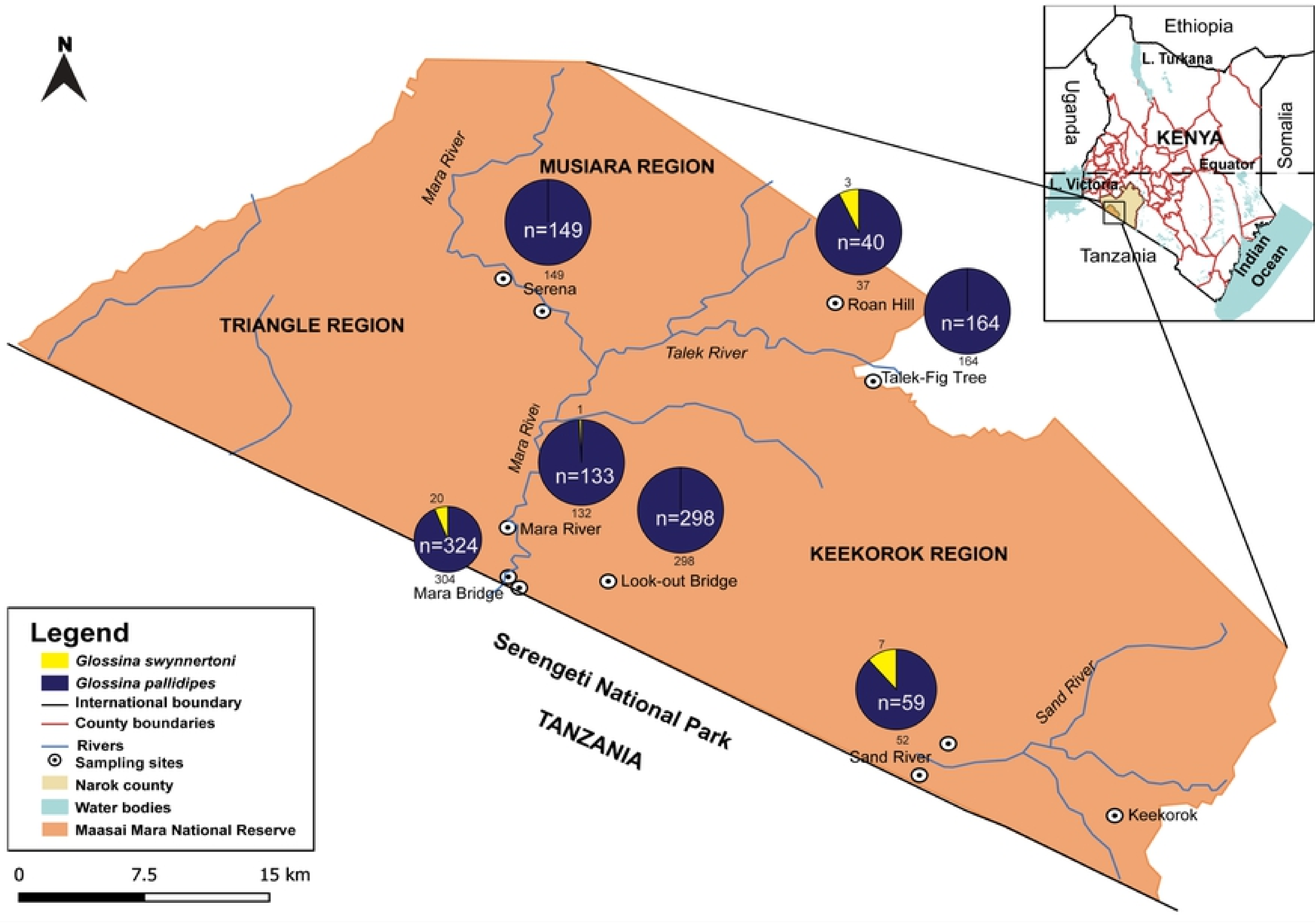
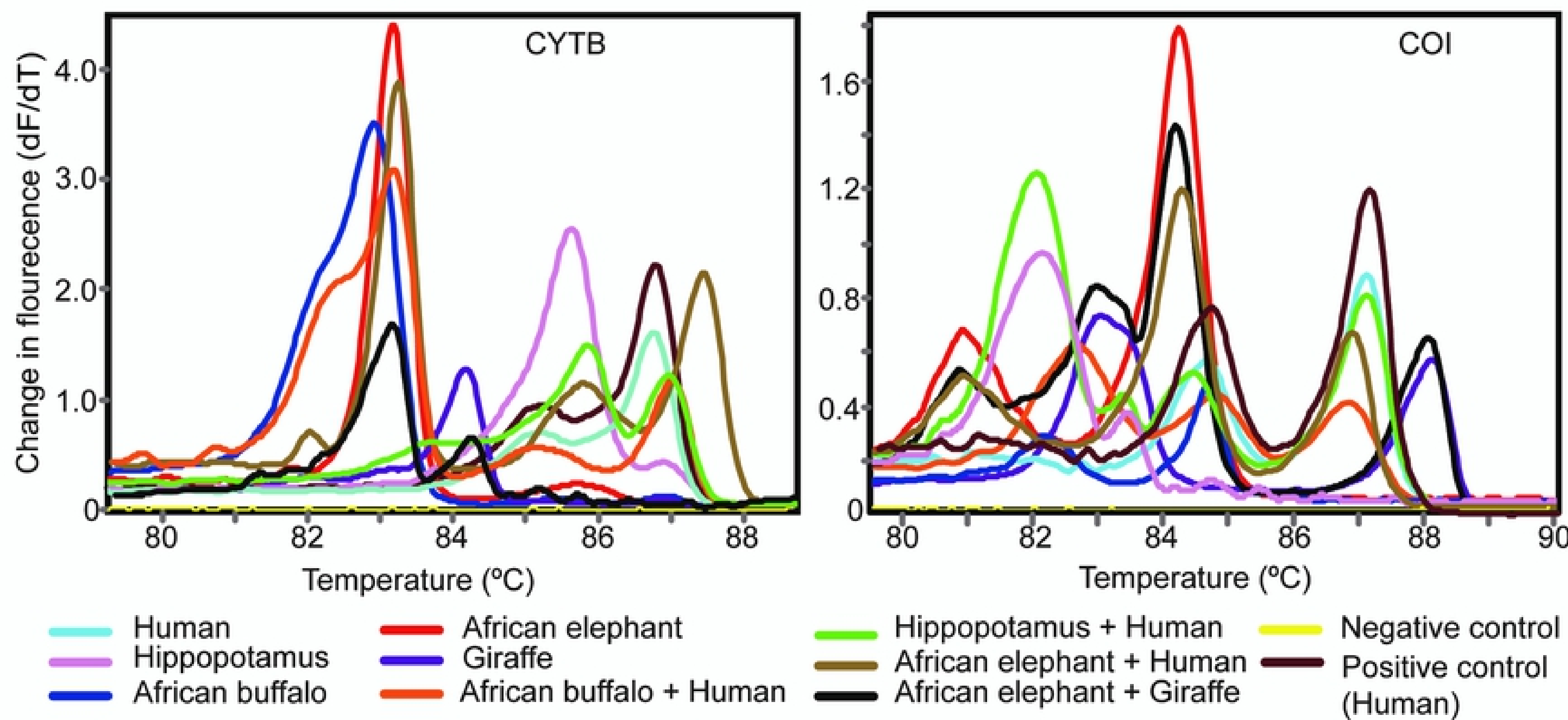


Fig 1

A



B

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Maasai Mara National Reserve overall blood-meal proportions

406
103
93
131
12
12
3

n = 760

By tsetse fly species

G. pallidipes

123
10
3
11
98
83

n = 328

G. swynnertoni

8
10
2
1
5

n = 26

LEGEND
bloodmeal not detected
Hippopotamus
African buffalo
Human
Giraffe
African elephant
Mixed blood-meal

Fig 2