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Received: 16 February 2022 | Revised: 25 April 2022 | Accepted: 17 May 2022

DOI: 10.1111/1755-0998.13670

MOLECULAR ECOLOGY RESOURCES WILEY

RESOURCE ARTICLE

Using haematophagous fly blood meals to study the diversity of blood-borne pathogens infecting wild mammals

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Funding information
Agence Nationale de la Recherche, Grant/Award Number: ANR-10-LABX-04-01; Agence Nationale de la Recherche, Grant/Award Number: ANR-11-LABX-0024; Horizon 2020 Framework Programme, Grant/Award Number: ZIKAlliance(no734548)

Abstract

Many emerging infectious diseases originate from wild animals, so there is a profound need for surveillance and monitoring of their pathogens. However, the practical difficulty of sample acquisition from wild animals tends to limit the feasibility and effectiveness of such surveys. Xenosurveillance, using blood-feeding invertebrates to obtain tissue samples from wild animals and then detect their pathogens, is a promising method to do so. Here, we describe the use of tsetse fly blood meals to determine (directly through molecular diagnostic and indirectly through serology), the diversity of circulating blood-borne pathogens (including bacteria, viruses and protozoa) in a natural mammalian community of Tanzania. Molecular analyses of captured tsetse flies (182 pools of flies totalizing 1728 flies) revealed that the blood meals obtained came from 18 different vertebrate species including 16 non-human mammals, representing approximately 25% of the large mammal species present in the study area. Molecular diagnostic demonstrated the presence of different protozoa parasites and

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Handling Editor: Sebastien Calvignac-Spencer

bacteria of medical and/or veterinary interest. None of the six virus species searched for by molecular methods were detected but an ELISA test detected antibodies against African swine fever virus among warthogs, indicating that the virus had been circulating in the area. Sampling of blood-feeding insects represents an efficient and practical approach to tracking a diversity of pathogens from multiple mammalian species, directly through molecular diagnostic or indirectly through serology, which could readily expand and enhance our understanding of the ecology and evolution of infectious agents and their interactions with their hosts in wild animal communities.

KEYWORDS
blood meals, invertebrate-derived DNA (iDNA), tsetse flies, wildlife infectious diseases, xenosurveillance

1 | INTRODUCTION

About 60% of emerging diseases in humans are of zoonotic origin (Jones et al., 2008). Emergence of these zoonotic diseases is linked to increasing contacts between humans and wild animals and their spread is amplified by the global intensification of international trade and travel (Machalaba & Karesh, 2017), as recently highlighted by the global SARS-CoV-2 pandemic, the regional pandemic of Zika across Latin America, two major Ebola outbreaks in central and west Africa and the establishment of *Plasmodium knowlesi* in South-East Asia as the fifth causative agent of human malaria (Schmeller et al., 2020; White, 2008). Domestic animals are also at risk from emerging infectious diseases as evidenced by numerous transfers of pathogens from wildlife reservoirs (e.g., Foot-and-mouth disease virus, Avian influenza virus, African swine fever virus, Rift valley fever virus, *Brucella* sp. bacteria) (Bengis et al., 2004; Wiethoelter et al., 2015). Outbreaks of such diseases constitute massive issues for human and animal health, necessitating active monitoring for signs of active outbreaks, as well as the epizootics and enzootic transmission processes that precede them, including rapid diagnosis of the pathogen involved. It is therefore crucial to anticipate and prevent potential epidemics and subsequent pandemics by developing new methods for the early detection and monitoring of pathogens in wild animal reservoirs (e.g., Hoffmann et al., 2016; Mörner et al., 2002).

Currently, surveys of wild animals for pathogens is mainly based on three methods (e.g., Ryser-Degiorgis, 2013): (i) the analysis of bushmeat and/or game meat; (ii) the direct trapping of animals for organ and tissue collection; or (iii) the analysis of noninvasive samples such as faeces. These methods have yielded valuable insights in many cases but also have limitations. Specifically, bushmeat and game meat represent only a fraction of the full range of fauna present in high biodiversity ecosystems, and is systematically biased towards species consumed or hunted for other purposes by humans. Live capture of wild animals is also associated with major difficulties, biases and dangers, which render sampling of some species either completely infeasible or prohibitively expensive. In particular, direct trapping often poses unacceptable risks to endangered target species and human investigators. Finally, noninvasive sampling of faecal droppings, for example, can be very difficult to collect fresh enough from cryptic and/or evasive animals and such specimens do not necessarily allow satisfactory detection of all pathogens. Furthermore, these samples often represent difficult media for pathogen detection due to nucleic acid or protein degradation and also because they contain inhibitors of the extraction or amplification reagents required for molecular analysis (Natarajan et al., 2021; Sarabia et al., 2020). New complementary approaches are therefore needed for monitoring and detecting pathogens in wildlife.

Recently, several studies have explored the possibility of trapping invertebrates that feed on living or dead vertebrate hosts to assess the presence of pathogens through "invertebrate-derived DNA" (iDNA) analysis (Alfano et al., 2020; Bitome-Essono et al., 2017; Calvignac-Spencer et al., 2013; Grubaugh et al., 2015; Hoffmann et al., 2017). This approach has been called xenomonitoring or xenosurveillance (e.g., Grubaugh et al., 2015). We showed for instance that haematophagous flies (such as tsetse flies) could be used as "flying syringes" to collect blood from wild vertebrates and survey pathogens found therein. During 16 weeks of sampling in the forests of Gabon (Bitome-Essono et al., 2017), more than 4000 haematophagous flies were collected, of which about a third were blood-engorged. Molecular analyses revealed a wide diversity of origin among their blood meals, with 20 vertebrate species represented. Blood sources identified included 10 mammalian species (e.g., forest buffaloes, elephants, hippopotamus, several antelopes, gorillas), some reptiles (crocodiles, tortoises and snakes) and birds. Further analyses of these samples also revealed diversity of extant malaria parasites, a group of parasites that has historically exhibited a propensity to emerge in and adapt to new hosts (e.g., *P. knowlesi*). It is notable that malaria parasites were detected in 10% of blood meals, even though the blood sucking flies used to obtain these samples are not vectors of these haemosporidia (Bitome-Essono et al., 2017). Some of the detected parasite species had previously been described, but others represented new species and this novel screening strategy also allowed us to identify previously unknown hosts of some *Plasmodium* lineages.

The findings demonstrated that the use of haematophagous flies as "flying syringes" could help identify and detect blood-borne pathogens of wild animals, representing a potentially invaluable tool for expanded and enhanced surveillance of wild pathogen

reservoirs in the future. Tsetse flies such as tsetse flies are particularly interesting for such surveillance for several reasons (Bitome-Essono et al., 2017): (i) they are easy to trap and a large proportion of trapped individuals are blood-engorged (unlike some other blood-sucking insects such as mosquitoes, both sexes are generally haematophagous), for example 30% in the samples we obtained in Gabon; (ii) the large size of their blood meal, ranging from 20 μl up to 100 μl for the largest species, enables sensitive pathogen detection; (iii) they are very opportunistic in their host choice, thus allowing the screening of a large diversity of vertebrate species, ranging from reptiles and birds through to several taxonomically divergent mammals (mostly large bodied species) (Muturi et al., 2011; Muzari et al., 2010; Späth, 2000); (iv) they occur across a wide range of environments, from forests to savannahs (Bitome-Essono et al., 2017; Muturi et al., 2011). By extending the array of pathogens tested for within their blood meals, and by screening a larger set of samples from different locations, these "flying syringes" could become an excellent tool for surveillance and studying the ecology and evolution of many different enzootic blood-borne pathogens, including viruses, bacteria, protozoa and macroparasites.

The objective of the study reported herein was to demonstrate the potential of this approach for screening a large variety of pathogens in an ecosystem with high densities of haematophagous flies and high biodiversity of vertebrates, as well as a known history of pathogen transfers between wild animals and from wild animals to humans and/or domestic animals and vice versa (e.g., Clifford et al., 2013). We also aimed to extend the approach by testing for antibodies against specific pathogens by applying serological tests to blood meals obtained from tsetse flies, as previously performed on similar or different invertebrate models (Barbazan et al., 2009; Cunningham et al., 1962; Stefanic et al., 2022). The addition of serology methods to the array of tests deployed was intended to determine which species had been exposed to a particular pathogen, so that broader surveys across wider geographic scales in the future could identify places where a pathogen species has been circulating, even though the pathogen itself cannot be ascertained through direct detection of active infections by molecular methods because outbreaks may often flare up and subside before or between surveys.

2 | MATERIALS AND METHODS

2.1 | Study area

The study was conducted in Ruaha National Park (RUNAPA) which is the second largest park in Tanzania and East Africa. It is located in south-central of Tanzania at approximately 7°30′S and 35°00′E, covering an area of about 20,226 square kilometres (Ruaha National Park, 2017) (Figure 1). The park is part of a more extensive ecosystem which includes the Rungwa Game Reserve, the Kizigo and Muhesi Game Reserves, and the MBOMIPA Wildlife Management Area. It has a semi-arid to arid climate with bimodal pattern of rainfall peaks occurring from December to February and March to April, with an average annual rainfall of 500 mm (Cusack et al., 2015). The dry season is from June to November.

The Ruaha National Park is home to a wide diversity of wild animals, especially large herbivore populations (e.g., savannah elephants, African buffaloes, giraffes and over a dozen of antelope species), which can serve as reservoirs for a large diversity of pathogens with potential for spillover into domestic animals or humans (Pastoret et al., 1988). This is especially so as the park is bordered by lands used for livestock grazing, farming and settlements. Farmers, pastoralists, livestock, and wildlife coinhabit the southern portion of the Ruaha ecosystem, where they all depend upon the same limited water

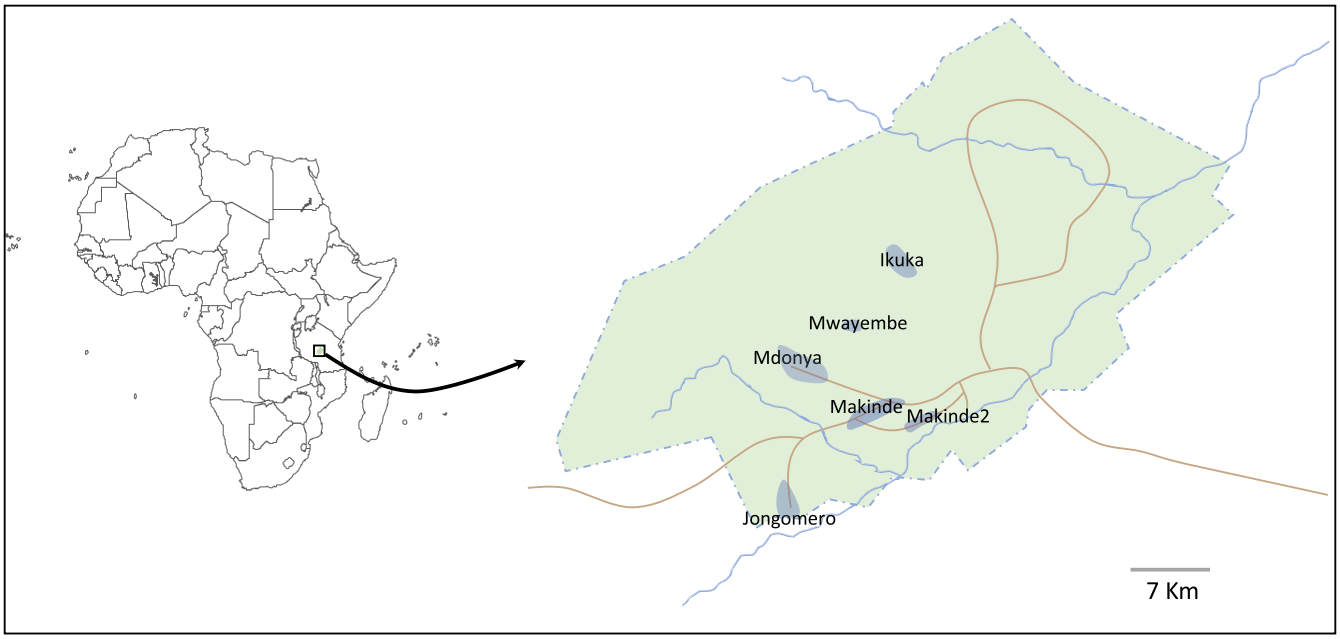

FIGURE 1 Location of the sampling sites in the park of Ruaha (Tanzania). In green, delimitation of the park. In blue, areas of sampling

sources in and around the Great Ruaha river and its tributaries, especially during the dry season (Clifford et al., 2013). This ecosystem has an epidemiological history that strongly suggests increased risk of zoonotic disease spillover and transmission across and between host taxon compartments (Mazet et al., 2009), presumably because of its role as an important interface between wild and domestic ecosystems. For example, evidence of Bovine tuberculosis, Brucellosis, Rift valley fever and interspecies transmission events have been frequently documented in a recent past (Roug et al., 2020).

This park is home to very high densities of haematophagous flies, especially tsetse flies (genus *Glossina*) (Muse et al., 2015). In east Africa, tsetse flies are known to feed on a large diversity of mammals including bovids (e.g., buffaloes, elands, kudus, gazelles, impalas, wildebeests), suids (e.g., warthogs and bush pigs), elephants, giraffes and nonhuman primates (e.g., baboons, vervet monkeys) (e.g., Muturi et al., 2011).

2.2 | Ethics statement

All research activities in Tanzania were reviewed, approved, and permitted by the Tanzania Commission on Science and Technology (COSTECH), the Tanzania Wildlife Research Institute (TAWIRI), Tanzania National Parks (TANAPA) and the Ethical Committee of Ifakara Health Institute.

2.3 | Fly collection

Flies were collected for 6 days using pyramidal traps in six different parts of Ruaha National Park (Table 1 and Figures 1 and 2). Sampling was done during the rainy season from 4–9 April 2019. During this 6 day period, 15 pyramidal traps were deployed each day (one day per site) of the park starting around 7:00 AM. Among them, 14 were hung from trees along dirt roads every 50–100 m from each other and left there over the course of the entire day. One trap was attached to the back of the car following a recommendation from our park ranger (Figure 2b). Caught tsetse flies were then recovered each evening around 5:00 PM and brought back to the park laboratory for dissection. The abdomen of each fly was separated from the rest of the animal using narrow-pointed forceps and preserved in 200 µl of RNAlater in pools of several individuals (generally 10) for molecular and serological analyses. All pools were crushed using sterile pellet pestles before freezing and were constituted by mixing only abdomens from the same day and site of collection. Forceps and pestles were cleaned after each use in several baths of bleach and water and reused once dried. A total of 356 pools of abdomens were obtained from 3378 flies. Two negative control tubes containing 200 µl of RNALater in which we plunged already used but cleaned forceps and the already used but cleaned pestles were conserved to assess the possibilities of inter-sample contamination during these steps. Samples were then preserved at Ifakara health institute in Tanzania at −80°C and then sent with dry ice to MIVEGEC laboratory in France for molecular and immunological analyses.

2.4 | Molecular analysis

Molecular analyses were performed using standard procedures to avoid as much as possible contaminations of our samples with foreign DNA or RNA. Rooms for extraction, preparation of PCR mix and amplification are separated. All materials used were previously autoclaved and all steps were performed under hoods that were previously decontaminated with RNAse AWAY and UV light.

2.5 | Nucleic acid extraction

Nucleic acids (both DNA and RNA) were extracted from only a subset of pools ($n = 182$, in total 1728 flies) for financial reasons. Among them, 138 were taken because traces of red blood (fresh) were observed while crushing the abdomens into the RNAlater.

For DNA and RNA extraction, 50 µl of the liquid from each pool was taken and diluted with 50 µl of phosphate buffer saline solution (PBS) and centrifuged at 17,000 *g* for 10 min. Supernatant was discarded and total nucleic acid extraction was performed on the pellet using the Mag FAST 384 kit (IDVET, France) according to the manufacturer's instructions. DNA and RNA extracts were preserved at −80°C until used for molecular analyses. Two negative extraction controls were added during extractions of the 182 pools.

2.6 | Molecular diagnostic of pathogens

Extracted nucleic acids were used as template to perform a set of molecular tests to identify the presence of different pathogens of interest including: (i) for parasites, the genera *Plasmodium*, *Babesia*, *Theileria* and *Leishmania*; (ii) for bacteria, the genera *Brucella* and *Mycobacterium* and (iii) for viruses, African swine fever virus (ASFV, a DNA virus), Foot-and-mouth disease virus (FMDV, an RNA virus), the Crimean congo haemorrhagic fever virus (CCHFV, an RNA virus), the Rift valley fever virus (RVFV, an RNA virus), the Peste des petits ruminants virus (PPRV, an RNA virus) and Bluetongue virus (BTV, also an RNA virus).

Conditions for qPCR or PCR used for the detection of this panel of pathogens are presented in Table S1. For certain RNA viruses (PPRV and BTV), RT-PCR was included in the kit used for molecular diagnostic while for others a reverse-transcription of RNA into cDNA was performed using the protocol described in Table S2 before diagnostic analysis. PCR negative controls were systematically added to all batches of amplification.

2.7 | Identification of blood meal origin

Identification of blood meal origin was performed by amplifying, from each fly pool DNA extract, a ~95 bp fragment of the vertebrate mitochondrial DNA (mtDNA) 16S locus and sequenced as a single pool of amplicons using Illumina Miseq Platform (Platform GenSeq, Montpellier, France). Amplicon libraries were constructed

following a two-step PCR protocol according to the Illumina 16S Metagenomic Sequencing Library Preparation Guide (Ref.15044223 Rev. B), replacing 16S rRNA primers with the mtDNA 16S primers (16S mam1 and 16S mam2) defined by Taylor (1996). Briefly, a first round of PCR was performed using locus-specific primers with 5′ nucleotide overhangs. The overhangs aimed at anchoring a second round of PCR that introduced indexes and completed Illumina adapters. The first round of PCR was carried out using the conditions described in Boessenkool et al. (2012). One negative control was added to this first round of PCR and further processed such as the samples. After a magnetic bead purification (Clean PCR, Proteigene), the second round of PCR was performed in a total volume of 18 µl (5 µl of first round PCR products, 9 µl Phusion High-Fidelity PCR Master Mix [NEB], 2 µl I5 index-adapter, 2 µl I7 index-adapter). Cycling conditions comprised a step at 95°C for 3 min followed by 10 cycles of 95°C 30 s, 55°C 30 s, 72°C 30 s and a final elongation step of 5 min at 72°C. A set of inhouse-designed index pairs (all including nine nucleotides and differing by at least three nucleotides) was used for multiplexing all samples on a single MiSeq run. Two negative controls were added to the second round of PCR. After purification with magnetic beads, final PCR products were multiplexed and sequenced on a MiSeq Illumina sequencer using MiSeq Reagent Kit version 3 (600-cycle; Illumina).

TABLE 1 Number of flies collected per site and per type of trap (back of the car, tree hanging or unknown). This latter category is provided as, for some sites, the information regarding the provenance of flies (back car trap or tree hanging trap) was not conserved

Sites	Back car ($n = 1$)	Unknown ($n = 15$)	Tree hanging ($n = 14$)	Total
Ikuka		869		869
Jongomero	117		163	280
Makinde	427		229	656
Makinde2	363		419	782
Mdonya		335		335
Mwayembe	72		384	456
Total	979	1204	1195	3378

Abbreviation: *n*, number of traps used.

2.8 | Molecular blood meals data processing and analyses

Reads were demultiplexed at the end of the sequencing using the embedded routine of the sequencer (Illumina Miseq) according to the dual index introduced during the library construction process. USEARCH version 11 pipeline (Edgar, 2010) was then employed to cluster all sequences into operational taxonomic units (OTUs) at cut-off value of 97% similarity. OTUs with <100 reads were discarded.

Remaining OTU sequences were then compared against the NCBI Genbank database (www.ncbi.nlm.nih.gov/) using BLASTn. Only the top hits, with at least 95% similarity were conserved. Taxonomic assignment was then manually performed according to the following procedure. If top hits corresponded only to one species and the species is present in Ruaha NP, then the OTU was assigned to the species. If the species is not present in Ruaha but a close species of the same genus is and is the only species of this genus, then we assigned this latter species to the OTU. If top hits corresponded to different species, then several situations occurred: (i) only the information of the genus was conserved if they belonged

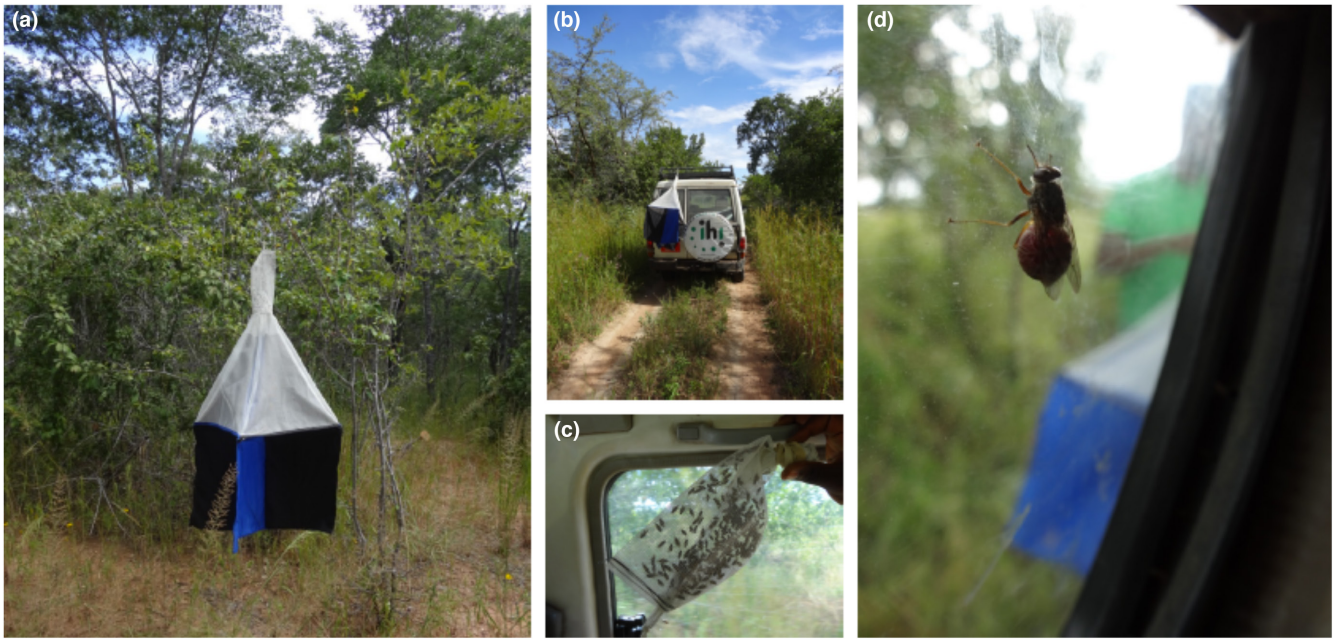

FIGURE 2 Pictures of the traps. (a) A pyramidal trap on the field in Ruaha national park (Tanzania). (b) A pyramidal trap placed at the back of the car. (c) A picture of the back-car trap after approximately 1 h of collection in a tsetse fly area. (d) An engorged tsetse fly inside the car during field sampling

to the same genus; (ii) only the species that is known to be present in Ruaha was conserved or (iii) the OTU was discarded because we could not assign without doubt a species or a genus to it. Finally, a species was considered to be absent from a pool if the reads belonging to this species represented <1% of the total number of reads assigned to this pool. This filtration was used to reduce the possible effect of intersample contamination. Blast results and final taxonomic assignment are provided in Table S3.

2.9 | Antibody detection

Because many of the blood samples were found to have originated from warthogs (*Phacochoerus africanus*), and because this species is known to be a natural reservoir of ASFV in East Africa (Hakizimana et al., 2021), screening for ASFV antibodies was opted. ASFV antibody detection was performed using the IDVET ID Screen African Swine Fever Indirect test kit (IDVET; Innovative Diagnostic). The kit provides a multiantigen indirect ELISA test for the detection of antibodies against P32, P62 and P72 ASFV antigens. The test was performed using 50 µl of the raw samples (the liquid part of crushed abdomens in RNA later, after allowing the homogenate to settle briefly) from a subset of fly abdomen pools, for which the presence of *Phacochoerus* blood was detected using molecular blood analysis and also on eight samples containing no *Phacochoerus* blood as negative controls. The positive and negative controls of the kit were also used to validate the performance of the kit. For each sample, absorbance was read at 450 nm. The cutoff for positivity was set at 0.30 as recommended by the kit manufacturer. However, the analysis of the distribution of optical density from the negative controls was used to redefine a lower, less conservative, cutoff value of 0.13 (according to the traditional formula for Elisa cutoff as 2 × mean + 2 standard deviations for the negative controls [Lardeux et al., 2016]).

3 | RESULTS

3.1 | Tsetse flies collection

After six days of collection in different locations of the park, a total of 3378 flies were obtained with an average of 563 flies captured per day. The number of flies captured with the mobile traps placed on the back of the car while driving through the sites of collection was on average far greater (mean number of flies trapped = 244.8 flies per trap per day) than for stationary traps placed along the road (mean number of 21.3 flies per trap).

3.2 | Diversity of host species detected in blood meals

Over the 182 pools of flies analysed, a total of 9,174,264 reads (average of 50,408 per sample [min: 1596; max: 83,667]) were obtained and trimmed. No difference in the amount of reads obtained from pools with or without fresh blood traces were observed. 148 OTUs were obtained from the 16S mtDNA sequences. 101 OTUs presented more than 100 reads and were then blasted on the global NCBI database (Table S3). Despite precautions taken during manipulation of the samples and molecular treatments (extraction, PCR), three negative controls out of six contained several thousands of reads. Two contained reads from *Homo sapiens* and one from *Phacochoerus africanus*, thus indicating that intersample contamination has occurred during the different steps (Table S4).

Taxonomic assignment of OTUs obtained from all pools allowed the identification of 18 different vertebrate host species, mainly mammals (Figure 3; Tables S3 and S4). Mammals included several species of Artiodactyla (*Tragelaphus strepsiceros*: greater kudu, *Tragelaphus imberbis*: lesser kudu, *Taurotragus oryx*: common eland, *Syncerus caffer*: African buffalo, *Sylvicapra grimmia*: common duiker, *Hippotragus equinus*: roan antelope, *Giraffa sp.*: giraffe, *Hippopotamus amphibius*: hippopotamus, *Potamochoerus larvatus*: bushpig and *Phacochoerus africanus*: warthog), as well as Carnivora (*Panthera leo*: lion, *Crocuta crocuta*: spotted hyena, *Mellivora capensis*: African honey badger), Primates (*Papio cynocephalus*: yellow baboon and *Homo sapiens*: human), of Rodentia (*Hystrix* sp.: porcupine) and Proboscidea (*Loxodonta Africana*: African savannah elephant). In addition, one bird species, *Bucorvus leadbeateri* (southern ground hornbill) was also identified, despite the fact that the primers used to amplify the 16S gene were supposed to be mammal-specific. The most frequently detected mammal species were warthog in about 73% of the pools, greater kudu in 49%, giraffe in 45% of the samples, African elephant in 31% and African buffalo in 25% of the pools. The 12 remaining species were all identified in <8% of the pools. Also, most of pools harboured more than one species, with 84% of pools containing at least two species.

Although it is plausible that the tsetse flies have fed on humans present in the park (rangers, staff of the park, researchers, tourists), human DNA is also a common laboratory/handling derived contaminant, as observed in some of our negative controls. Whether the presence of human DNA in this study originates from the blood meal of tsetse, or is due to contamination, cannot be easily determined.

3.3 | Pathogen detection

Twelve pathogen species of medical and/or veterinary interest (protozoa, bacteria and viruses) were screened for among the pools of host blood upon which tsetse flies had fed. From all 182 pools analysed, no DNA or RNA viruses were detected out of the six virus species of interest searched tested for. Positive pools were only obtained for protozoan and bacterial pathogens. None of the negative controls was observed positive to any of the pathogens screened.

The protozoan parasites detected in the pools included *Plasmodium* spp. and *Theileria* spp. None of the pool samples tested were positive for *Leishmania* or *Babesia*. *Plasmodium* parasites were detected in two pools of tsetse abdomens containing blood of greater kudu (*T. strepsiceros*) only (Figure 4). The BLAST analyses of the

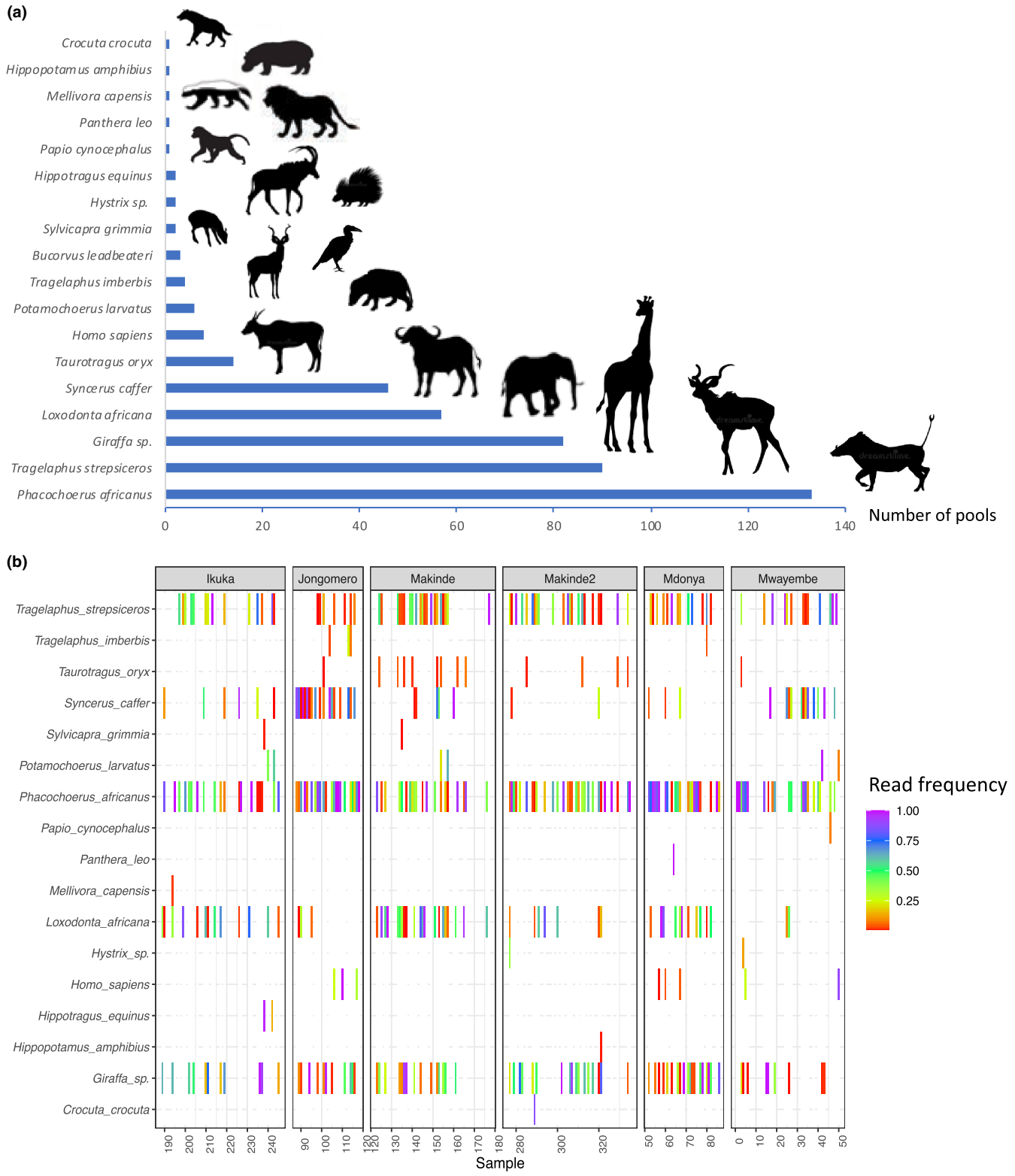

FIGURE 3 Diversity of host species found in the blood meals of the tsetse flies collected in Ruaha National Park. (a) Number of pools containing each detected host species. (b) Heat map of the frequencies of reads (proportion of reads belonging to one host species/total number of reads in a pool) blasting to the different species found in the different pools of tsetse flies within each sampling site (Ikuka, Jongomero, Makinde, Makinde2, Mdonya, Mwayembe)

Plasmodium sequences revealed a strong similarity with *Plasmodium* of ungulates found in Africa (Bitome-Essono et al., 2017; Boundenga et al., 2016; Templeton et al., 2016).

Theileria parasites were detected in 14 pools (Figure 4). BLAST analyses revealed a similarity of our sequences with several distinct *Theileria* species (e.g., *Theileria giraffi*, *Theileria taurotragi*, *Theileria*

ovis and *Theileria parva*). The linkage of parasite to a particular single host species was not possible because pools from which they were recovered contained blood of several host species (Figure 4). Among them, greater kudu, warthog, giraffe and elephant were the most frequent.

With regard to bacteria, both *Mycobacterium* spp. and *Brucella* spp., respectively were detected in two and four samples. Sequences obtained were not long enough to discriminate between the different species of bacteria. Again, the linkage of the pathogens to a particular single host species was not possible because the positive pools contained blood from multiple host species, except for one pool that was positive for *Brucella* that contained only giraffe blood. For *Mycobacterium* spp., the candidate hosts were giraffe, elephant, warthog and greater kudu, while for *Brucella*-positive pools containing multiple hosts, the candidate hosts were giraffe, warthog and greater kudu.

Note that three pools were positive for several pathogens (e.g., *Plasmodium* and *Theileria*) (Figure 4) but we were unable to determine whether these arose from coinfections of a single host animal blood or infections from different host animal bloods within each pool.

Overall pools of flies, pathogens were more probably detected in pools containing traces of fresh blood than in the other flies (Fisher's exact test, p-value = .0082).

3.4 | Antibody detection

Using the manufacturer-recommended cutoff value for the IDVET kit (OD = 0.30), six pools exhibited a positive antibody response to ASFV out of the 80 warthog-positive pools tested (7.5%). With our less conservative cutoff value of 0.13 (based on the mean and distribution of negative controls), we identified 17 positive pooled samples (21.3%) out of the 80 tested (Figure 5).

4 | DISCUSSION

The use of blood-feeding invertebrates to survey wild vertebrate diversity has now been used in different contexts and ecosystems (e.g., Drinkwater et al., 2020; Kocher et al., 2017; Massey et al., 2022). Nevertheless, the use of the same invertebrates to investigate the pathogen diversity associated to wild hosts is still in its infancy and has rarely been performed (Calvignac-Spencer et al., 2013). To date, only few studies have used such an approach for pathogens detection from wild animals. For instance, one study has been carried out on tsetse flies, in a forest ecosystem in Gabon with a limited and specific focus on detection of *Plasmodium* parasites (Bitome-Essono et al., 2017). Another study used leeches in Borneo (Alfano et al., 2020) while others used carrion flies to determine the infection causing mortality in some wild animals (Gogarten et al., 2019; Hoffmann et al., 2016, 2017; Patrono et al., 2020). Overall, the study reported herein provides a further successful example of the potential use of blood-feeding flies such as tsetse for xenosurveillance, as a means to collect tissues (in this case, blood) from wild animals and detect the presence and diversity of circulating pathogens from these tissue specimens. Also, this study demonstrates that it is possible to extend the principle of xenosurveillance based on samples of blood-fed insects to the detection of circulating antibodies against specific infectious diseases even after outbreaks may have come and gone.

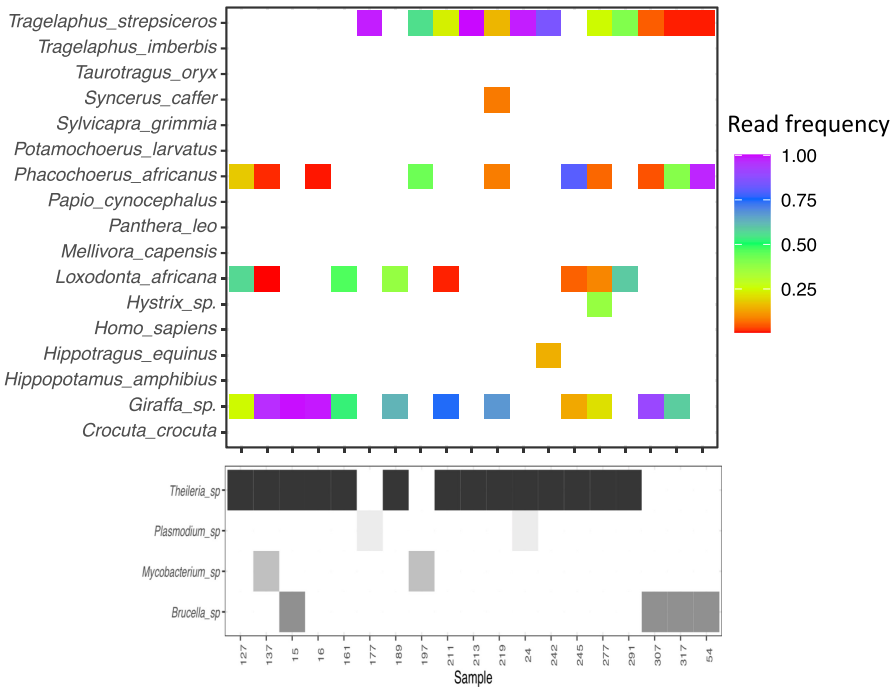

FIGURE 4 Top. Heat map of the frequencies of reads (proportion of reads belonging to one host species/total number of reads in a pool) blasting to the different species found in the infected pools. Bottom. Presence/absence of the pathogen from the pool

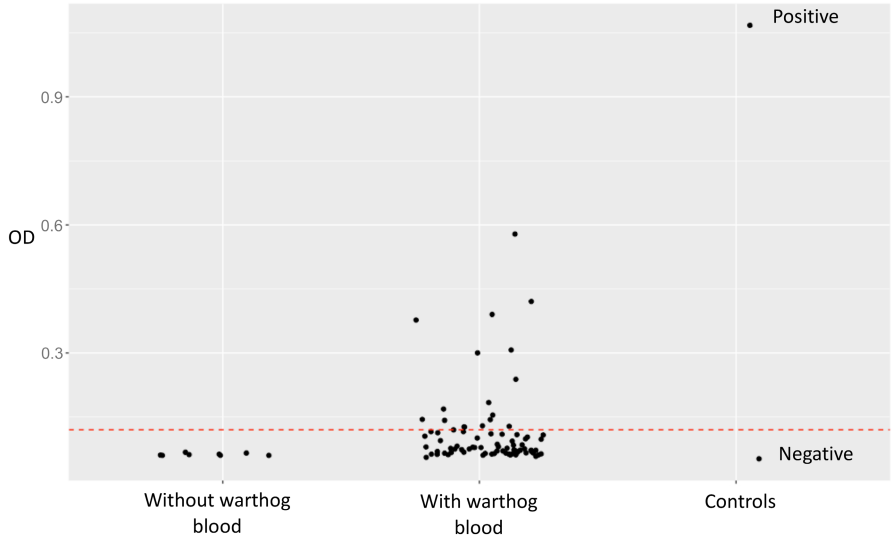

FIGURE 5 ELISA results for the detection of antibodies against ASFV. OD, optical density. The cutoff value of 0.13 is represented with the red dashed line

4.1 | An efficient tool to screen pathogens in a large range of mammal species

Tsetse flies (and more generally haematophagous flies) represent an interesting target for xenosurveillance of wild animals in areas where they are abundant for several reasons. First and foremost, they are easy to trap at low cost with little or no specific expertise. For example, in the present study, around 600 flies were captured every day using only 15 pyramidal traps deployed in areas of known abundance. Interestingly, we greatly increased the success of capture by placing a trap at the back of the car while driving into the areas targeted for tsetse fly capture, which were mostly Miombo forest areas in the west of the park. This is not surprising as tsetse are known to be attracted by moving objects and it is known that passengers of vehicles are particularly at risk of being bitten (Rayaisse et al., 2015).

Trapping can easily be done by anyone after a little basic training and the tsetse do not need any taxonomical identification beyond simple differentiation from flies from other taxa unless their role as a vector of transmission per se is of interest. Second, the proportion of flies collected with traces of blood was high even though the pyramidal traps used specifically target host-seeking insects. In a previous study in a Gabonese forest ecosystem, about 30% of the captured flies were blood engorged (both males and females) (Bitome-Essono et al., 2017). In the present study, the presence of fresh red blood was observed in 167 of the 355 pools of flies which corresponds, if we consider that only one fly was blood-engorged per pool, to approximately 10% of flies with fresh blood. However, it should be noted that this is probably an underestimation because many more flies had traces of brown or black blood (as observed during the crushing of the abdomens) from older blood meals that had been already partly digested. Also, the range of vertebrate host species identified across all blood meal pools was encouragingly high. Only six days of sampling and 1728 flies analysed yielded blood samples from 17 mammal species, including a large number of samples from host species of particular epidemiological interest, such as warthog (133 pools or 73% of those tested), African buffalo (46 pools or 25% of those tested) or greater kudu (90 pools or 49% of those tested). These species are particularly important because they are considered to be reservoirs of several important infectious diseases of domestic animals such as African swine fever, Bovine tuberculosis or Foot-and-mouth disease (Pastoret et al., 1988). Obtaining such a quantity and diversity of blood samples through more traditional collection techniques, such as live capture and anaesthesia or bushmeat and carcass analyses, would be essentially impossible and any attempt to do so would require far more time and sampling effort at far greater cost. The number of animal species collected in one short session of sampling represents approximately 25% of the mammal species of the park excluding small mammals (e.g., bats, rodents, shrews), and we expect even more biologically diverse blood samples could be obtained with a modestly extended and expanded sampling effort. Indeed, tsetse flies have been observed to feed on a broader range of mammals (including small mammals, Gaithuma et al., 2020) but also on birds and reptiles (e.g., Bitome-Essono et al., 2017; Sawabe et al., 2006). It is notable that one bird species was detected in the present study even though our primers are designed to be mammal specific. Using other primers (less specific to mammals) (see e.g., Riaz et al., 2011) could allow to detect other groups of vertebrates, including the >400 species of birds that may be found in Ruaha.

One limitation of our approach was the detection of contaminants in two out of six of the negative controls (*Homo sapiens* and *Phacochoerus africanus* contaminant reads). We tried to reduce the impact of contamination on animal detection rates as much as possible by discarding the species that represented <1% of the reads obtained per pool, but it is still possible that some species were wrongly detected in some pools because of it. The estimates of the frequency of each mammal species detected in blood meals must therefore be considered with caution.

4.2 | Pathogen detection and host association

In the present study, we were able to detect a wide array of pathogens in the blood meals of tsetse flies, ranging from protozoa to bacteria, thus demonstrating potential for concomitant surveillance

of a diversity of pathogens. Although viruses were not detected in our samples, a complementary recent study (Franck Prugnolle/Christophe Paupy personal communication) demonstrated experimentally that blood meals containing different viruses of medical or veterinary importance (i.e., chikungunya, zika, dengue viruses, ASFV, BTV and PPRV), at viral titers similar to those observed in nature, could be detected with high probabilities. Although the detection probability decreased with time, high detection rates were observed even 6 days after feeding when bloodmeals are almost entirely digested (F.P./C.P. personal communication). The absence of viruses in our study is therefore probably the consequence of the dynamic of viral infections in reservoir hosts that is often characterized by important variation of the virus prevalence over space and time due to the structure, connectivity and dynamic of the host populations as well as external factors (such as climate) (Becker et al., 2019). Spatiotemporal sampling designs could help to capture these spatial and temporal variation in virus shedding (Becker et al., 2019) and thus ensure their surveillance in the wild reservoirs.

In this study, we searched for certain pathogens of veterinary/medical interest with molecular methods adapted to each pathogen. Such an approach has the disadvantage to require prior knowledge about which pathogens may be circulating in the area. In addition, blood meals retrieved from the midgut of flies are not always very fresh, so nucleic acids may suffer degradation thereby increasing the rate of false negatives obtained with PCR-based detection methods. This is in line with the fact that the rate of detection of pathogens was significantly higher in pools containing fresh blood than in others. One possible alternative is to use more generalist approaches such as metagenomic and metatranscriptomic approaches using high throughput sequencing technologies as applied to leeches' (Alfano et al., 2020) and mosquitoes' blood meals (Grubaugh et al., 2015). In Alfano et al. (2020), for instance, the investigators used some oligonucleotide capture methods to enrich their sample in viral RNA content and then deep-sequenced the samples, allowing them to identify several viruses, including new viruses. With the ongoing reductions in the cost of deep sequencing and the concomitant increase in the number of sequences obtained per run, this approach could enable unprecedented and profound insights into the natural dynamics of pathogen populations in wild animal reservoirs. Limitations may exist, however, in the number of samples that can be pooled together before proceeding to the sequencing stage because viral sequences from such samples are often in low abundance compared to other kind of RNA/DNA sequences. Also, the processing and analysis of the resulting data requires advanced skills in molecular genetics and the application of bioinformatic tools that remain underdeveloped in many settings, notably in the low-income tropical countries where such xenosurveys are of most direct national relevance.

Another limitation of our study is that samples were pooled before nucleic acid extraction, so it was often difficult and usually impossible to associate a particular pathogen with a specific host. This is because some species (such as warthog and giraffe) are very prevalent in the tsetse blood meals and are thus present in most pools. In future studies where circumstances and resources allow, pooling should ideally be performed after nucleic acid extractions rather than during sampling. However, this implies far greater sample processing effort in the field and at the laboratory, which is time consuming and costly. Alternatively, statistical approaches could allow to quantify host-pathogen associations provided sufficient sample size.

4.3 | Serology: A new perspective for xenosurveillance

This study also validates the potential of tsetse blood meal analyses (or other blood fed invertebrates) to detect the circulation of antibodies against specific pathogens in wild animals, as already suggested by previous studies (Barbazan et al., 2009; Cunningham et al., 1962; Stefanic et al., 2022). As a proof of concept, we tested for antibodies against African swine fever virus (ASFV) in a subset of sample pools known to contain warthog blood. Warthogs are known to be reservoirs of ASFV, which is a tick-borne disease in East Africa for which seroprevalence can be very high (Hakizimana et al., 2021).

This methodological validation opens up new opportunities for xenosurveillance of pathogens with volatile population dynamics, because serological signals often persist far longer than the brief outbreaks that cause them. Further studies are nevertheless required to validate and develop this xeno-serosurveillance approach using tsetse, including experimental assessments to determine the probability of detection of the antibodies with respect to the age, digestion level of blood meals and host species. A recent study (Stefanic et al., 2022) indeed demonstrated differences in sensitivity in the detection of antibodies between different host species using experimentally blood fed mosquitoes and a limit of detection of 72 h post-feeding. The detection of different categories of antibodies (IgG or IgM for instance) could allow to distinguish between recent versus more ancient infections (e.g., Hsueh et al., 2004). Another aspect that needs to be considered is the problem of cross-reaction with ELISA tests (Tighe et al., 2015), that is the fact that the antigens used can sometimes be recognized by antibodies that were produced for another pathogen (and so another antigen) leading to false positives. This problem needs to be considered for each pathogen species of interest. Also, alternative sample preservation media will need to be identified and optimized because RNALater (the medium used in our study to conserve our pools of flies) tends to precipitate proteins and can interfere with ELISA analyses (Keele et al., 2006). Methods allowing the detection of antibodies against a large set of pathogens such as VirScan chip (Xu et al., 2015) could also be used to increase the range of pathogens surveyed and sample throughput rates.

5 | CONCLUSION

This study demonstrates how samples of field-caught haematophagous flies, such as tsetse can be effectively used to collect blood

specimens from wild animals and detect a large array of blood-borne pathogens of medical and/or veterinary interest directly, or to detect circulating antibodies against specific pathogens as an indicator of prior exposure. In a context where interfaces between wild animals, humans and domestic animals are rapidly increasing, this method could be used to enhance our understanding of the population dynamics of pathogens in wild vertebrate communities and therefore allow their surveillance in wild reservoirs. More generally, this tool could be used to better understand the ecology (population dynamic, community composition…) and evolution of pathogens (genomic of the pathogens from these samples could be envisaged) in wild animals, a compartment that is often neglected because of the inherent complexity to obtain samples. In a similar way, blood samples obtained from the flies could be used to analyse not only the diversity of vertebrate species present in an area (as was previously done using other haematophagous invertebrates) but also to perform genomic or genetic studies on some of the host species for which tissue samples can be otherwise difficult to obtain from wild populations.

AUTHOR CONTRIBUTIONS

S.M., V.R., C.P., R.S. and F.P. designed the study. S.M. and F.P. collected the samples. S.M., V.R., C.A., L.B., E.M., P.D., D.J., A.M., S.M., A.O., Y.S., B.R., G.K., F.C., P.Y.B.-E., F.B., H.M., C.P., R.S., F.P. performed research. S.M., V.R., C.P. and F.P. wrote the original manuscript, with input and revisions from all the coauthors. All authors contributed to and approved the final manuscript.

ACKNOWLEDGEMENTS

The authors thank the three reviewers as well as the editor for their very pertinent comments on a previous version of the manuscript. Data used in this study were (partly) produced through the GenSeq technical facilities of "the Institut des Sciences de l'Evolution de Montpellier" with the support of LabEx CeMEB, ANR "Investissements d'avenir" programme (ANR-10-LABX-04-01), Horizon 2020 Framework Progamme "ZIKAlliance (no. 734548) and the French LabEx ParaFrap (ANR-11-LABX-0024) and the National Reference Centre for Leishmaniasis. Authors also thank the CNRS, IRD, UM, CIRMF, IHI and Pandora Id Net consortium for their help and support. A special thanks to Christoline Motta, our park ranger, without which the tsetse collection could not have been done. We also thank Pascal Grébault for providing the traps.

CONFLICT OF INTEREST

Authors declare there are no conflict of interests.

DATA AVAILABILITY STATEMENT

Raw sequence reads from 16S mtDNA amplification of blood meals have been deposited in the SRA (BioProject PRJNA830652). Sequences obtained from pathogens (*Plasmodium* sp. and *Theileria* sp.) have been deposited in Genbank (accession nos. ON652617-ON652635). Metadata are also stored in the SRA (BioProject PRJNA830652).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Mwakasungula, S., Rougeron, V., Arnathau, C., Boundenga, L., Miguel, E., Boissière, A., Jiolle, D., Durand, P., Msigwa, A., Mswata, S., Olotu, A., Sterkers, Y., Roche, B., Killeen, G., Cerqueira, F., Bitome-Essono, P. Y., Bretagnolle, F., Masanja, H., Paupy, C. … Prugnolle, F. (2022). Using haematophagous fly blood meals to study the diversity of blood-borne pathogens infecting wild mammals. *Molecular Ecology Resources*, *00*, 1–13. https://doi.org/10.1111/1755-0998.13670