Distribution and phenotypic population structure of the tsetse flies *Glossina morsitans morsitans* Westwood and *Glossina morsitans centralis* Machado in Zambia.

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Declaration

I, Jackson Muyobela, declare that the thesis which I hereby submit for the degree of Doctor of Philosophy in Entomology at the University of Pretoria, is my own work and has not been previously submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: ……

DATE: …….

Dedication

I dedicate this to my parents, the late Benny Mwamba Muyobela and Margret Nyasenda Lungu.

To my dear wife, Suzyo Bwalya Mbambara; Children, Jackson Mwamba Muyobela, James Kumbukani Muyobela, Patrick Bwalya Muyobela and Christian Lutanda Muyobela for your moral and spiritual support.

Above all I thank God for divine favour.

Publications and thesis organisation

Chapters in this thesis are presented in the form of publications. Hence, there is overlap of information and differences in the format of presentation.

Chapter 2. Muyobela J, Pirk CWW, Yusuf AA, Mbewe NJ, Sole CL (2021) A novel vehicle-mounted sticky trap; an effective sampling tool for savannah tsetse flies *Glossina morsitans morsitans* Westwood and *Glossina morsitans centralis* Machado. PLoS Negl Trop Dis 15(7): e0009620. https://doi.org/10.1371/journal.pntd.0009620

Chapter 3. Muyobela J, Pirk CWW, Yusuf AA, Sole CL (2023) Spatial distribution of *Glossina morsitans* (Diptera: Glossinidae) in Zambia: A vehicle-mounted sticky trap survey and Maxent species distribution model. PLoS Negl Trop Dis 17(7): e0011512. https://doi.org/10.1371/journal.pntd.0011512

Chapter 4. Muyobela J, Pirk CWW, Yusuf AA, Sole CL (2024) Phenotypic divergence of *Glossina morsitans* (Diptera: Glossinidae) populations in Zambia: Application of landmark-based wing geometric morphometrics to discriminate population-level variation. Ecology and Evolution. 2024;14: e70348. https://onlinelibrary.wiley.com/doi/full/10.1002/ece3.70348

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Abstract

Glossina morsitans s.l. is an efficient vector of African trypanosomiasis, a debilitating and fatal disease of humans and livestock. This study aimed at investigating the current distribution and phenotypic population structure of *G. m. centralis* Machado and *G. m. morsitans* Westwood to inform effective vector management strategies in Zambia. To achieve these objectives, the study also evaluating a vehicle-mounted sticky trap (VST) for effective and rapid sampling of *G. morsitans* over large geographic areas. Randomised block design experiments were used to establish the optimal design of VST. An extensive VST based tsetse survey was then conducted in all tsetse belts in Zambia. The occurrence records obtained from this survey were used to model the distribution of *G. m. centralis* and *G. m. morsitans* using a Maxent species distribution model. Landmark-based wing geometric morphometrics was undertaken to investigate the population-level phenotypic variation of the two subspecies. There were no significant differences in catch indices of VST constructed using an all-blue, all-black and 1:1 blue-black panel. Overall, the VST oriented in-line and baited with butanone and 1-octen-3-ol, caught 2.42 *and* 2.60 times more *G. m. centralis* and *G. m. morsitans* respectively, than the standard mobile trapping device, the black-screen fly round. The VST survey captured a total of 15,602 flies with *G. m. morsitans* (58%) and *G. m. centralis* (39%) being the most abundant. The predicted potential distribution for *G. m. centralis* was 80,863 km^2 while that of *G. m. morsitans* was 70,490 km^2 representing a 47 and 29% reduction compared to their historical distributions, respectively. Significant differences in wing centroid size and shape were observed between *G. morsitans* sexes, subspecies and sample locations within each subspecies range. The populations of *G. morsitans* were found to exhibit significant population-level variation in fly size and wing shape which suggests high levels of population structuring. The main drivers of this structuring could be random genetic drift in *G. m. centralis* demes and local adaptation to environmental conditions in *G. m. morsitans* populations.

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Chapter 1: General Introduction

1.1 Background

Tsetse flies (Diptera: Glossinidae) are obligate blood-feeders that transmit pathogenic trypanosomes which cause the disease complex African trypanosomiasis that affects both humans and livestock (Jordan, 1993). The human disease is called human African trypanosomiasis (HAT) or sleeping sickness and occurs either as a chronic or acute disease (Gibson, 2005). The chronic form of HAT is caused by *Trypanosoma brucei gambiense* and occurs in West and Central Africa with humans being the principal reservoirs (Aksoy et al., 2017). *Trypanosoma b. gambiense* has however been isolated from pigs, dogs, and sheep but the epidemiological significance of these domestic hosts remains unknown (Welburn et al., 2001). The acute zoonotic form of HAT is caused by *T. b. rhodesiense* and occurs east of the Rift valley, in Eastern and Southern Africa (Franco et al., 2017). An estimated 70 million people occupying an area of 1.55 million km^2 are at risk of contracting HAT in sub-Saharan Africa (Simarro et al., 2012).

African animal trypanosomiasis (AAT) or *nagana* is a chronic wasting disease that precludes most animal agriculture in much of sub-Saharan Africa (FAO, 2014). The tsetse-transmitted agents of AAT include *T. brucei* (including its 'subspecies'), *T. congolense*, *T. vivax* and *T. simiae* (Krafsur and Maudlin, 2018). *Nagana* is of much economic importance causing three million cattle deaths annually with 35 million doses of trypanocidal drugs being used yearly to maintain susceptible livestock in tsetse infested areas (FAO, 2014). Wherever AAT is prevalent, meat, milk, dung, and draft power production are greatly reduced. Therefore, the presence of tsetse and trypanosomiasis constitutes a major constraint to improved livestock production and productivity in sub-Saharan Africa (Bourn et al., 2005).

In Zambia, an estimated 277,000 km^2 (or 38%) of the landmass is infested with tsetse flies (Evison and Kathuria, 1982). Four species occur namely *Glossina fuscipes martini* Zumpt, *G. brevipalpis*

Newstead, *G. pallidipes* Austin and two subspecies of *G. morsitans*: *G. m. centralis* Machado and *G. m. morsitans* Westwood (Ford and Katondo, 1977). *Glossina morsitans* is the most widely distributed covering the entire tsetse infested range of the country (Evison and Kathuria, 1982). The other three species exist within *G. morsitans* range but to a much lesser extent. Being an efficient trypanosome vector, *G. morsitans* is implicated as the major vector for both the rhodesiense form of HAT and AAT in Zambia. Franco et al. (2022) indicated that the incidence of HAT is relatively low in the country with one (1) to 15 cases reported annually. These cases are mainly associated to the wildlife reservoir and generally occur in communities living near and/or working in wildlife protected areas (Franco et al., 2020). Over 60% of Zambia's cattle population is at risk of trypanosomiasis (Mbewe et al., 2015) with AAT prevalence estimates ranging from one (1) to as high as 90% (Mulenga et al., 2021). As such, AAT has been ranked the second most important livestock zoonotic disease in Zambia second only to anthrax (Wezi et al., 2023).

Although several methods to control African trypanosomiasis are available – screening and curative treatments for HAT, prophylactic and curative treatment of animals, and promotion of trypanotolerant cattle – vector control remains the most sustainable approach to disease management, especially for the zoonotic form of the disease (Bouyer et al., 2010; Vreysen et al., 2013). Since the spatial distribution pattern of tsetse is a key determinant of trypanosomiasis epidemiology and transmission dynamics (Dicko et al., 2015), accurate knowledge of tsetse distribution is a critical prerequisite to the implementation of effective evidence-based vector management strategies (Diall et al., 2017). Despite this critical need, existing distribution maps for *G. m. centralis* and *G. m. morsitans* in Zambia are more than 40 years old (Ford and Katondo, 1977) and were based on coarse spatial resolution data obtained from surveys done in the colonial era (Evison and Kathuria, 1982). Given that the distribution of tsetse is influenced by climate (Hargrove, 2001), vegetation (Van den Bossche et al., 2010) and host availability (Vreysen et al., 2013), factors which have all reportedly undergone

significant changes in the last 40 years (Ducheyne et al., 2009; NCEI, 2022), the existing distribution maps of *G. m. centralis* and *G. m. morsitans* cannot reliably inform their control in Zambia.

Despite over a 100 years of tsetse control on the African continent, local vector eradication success has been limited to less than 2% of the total infested area (Bouyer et al., 2015). This has been attributed to either resurgence of residual populations omitted from the eradication campaign or reinvasion from neighbouring infested areas (Hargrove, 2003). Two recent vector management campaigns against *G. austeni* Newstead (Vreysen et al., 2000) and *G. m. centralis* (Kgori et al., 2006) achieved sustained elimination by targeting isolated tsetse populations as a whole (Bouyer et al., 2015). The success of these two campaigns highlighted that the identification of biogeographic (Getahun et al., 2014) or ecological (Solano et al., 2010) island populations that are under restricted gene flow (Ostwald et al., 2023) is essential for sustainable tsetse control (Bouyer et al., 2010). Therefore, the implementation of tsetse control using an area-wide integrated vector management (AW-IVM) approach that targets an entire discrete tsetse population is technically and economically desirable (PATTEC, 2001). Information on the population structure and degree of isolation of *G. m. centralis* and *G. m. morsitans* in Zambia is however currently unavailable.

Accurate designation of tsetse populations as isolated requires the estimation of the number of migrants per generation or the levels of gene flow between them (Bouyer et al., 2007). However, these studies require high technical expertise and are both lengthy and costly (Krafsur, 2009). The analysis of population-level phenotypic variation using geometric morphometric methods provides a cheaper, faster and effective method to investigate tsetse population structure (Dujardin, 2008). Furthermore, morphometric studies have reportedly produced results comparable to approaches based on molecular markers (Kaba et al., 2012) and have been used to investigate the natural population variation in several species of *Glossina* (De Beer et al., 2019; Mbewe et al., 2018; Solano

et al., 1999). Phenotypic variation in natural populations of *G. m. centralis* and *G. m. morsitans* is yet to be investigated.

A prerequisite to determining the extent of tsetse infested areas, updating of outdated distribution maps and the identification of isolated tsetse populations, is extensive systematic sampling (Leak et al., 2008). Currently, extensive geographical sampling of natural tsetse populations is limited due to high survey costs and poor accessibility to certain areas (Krafsur, 2009). Consequently, there is need for further exploitation of tsetse host-orientation behaviour to facilitate the development of an efficient, cost-effective trapping device that may aid rapid systematic sampling of tsetse infested areas (Torr et al., 2011). To mitigate the challenge of accessibility for developed sampling devices, species distribution models (SDMs), defined as numerical tools that combine observations of species occurrence or abundance with environmental estimates (Elith and Leathwick, 2009), may be used to predict tsetse distributions across the landscape (Dicko et al., 2014).

Taxonomy and distribution

The taxonomy of tsetse flies as outlined by McAlpine (1989) and Grimaldi and Engel (2005), is shown in Fig 1.1. *Glossina* Weidemann 1830, is the only genus that is assigned to the Glossinidae. Characteristic of all Pupiparia is adenotrophic viviparity and exclusive blood feeding adults (Krafsur, 2009).

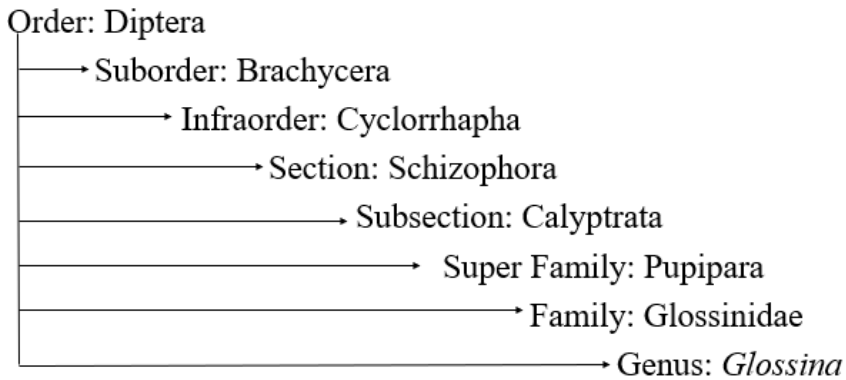

Fig 1.1. Taxonomy of tsetse flies

The distribution of tsetse is restricted to the African continent with the northern limit of their range corresponding to the southern edges of the Sahara and Somali Deserts (Jordan, 1993). In the South, no tsetse flies have been recorded south of the Kalahari and Namibian deserts while in the East no records exist below latitude 29°S Fig 1.2. The genus *Glossina* has been divided into three extant subgenera, *Austenina* (*Fusca* or forest group) Townsend, *Nemorhina* (*Palpalis* or riverine group) Robineau-Desvoidy, and *Glossina* (*Morsitans* or Savannah group) Wiedeman, based on the structure of male and female genitalia, their habitat requirements and host preferences (Gooding and Krafsur, 2005). The continental distribution of each subgenus is shown in Fig 1.2.

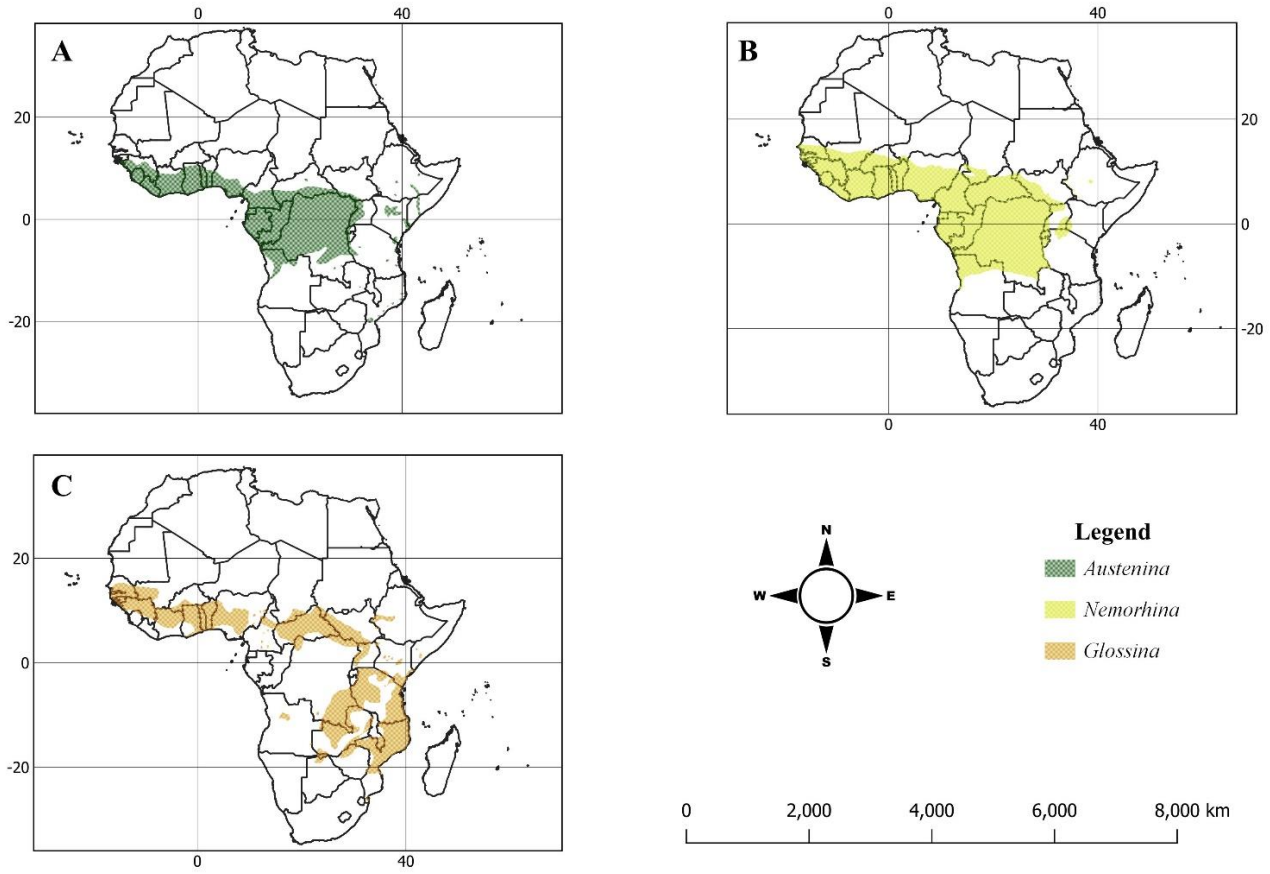

Fig 1.2. Distribution of the three subgenera of the genus *Glossina*. (A) Distribution of the subgenus *Austenina* – forest group tsetse. (B) Distribution of the subgenus *Nemorhina* – riverine tsetse. (C) Distribution of the subgenus *Glossina* – Savannah tsetse. Data on each subgenus in Jordan (1993). The base map layer was obtained from the Database of Global Administrative Area GADM (https://geodata.ucdavis.edu/gadm/gadm4.1) and under the license https://gadm.org/license.html. The figure was created using QGISv3.0 (http://qgis.org/en/site/)

Glossina morsitans s.l. (*G. m. submorsitans* Newstead, *G. m. centralis* and *G. m. morsitans*) and *G. pallidipes* are the most important Savannah group species being the major vectors of AAT and HAT in Eastern and Southern Africa (Krafsur and Maudlin, 2018). Their distribution is restricted to savannah woodlands (Leak et al., 2008) and they exhibit high sensitivity to habitat fragmentation caused by human encroachment – their abundance decreases when the human population exceeds 5 people/km^2 (Van den Bossche et al., 2010). Given that *G. morsitans* is known to be the most economically important tsetse species in Zambia (Evison and Kathuria, 1982) updating its current distribution is crucial for the implementation of evidence-based vector control.

Tsetse biology

All tsetse flies reproduce by a K-selected reproductive strategy known as adenotrophic viviparity (Vreysen et al., 2013). Egg and larval development occur within the uterus of an inseminated female and requires 7-12 days depending on temperature. Upon parturition, mature (3rd instar) larvae do not feed but burrow into the ground and pupate. Adults emerge approximately 30 days later depending on temperature (pupal development fails below 17°C and above 32°C). Females become inseminated (females' mate only once) by the end of their first week of adulthood and will produce their first mature offspring 16 to 17 days after emergence. As such, each female produces only one offspring at a time and can produce up to 12 offspring during her 2-3 months adult lifespan, at 9–10-day intervals. Consequently, tsetse flies have a low intrinsic population growth rate, with the maximum rate of population increase estimated to be no more than 10 to 15 times per year (Hargrove, 1988).

The entire nutritional intake of tsetse flies is limited to vertebrate blood which supplies the amino acid proline that is partially metabolised to facilitate flight in adult insects. When exhausted, tsetse must rest to reconstitute the limited proline reserves. Consequently, tsetse are unable to fly for long periods but instead, fly in short bursts, resulting in a relatively low capacity for active dispersal. Flights speeds of up to 4 m/s (14.4 km/hr) on laboratory mills have been observed for *G. m. morsitans* (Hargrove, 1975), although mean speeds of 6 m/s (22 km/hr) and maximum speeds of 10 m/s (36

km/hr) have been recorded in the field (Gibson and Brady, 1988). Therefore, any mobile device developed to sample tsetse populations should not exceed 20 km/hr.

Flight time of tsetse is estimated to be about 15-30 min per day, with the duration of each flight being 30-60 seconds (Bursell and Taylor, 1980). The recorded duration of flight in the field was between 30-60 seconds with an average flight speed of 5 m/s, and each flight covering between 150 and 300 m. Thus, a tsetse fly can fly between 4.5 and 9 km in a day. A random walk model with a step length of 50 m and a total flight distance of 4.5 km gives a root-mean-square displacement in one day of 167 m, while a step length of 200 m and a total flight distance of 9 km give a root-mean-square displacement in one day of no more than 1.3 km (Williams et al., 1992). The implication of the foregoing discussion is that dispersal rates of tsetse are low compared to other insect species but are sufficiently high for significant gene flow between population demes (Krafsur, 2009).

Host orientation and trap development.

Tsetse survival depends on regular detection of a suitable host on which to take a blood meal; a task accomplished by its host-seeking behaviour. Host-seeking behaviour in tsetse is influenced by both endogenous (a circadian rhythm of activity, level of starvation, age, sex, and pregnancy status) and exogenous (temperature, vapour pressure deficit, visual, mechanical and olfactory stimuli) factors (Colvin and Gibson, 1992). Two sensory cues of host-seeking behaviour, olfaction and vision have been exploited and combined together for efficient trap design (Colvin and Gibson, 1992). Olfaction is used to attract as many tsetse flies as possible while vision aids the efficient capture of a significant proportion of the tsetse flies attracted (Vale, 1982). Elucidation of host-orienting stimuli that influences activation, short- and long-range responses to a host, is therefore essential for rational trap design.

Both endogenous and exogenous factors influence the activation of resting tsetse. A review of the daily activity pattern of 18 species of tsetse revealed that the insects were mostly active in the morning

and/or evening, relatively inactive through mid-day, and almost totally inactive at night (Brady and Crump, 1978). Brady (1988), indicated that *G. morsitans* exhibits a V-shaped diurnal activity pattern and is mostly active in the morning and evening. Since the probability of capture of any tsetse trap depends on the daily activity pattern, for *G. morsitans*, it varies throughout the day and is highest in the morning and evening. Diurnal patterns of activity are influenced by environmental temperature with activity levels positively correlated to temperature over the lower half of habitat range (18°C and above) and negatively correlated at temperatures above 28°C (Brady, 1988). However, Brady and Crump (1978) showed that 80% of the daily pattern of activity in nature is due to an endogenous rhythm rather than environmental control, despite this correlation. Starvation levels were associated with the most active and persistent host-seeking behaviour, and visual responsiveness from rest is correlated with the nutritional condition (Brady, 1975). Torr and Solano (2010), highlighted that olfaction has a less of an effect on activation.

The importance of long-distance olfactory cues for locating stationary hosts has been demonstrated in *morsitans* group tsetse (Torr and Solano, 2010) where they mediate long-range (~90 m) anemotaxis responses (Griffiths et al., 1995). The components in host odour found to be attractive are carbon dioxide, acetone, butanone, 1-octen-3-ol, and other phenolic derivatives, such as 4-methlyphenol and 3-n-propylphenol (Colvin and Gibson, 1992). These compounds were identified as components of host breath, urine and skin secretions and are now available for increasing trap efficiency for some tsetse species (Leak et al., 2008). Different components of host odour are known to elicit different behaviours. For example, acetone and octenol increase visual responsiveness of tsetse to the host (Torr, 1990) while carbon dioxide elicits a landing response (Torr, 1988). Butanone and 1-octen-3-ol are routinely used to improve the efficiency of sampling devices used to trap *G. morsitans* due to their relatively low cost (Willemse, 1991). It has been demonstrated that tsetse tend to avoid taking blood meals from certain vertebrates such as waterbuck, zebra, wildebeest and impala despite their

high abundance in tsetse habitat (Auty et al., 2016; Clausen et al., 1998; Muturi et al., 2011). This observation led to the discovery of allomones emanating from the skin of non-preferred vertebrate hosts (Gikonyo et al., 2000) and forms the basis of tsetse repellent technology. For example, a potent tsetse waterbuck repellent (WRC) consisting of geranylacetone, guaiacol, pentanoic acid and δ-octalactone (Bett et al., 2015; Gikonyo et al., 2000) has been developed and is now in use as a tsetse repellent collar to protect livestock (Saini et al., 2017). Furthermore, Olaide et al. (2019) showed that a blend of seven compounds identified from zebra odour. 6-methyl-5-hepten-2-one, acetophenone, geranylactone, heptanal, octanal, nonanal and decanal had significant tsetse repellent effects against *G. pallidipes.*

Tsetse short-range (~15 m) responses to a host are governed by visual cues such as colour, shape, orientation, brightness, contrast and movement (Colvin and Gibson, 1992). Colour investigations have shown that phthalogen blue (with a reflectance wavelength of 450 nm) is the most attractive (Green and Flint, 1986), followed by black (Vale, 1982) and then white (Dransfield et al., 1982). Colour was also demonstrated to be important in eliciting landing behaviour. In *G. pallidipes* and *G. morsitans s.l.*, landing tendency was strongest with black, intermediate with blue, and lowest with white (Green and Flint, 1986). The attractiveness of shape to host-seeking tsetse in order of decreasing importance was observed to be circle, square and rectangle (Torr, 1989). The orientation of shapes and their sizes is also important with horizontal placed rectangles appearing to be more attractive to tsetse than those placed vertically (Byamungu et al., 2018) and large objects are more attractive than smaller ones. However, sticky small targets (0.25 × 0.25 m) were shown to be highly attractive to *G. f. fuscipes* (Mbewe et al., 2018). Movement has been observed to significantly increase the attractiveness of an object. Vale (1974), showed that mobile baits caught up to 16 times as many male *G. m. morsitans* as stationary baits.

The exploitation of the above host-seeking behaviour has led to the development of several stationary blue and black coloured traps that may be baited with host attractants. For Savannah tsetse flies, the F3 trap, NGU, Epsilon and NZI traps (Fig 1.3) are recommended (Leak et al., 2008). Although stationary traps provide a standardised system of sampling *Glossina*, they are not sensitive enough to readily detect low populations of *G. morsitans* and alternative mobile trapping systems such as the Black-Screen Fly Round (BFR) (Fig 1.3) are regularly used to trap this tsetse (Leak et al., 2008). However, BFR are highly dependent on the ability of operators to capture tsetse with hand nets. The vehicle-mounted electric target (Fig 1.3) is currently the only alternative mobile tsetse sampling tool but its use has mainly been limited to research as it is expensive and requires high levels of maintenance (Leak et al., 2008). Consequently, the development of a cost-effective, mobile sampling device, which is efficient throughout the daily range of probabilities of capture of *morsitans* group tsetse, and independent of operator efficiency would allow a cheaper means of determining their area-wide distribution of *G. morsitans* and facilitate efficient mapping of isolated populations.

Geometric morphometric analysis

The quantification and analysis of phenotypic variation of organisms using morphometric methods has been part of biological research for over a century. Since phenotypic variation is environmentally and/or genetically determined, morphometric analysis provides a tool for detecting local adaptations and genetic divergence among populations (Dujardin and Slice, 2007). Traditionally, morphometrics involved the estimation of distances between anatomical points called landmarks. Modern or geometric morphometrics utilises the coordinates of these landmarks in a given system of orthogonal axes to provide not only size information, such as distance between landmarks but also shape data, such as their relative position (Adams et al., 2004; Dujardin et al., 2010). The visualisation of shape variation among individuals or groups employing informatics is probably the most spectacular application of modern morphometric analysis (Dujardin, 2008).

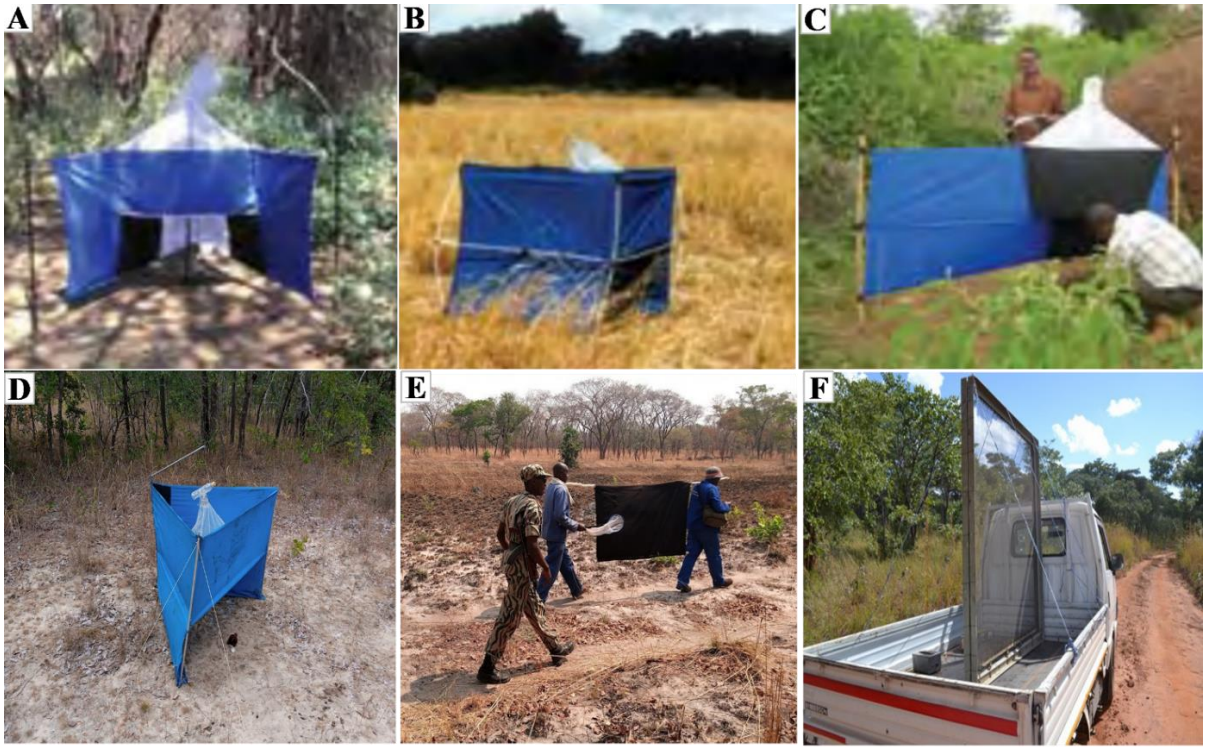

Fig 1.3. Sampling tools used to sample Savannah tsetse flies. (A) Nzi trap. (B) F3 trap. (C) Ngu trap. (D) Epsilon trap. (E) Black screen fly round. (F) Vehicle-mounted electric target.

In practice, morphometric techniques measure size and shape in reference to a measurable part of the organism under study. This is acceptable because the objective of morphometrics is not to provide a complete description of an organism's dimensions, but rather to allow valid comparisons of a given phenotype among individuals, local populations and/or species (Klingenberg et al., 2002).

As it can detect minimal morphological variation, modern morphometric analysis has been extensively used in insect population studies. As a taxonomic tool, it has contributed to the detection of new and cryptic species (Dujardin et al., 2009), assessment of natural population structure (Kaba et al., 2012) and identification of sources of re-infestation after control (Mbewe et al., 2018). As a population marker, wing geometry has produced results comparable to molecular markers in *G. p. gambiensis* (Bouyer et al., 2007 and Solano et al., 1999) and *G. p. palpalis* (Kaba et al., 2012). Studies characterising natural morphometric variation in *morsitans* group tsetse are currently limited (Mustapha et al., 2018).

1.2 Problem Statement

African trypanosomiasis continues to negatively affect the human and livestock health of rural communities in tsetse infested areas across Zambia. This has been primarily attributed to inadequate vector control strategies as previously cleared areas are rapidly reinvaded once control measures cease. It is for this reason that the Tsetse and Trypanosomiasis Control Unit Zambia has adopted an area-wide integrated vector management (AW-IVM) approach, but its implementation is currently being hampered by the lack of current data on the spatial distribution and population structure of the major trypanosomiasis vectors *G. m. centralis* and *G. m. morsitans*. Updating the distribution and generating data on population structure will facilitate AW-IVM of *G. morsitans* in Zambia by ensuring that entire population are treated thus preventing the reinvasion of controlled area.

1.3 Rationale of the Study

The core motivation of this study is the lack of information on their current spatial distribution and the degree of population isolation of *G. m. centralis* and *G. m. morsitans* populations in Zambia. Without this information, the effective design and implementation of an AW-IVM approach for the suppression and/or eradication is not feasible and gains made from vector control will continue to be eroded due to reinvasion.

To facilitate effective and efficient data collection over the large geographic area required for this study, there is need to develop a vehicle-mounted sticky trap. While development of this trap is not the central focus of the study, it is required to overcome previous challenges to tsetse sampling such as the high cost of surveying large areas.

1.4 Justification of the study

Updating the current distribution limits and identifying any significant population structuring of *G. m. centralis* and *G. m. morsitans* populations will facilitate improved understanding of the risk and impact of HAT and AAT in Zambia. This information will enable improved planning of future control

programmes by aiding the selection of priority intervention areas with low risk of reinvasion and guide the decision whether to undertake suppression or elimination. Furthermore, the novel sampling method developed by this study provides a useful tool for future studies.

1.5 Aim of the Study

The study aimed at investigating the current distribution and phenotypic population structure of *G. m. centralis* and *G. m. morsitans* in Zambia to inform effective vector management strategies.

1.6 Specific Objectives

Objective 1: To update the spatial distribution maps of *G. m. centralis* and *G. m. morsitans* in Zambia using vehicle-mounted sticky traps.

Objective 2: To assess the phenotypic variation and population structure *G. m. centralis* and *G. m. morsitans* subspecies across different regions in Zambia.

Objective 3: To evaluate the effectiveness of a vehicle-mounted sticky trap as a sampling tool for collecting data on tsetse fly populations.

1.7 Research Questions

Research question 1: What are the current distribution patterns of *G. m. centralis* and *G. m. morsitans* in Zambia and how have they changed since the 1977 distribution map by Ford and Katondo?

Research question 2: Is there significant phenotypic variation among populations of *G. m. centralis* and *G. m. morsitans* in Zambia that indicates population structuring?

Research question 3: What is the most efficient colour design, orientation and combination of synthetic olfactory attractants that can be used to efficiently trap *G. m. centralis* and *G. m. morsitans* tsetse using a vehicle mounted sticky trap?

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Chapter 2: A novel vehicle-mounted sticky trap; an effective sampling tool for savannah tsetse flies *Glossina morsitans morsitans* Westwood and *Glossina morsitans centralis* Machado

2.1 Overview

In this thesis, the distribution and phenotypic population structure of *Glossina morsitans morsitans* and *Glossina morsitans centralis* in Zambia are explored to inform future vector management strategies. A key challenge in achieving these objectives is the efficient and cost-effective sampling of tsetse populations across vast and diverse geographical areas. To address this, a novel vehicle-mounted sticky trap was developed and detailed in the publication: Muyobela J, Pirk CWW, Yusuf AA, Mbewe NJ, Sole CL (2021). A novel vehicle-mounted sticky trap; an effective sampling tool for savannah tsetse flies *Glossina morsitans morsitans* Westwood and *Glossina morsitans centralis* Machado. PLoS Negl Trop Dis 15(7): e0009620. https://doi.org/10.1371/journal.pntd.0009620. The paper focused on the design, testing and validation of the trap in sampling tsetse populations and is key to gathering the data presented in subsequent chapters.

As a result of the effectiveness of this trap, robust data collection has been possible, supporting the broader objective of improving our understanding of tsetse ecology in Zambia as well as supporting the development of AW-IVM strategies.

Chapter two therefore, is the methodological foundation of the thesis, that covers how the vehicle mounted sticky trap, which is a crucial tool for achieving the study's primary goals of understanding tsetse fly distribution and phenotypic population structure, was developed.

2.2 Introduction

Tsetse flies (Diptera: Glossinidae) are the sole vectors of trypanosomes that cause human African trypanosomiasis (HAT or sleeping sickness) and animal African trypanosomiasis (AAT or nagana) (Aksoy et al., 2017). Increased treatment and vector control in the last two decades has resulted in a substantial reduction in reported HAT cases, from over 30,000 in 1999 (Büscher et al., 2017) to

below 1000 in 2018 (Franco et al., 2020). Despite this effort to eliminate HAT, the disease remains a considerable burden to rural communities (Franco et al., 2017). Animal African trypanosomiasis is a significant cause of poverty and malnutrition in sub-Sahara Africa and is estimated to cause three million cattle deaths annually (FAO, 2014). This study focuses on two subspecies of the savannah tsetse fly *Glossina morsitans*, *G. m. morsitans* Westwood and *G. m. centralis* Machado. Both subspecies are efficient vectors of sleeping sickness and nagana (Rogers, 2000), with HAT foci persisting within their geographic range (Simarro et al., 2010).

Effective tsetse sampling tools are essential in the control of African trypanosomiases (Takken and Knols, 2010), as they provide critical information relating to distribution limits of tsetse populations, risk of trypanosomiasis to livestock and humans, and the outcome of vector control interventions (Kuzoes and Schofield, 2005). Several trapping devices that exploit the host-seeking behaviour of tsetse, particularly, those related to visual and olfactory stimuli, have been developed. Trap development has been facilitated by the observation that *Glossina* can perceive shape, colour and movement (Colvin and Gibson, 1992). Experimentation has shown that *G. morsitans and G. pallidipes* discriminate colour, with phthalogen blue (reflectance peak 450 nm) being the most attractive whereas black and UV-reflecting white stimulate landing (Green, 1988; Green and Flint, 1986; Vale, 1982). The attractiveness of shapes increases in the order circle, square and rectangle (Torr, 1988), with horizontal rectangles being more attractive than vertical ones (Byamungu et al., 2018). An array of traps with varying combinations of blue and black cloths have been developed for sampling *Glossina* (Leak et al., 2008). Stationary traps developed for sampling *G. morsitans* include the NGU (Brightwell et al., 1987; Malele et al., 2016) and NZI (Mihok, 2002) traps used in East Africa, and the epsilon trap recommended for use in southern Africa (Hargrove and Langley, 1990). Pyramidal traps have recently been shown to be effective against *G. m. centralis* (Byamungu et al., 2018).

Host olfactory cues have been shown to increase trap catches of savannah tsetse species (Torr and Solano, 2010). Components of host odour found to be attractive are carbon dioxide, acetone, butanone, 1-octen-3-ol, and several phenolic derivatives, such as 4-methlyphenol and 3-n-propylphenol (Kuzoes and Schofield, 2005). Butanone and 1-octen-3-ol are generally used to bait traps during surveys and monitoring operations in the field due to their relatively low cost (Chahda et al., 2019; Willemse, 1991).

Stationary traps provide a standardised system of sampling, catch a higher proportion of females and can operate throughout the tsetse's activity period (Leak et al., 2008), but their use has limitations. Leak et al. (2008) highlights the following as the major disadvantages of stationary traps: (i) they need to be well constructed and maintained to provide a standard sample; (ii) their effectiveness is dependent on their deployment site; (iii) they give a misleading picture of daily activity rhythm (Hargrove and Brady, 1992); and (iv), they are not sensitive enough to detect readily low-density populations of certain species such as *G. morsitans*. Furthermore, their high cost of deployment over large areas tends to limit their use in large scale surveys (Bouyer et al., 2015; Krafsur, 2009; Kuzoes and Schofield, 2005; Leak et al., 2008). Consequently, there has been limited geographical sampling of natural tsetse populations (Krafsur, 2009), which hinders the understanding of several aspects of their population structure such as genetic sub-structuring (Krafsur and Maudlin, 2018).

Translocation has been demonstrated to significantly increase the attractiveness of an object to *G. morsitans* (Vale, 1974) and mobile baits are more effective in trapping *G. m. morsitans* than stationary baits (Malele et al., 2016; Mweempwa et al., 2020). Mobile baits used for sampling *G. morsitans* include the vehicle-mounted electric target (Hargrove et al., 2019) and black-screen fly round (BFR) (Chilongo et al., 2020; Robinson, 1995). The use of vehicle-mounted electric targets has mainly been limited to research studies as they are expensive and require high levels of maintenance (Leak et al., 2008). Black-screen fly rounds, consisting of a catching party of two individuals with hand nets and

a baited 1 × 1.5m black screen, produce samples that depend on the varying ability of people to catch tsetse flies with hand nets (Leak et al., 2008).

Therefore, the development of an effective, mobile sampling device that is not influenced by the operator's ability to capture *G. morsitans*, particularly at low population densities, is desirable. In this study, VST was evaluated for effective and rapid sampling of *G. morsitans*. Specifically, the aim was to identify the optimal trap colour and orientation and assess the need for olfactory attractants. Further, VST effectiveness was evaluated against the BFR, for sampling *G. m. morsitans* and *G. m. centralis*.

2.3 Materials and methods

Study Sites

The study was conducted in Zambia, between the longitudes 22 and 34°E, and latitudes 8 and 18°S. *Glossina morsitans* is the most widely distributed tsetse in Zambia covering an estimated 277,000 km^2 (or 38% of the total landmass). *Glossina m. morsitans* occupies the hotter eastern part while *G. m. centralis* occurs in the cooler western and Northern parts (Rogers and Robinson, 2004). Studies were conducted at two sites, one for each subspecies. A brief description of each site is given below.

Luano Game Management Area. Mopane woodland is the dominant vegetation with small pockets of farmland, used mainly for growing maize and groundnuts. The livestock population is low with goats and chickens being the main species. Wildlife is abundant and represents the major blood meal source for the resident *G. m. morsitans* (Munangandu et al., 2012). Experiments were conducted over 9 days in July 2020 along a 13 km motorable track north-east of Lubalashi village.

Mumbwa South Game Management Area. Miombo woodlands interspaced with large riverside dambos (grassy wetlands) is the dominant vegetation. No human settlements occur within a 50 km radius of the study site. Wildlife is diverse and abundant and represents the major blood meal source

for *G. m. centralis* (Gaithuma et al., 2020). Experiments were conducted over 9 days in September 2020 along a 16 km motorable hunting track.

Trapping devices and materials

Two fabrics were used in trap construction. These were blue polyester (PermaNet, Vestergaard Frandsen, Denmark) and 100% polyester black (225 g/m^2, Q15093 Sunflag, Nairobi). They were cut and fixed onto one side of a 5 mm × 1 m × 1.5 m plywood board to produce a 1 × 1.5 m all-blue, all-black and 1:1 blue-black panel. A 5 cm strip of black duct tape was placed around the edge of the panel to secure the cloth onto the plywood board (Fig 2.1). A 0.5 × 1.5 m of the same 100% polyester black cloth was used to make the black screen used in BFRs.

To enumerate flies landing on trap panels, one-sided adhesive film (30 cm wide rolls, Rentokil FE45, Liverpool, UK) was fastened above the cloth, using black duct tape (Fig 2.1). Spectral reflectance of the cloth is not affected by the adhesive film except in the ultra-violet (UV) spectrum (Byamungu et al., 2018). Virtually all UV wavelength below 380 nm is absorbed due to the addition of a UV absorber in the glue. Spectrophotometric measurements of light reflected from the adhesive film applied to fabrics similar to those used in this study indicated that all wavelengths in the UV range were absorbed by the fabrics (Oloo et al., 2014). New adhesive film was used at the start of each experiment.

Butanone and 1-octen-3-ol were used as attractants according to methods described by Torr *et al.* (Torr et al., 1997). A 500 ml glass bottle with a 2 mm aperture in the stopper was used to dispense butanone at a rate of 150 mg/hr, while polyethylene sachets of 4 × 5 cm 500 gauge/0.125 mm dispensed 3 g 1-octen-3-ol at 0.5 mg/hr.

Fig 2.1: Vehicle-mounted sticky panel traps. (A) All-black sticky panel trap. (B) All-blue sticky panel trap. (C) 1:1 blue-black sticky panel trap. (D) All-blue sticky panel trap mounted in-line orientation (E) All-blue sticky panel trap mounted in transverse orientation (F) All-blue sticky panel in transverse orientation covered with non-stick baking paper.

A 1 × 1.5 m rectangular steel frame with horizontal support legs (Fig 2.1) was used to mount the sticky trap panel on the back of a Nissan Hardbody, twin cab, 4×4 vehicle. To construct a two-sided sticky trap, two panels were fixed on either side of the steel frame. The same driver was used throughout the study. The rectangular trap panel was set up such that its longest side was horizontal to the ground in all experiments. Non-stick baking paper was used to cover the sticky surface of the trap when not in use (Fig 2.1).

Experimental Design

In all experiments, randomised block designs (Mbewe et al., 2018) were used to compare treatment effects. Different blocks constituted 1 km transects, set 300 m apart (Vale and Phelps, 1978), with groups of adjacent days as experimental units (Torr, 1990). Treatments were randomly allocated to days within blocks such that each transect was traversed at the same time, in consecutive days. Thus, site and time of sampling variation was blocked.

The vehicle traversed transects hourly with closed windows at a maximum speed of 10 km/hr following recommendations for vehicle electric nets (FAO, 1982), from 7:00 to 17:00 hr for *G. m. centralis* and 9:00 to 17:00 hr for *G. m. morsitans*. This resulted in 11 and 8 experimental replicates respectively. The average time taken to complete a 1 km transect was 10 min. A 60 s waiting period was undertaken at the end of the transect to allow the trapping of trailing tsetse before milking of the trap. Trapped flies were removed from the sticky film with forceps, killed by squeezing the thorax, identified, sexed and enumerated.

After sampling, the vehicle was moved to the starting point of the next transect, with the sticky surface covered with non-stick baking paper. On average, a 25 min waiting period was undertaken before the commencement of a subsequent transect. This prevented the entry of flies from one transect into another, thus ensuring the independence of blocks.

In the colour experiment, one panel of the all-blue, all-black and blue-black was randomly placed at the left side of the steel frame in in-line orientation, to compare the mean catch of treatments. Tsetse catches on the blue and black sections of the blue-black panel were recorded separately to facilitate their comparison. Two panels of the most effective colour were placed on either side of the steel frame in the orientation experiment, comparing in-line and transverse orientations (Fig 2.1). Data for the left and right (in-line orientation), and front and back (transverse orientation) was recorded separately to compare one versus two-panel traps. Octenol sachet was fixed at the top while butanone

was placed at the bottom of the leading edge of the trap to compare a baited and un-baited trap in olfactory experiments. When moving from one transect to another, odour dispensers were sealed in airtight containers and were reopened at the commencement of the next transverse.

The effectiveness of the optimised VST was finally compared with the BFR in a similar design. BFR was conducted as described by Robinson (Robinson, 1995), where the catching party consisted of two men, each with a hand net, carrying a 0.5 × 1.5 m black cloth suspended from a 2 m pole and baited with octenol and butanone. The two men selected for the study had a combined 65-year experience in operating BFR and manning tsetse pickets. In each transect, 5 stops, 200 m apart, were identified. The catching party walked at normal speed to each stop where they caught tsetse flies that had landed on the screen or each other. After 3 min, the catching party moved to the next stop. On average, it took the catching party 35 min to traverse each transect. As the efficiency of BFRs reduces at high tsetse densities (Leak et al., 2008), this experiment was done in transects with low tsetse densities.

Statistical Analysis

To analyse changes of fly catches in the treatments, a negative binomial model with a log link was used in R (R Core Development Team, 2015). The general formula of the model used was:

$$\log(\mu) = In(t) + B_0 + B_1x_1 + B_2x_2 + B_3x_3,$$

with μ representing the mean, $In(t)$ the dispersion parameter, B_0 the intercept, $B_1x_1$the treatment, B_2x_2 the block and B_3x_3 the day effects of the model. Thus, mean tsetse catches were modelled on treatments for each experiment whilst taking into account the block and day effects of the study (Mbewe et al., 2018). To provide a common index of effect, the mean tsetse catch of a treatment was expressed as a proportion of a reference treatment or control, the resultant value termed catch index. Model coefficients were used to estimate catch indices of treatments, which were compared using the likelihood ratio test. The significance of each comparison was determined when the number 1 was

not included in the 95 % confidence interval (CI) of the ratio test. Catch indices of 2 or 0.5 indicated that a treatment caught twice or half as many flies as the control. The "*effects*" package in R (Fox, 2003) was used to obtain de-transformed means of treatments. All reported estimates are accompanied by 95% CI and the alpha level was set at 0.05 for statistical significance. Computations were conducted using R (R Core Development Team, 2015).

2.4 Results

A total of 11,342 tsetse were captured during the study (Table 2.1) with 29% being females. Only the two *G. morsitans* subspecies, *G. m. morsitans* and *G. m. centralis* were collected. For both subspecies, no transect with zero catch in all sampling hours was recorded.

Table 2.1 Total tsetse catches for each experiment segregated by sex.

Subspecies	Experiment	Female	Male	Total
G. m. morsitans	Colour	170	730	900
	Orientation	340	1,068	1,408
	Olfaction	707	1,514	2,221
	BFR vs VST	176	681	857
G. m. centralis	Colour	347	912	1,257
	Orientation	419	955	1,374
	Olfaction	987	1,984	2,973
	BFR vs VST	123	229	352
Total		**3,269**	**9,842**	**11,342**

Colour Comparison

As shown in Table 2.2 and 2.3, overall catch indices of the all-black, all-blue and blue-black panels for both *G. m. morsitans* and *G. m. centralis* did not differ significantly. For female *G. m. morsitans*, the catch index of the all-blue panel was significantly lower than the all-black panel, catching 39% fewer flies. Catch indices for males of all three panels were not significantly different. Male and female catch indices for *G. m. centralis* were not significantly different for all three panels (Table 2.3). In both subspecies, the catch index of the blue and black sections of the blue-black panel was significantly different, with the black section catching more male and female tsetse (Table 2.2 and Table 2.3). An all-blue panel was selected for use in all further experiments.

Orientation Comparison

Overall, male and female catch indices of sticky trap panels oriented in-line or transverse to the movement of the vehicle were not significantly different for both *G. m. morsitans* and *G. m. centralis* (Table 2.2 and 2.3).

Comparison of One Versus Two Panel Design

The in-line orientation did not show a significant difference between the overall, male and female catch indices of the left and right trap panels for data collected throughout the day and for separate morning and afternoon analysis for *G. m. morsitans* (Table 2.2). Overall catch indices of the front trap panel in transverse orientation were significantly higher for data collected in the afternoon and throughout the day (Table 2.2), catching 170 and 72% more flies, respectively.

Left and right panel catch indices of the in-line oriented trap for data collected throughout the day were not significantly different for *G. m. centralis* (Table 2.3). A separate analysis of data collected in the morning and afternoon periods showed that the right panel caught 4.42 (95% CI: 1.39 – 14.10 and $P < 0.05$) times (or 342%) more flies than the left panel, in the morning. In the afternoon, the catch index of the right panel significantly reduced to 0.47 (95% CI: 0.27 – 0.81 and $P < 0.05$) times that of the left panel, catching 53% fewer flies. As shown in Table 2.3, the catch index of the front panel of the transverse-oriented trap was significantly lower than that of the back panel, with the front panel catching 41% fewer flies. Two-sided in-line oriented trap panels were selected for use in all subsequent experiments.

Olfaction

No significant difference was observed between the overall catch indices of the un-baited and baited panel trap for *G. m. morsitans* (Table 2.2). For *G. m. centralis*, the overall catch index of the baited trap panel was significantly higher (Table 2.3), catching 38% more flies than the un-baited trap. The

sticky trap panel used in the subsequent experiment was baited with octenol and butanone for *G. m. centralis* while that for *G. m. morsitans* was un-baited.

Comparison of black screen fly round (BFR) and vehicle-mounted sticky trap panel (VST)

A significant difference between the overall catch indices of the BFR and VST was observed for both *G. m. morsitans* and *G. m. centralis* (Table 2.2 and 2.3). The VST caught 160 and 142% more flies than the BFR, respectively. VST female catch index for both subspecies was also significantly higher, catching 598 and 248% more female *G. m. morsitans* and *G. m, centralis*, respectively.

2.5 Discussion

The results of the colour experiments indicated that there were no significant differences in the overall catch indices of all-blue, all-black and blue-black trap panels for both *G. m. morsitans* and *G. m. centralis*. This result suggests that the colours used in this study had an equal effect in eliciting close-range orientation towards sticky surfaces, an observation supported by the finding that blue and black surfaces are equally attractive to tsetse (Green and Flint, 1986; Lindh et al., 2012). Differences in landing responses were observed on the blue-black panel with the black section having a higher catch index, a result also consistent with other findings (Green, 1994; Green and Flint, 1986; Vale, 1974). Further research is recommended to evaluate the effect of other colours, panel sizes and rates of movement on the mean tsetse catch of trap panels.

Table 2.2. De-transformed means and catch indices as estimated by negative binomial regression for female and male *G. m. morsitans*.

Experiment	Treatment	Female			Male			Overall		
		Mean Catch (95% CI)	Catch Index (95% CI)	*P-value*	Mean Catch (95% CI)	Catch Index (95% CI)	*P-value*	Mean Catch (95% CI)	Catch Index (95% CI)	*P-value*
Colour	All Black (Control)	7.71 (5.74 – 10.35)	1		22.90 (18.74 – 27.99)	1		30.20 (25.04 – 36.42)	1	
	All Blue	4.68 (3.24 – 6.75)	0.61 (0.40 – 0.92)	0.018	28.84 (23.69 – 35.08)	1.26 (0.98 – 1.62)	0.073	33.67 (27.85 – 40.71)	1.12 (0.87 – 1.42)	0.373
	Blue - Black	5.64 (3.98 – 8.01)	0.73 (0.48 – 1.10)	0.133	27.90 (22.90 – 33.99)	1.22 (0.94 – 1.57)	0.122	34.25 (28.36 – 41.37)	1.13 (0.89 – 1.43)	0.299
Blue - Black	Black (Control)	2.31 (1.31 – 4.08)	1		19.65 (16.13 – 23.93)	1		27.62 (17.99 – 42.40)	1	
	Blue	3.11 (1.87 – 5.19)	1.34 (0.78 – 2.33)	0.278	8.17 (6.15 – 10.84)	0.42 (0.32 – 0.54)	0.000	12.75 (8.08 – 20.12)	0.46 (0.25 – 0.82)	0.008
Orientation	In-line (Control)	7.75 (5.49 – 10.92)	1		27.24 (20.58 – 36.05)	1	1	36.02 (21.69 – 46.87)	1	
	Transverse	7.24 (5.10 – 10.27)	0.93 (0.56 – 1.55)	0.780	21.13 (15.79 – 28.28)	0.78 (0.52 – 1.17)	0.212	29.14 (22.24 – 38.18)	0.81 (0.55 – 1.19)	0.263
In-line	Left (Control)	4.87 (3.39 – 6.98)	1		21.60 (17.38 – 26.83)	1		26.79 (21.38 – 33.55)	1	
	Right	6.08 (4.33 – 8.55)	1.25 (0.92 – 1.71)	0.158	23.44 (18.96 – 28.98	1.09 (0.85 – 1.39)	0.518	31.99 (25.75 – 39.74)	1.19 (0.92 – 1.55)	0.179
Transverse	Back (Control)	7.73 (5.25 – 11.37)	1		14.23 (9.92 – 20.40)	1		23.22 (17.35 – 31.08)	1	
	Front	7.64 (5.19 – 11.26)	0.99 (0.62 – 1.58)	0.962	32.58 (23.61 – 44.97)	2.29 (1.50 – 3.51)	0.000	39.98 (30.50 – 52.40)	1.72 (1.22 – 2.43)	0.001
Olfactory	Un-baited (Control)	36.82 (27.16 – 49.92)	1		71.92 (51.72 – 100.03)	1		110.11 (80.27 – 151.05)	1	
	Baited	40.40 (29.80 – 54.78)	1.10 (0.77 – 1.56)	0.598	89.28 (64.30 – 123.96)	1.24 (0.84 – 1.84)	0.256	130.31 (95.04 – 178.65)	1.18 (0.81 – 1.73)	0.356
BFR vs VST	BFR (Control)	2.91 (1.56 – 5.42)	1		32.01 (21.93 – 46.72)	1		35.34 (24.20 – 51.62)	1	
	VST	20.30 (14.73 – 27.99)	6.98 (4.54 – 11.33)	0.000	70.32 (49.39 – 100.12)	2.20 (1.50 – 3.21)	0.000	91.86 (64.55 – 130.71)	2.60 (1.78 – 3.81)	0.000

Table 2.3. De-transformed means and catch indices as estimated by negative binomial regression for female and male *G. m. centralis*.

Experiment	Treatment	Female			Male			Overall		
		Mean Catch (95% CI)	Catch Index (95% CI)	*P-value*	Mean Catch (95% CI)	Catch Index (95% CI)	*P-value*	Mean Catch (95% CI)	Catch Index (95% CI)	*P-value*
Colour	All Black (Control)	8.04 (6.29 -10.28)	1		18.28 (14.78 - 22.61)	1		26.99 (22.09 -32.99)	1	
	All Blue	7.01 (5.15 - 9.55)	0.87 (0.60 -1.26)	0.467	14.78 (11.32 - 19.31)	0.81 (0.58-1.12)	0.187	21.78 (17.05 - 27.82)	0.81 (0.59 - 1.10)	0.158
	Blue - Black	10.35 (8.05 - 13.32)	1.29 (0.95 - 1.75)	0.106	16.28 (12.70 - 20.86)	0.89 (0.67 - 1.18)	0.420	28.45 (22.81 -35.48)	1.10 (0.80 - 1.38)	0.699
Blue - Black	Black (Control)	6.21 (4.67 – 8.26)	1		11.00 (8.72 – 13.88)	1		18.87 (15.32 – 23.24)	1	
	Blue	3.81 (2.70 – 5.39)	0.61	0.008	5.81 (4.39 -7.69)	0.42 (0.32 – 0.54)	0.000	9.77 (7.59 – 12.56)	0.52 (0.40 – 0.68)	0.000
Orientation	In-line (Control)	11.37 (8.74 – 14.80)	1		26.86 (21.55 – 33.49)	1		39.70 (32.14 – 49.05)	1	
	Transverse	10.05 (7.64 – 13.20)	0.88 (0.65 -1.20)	0.435	20.64 (16.33 – 26.08)	0.77 (0.58 – 1.00)	0.051	30.53 (24.45 – 38.11)	0.76 (0.56 – 1.00)	0.049
In-line	Left (Control)	5.59 (3.27 – 9.56)	1		12.39 (6.99 – 21.95)	1		18.33 (10.67 – 31.48)	1	
	Right	6.00 (3.53 – 10.19)	1.07 (0.52 -2.21)	0.831	16.43 (9.37 – 28.83)	1.33 (0.56 – 3.20)	0.431	22.41 (13.12 – 38.28)	1.22 (0.54 – 2.82)	0.550
Transverse	Back (Control)	6.36 (4.39 – 9.23)	1		12.14 (8.22 – 17.93)	1		17.19 (11.48 – 25.74)	1	
	Front	2.62 (1.63 – 4.22)	0.41 (0.25 – 0.68)	0.004	6.86 (4.46 – 10.56)	0.57 (0.34 – 0.94)	0.021	10.59 (6.59 – 15.62)	0.59 (0.34 – 1.03)	0.043
Olfactory	Un-baited (Control)	26.94 (22.08 – 32.86)	1		44.35 (36.23 – 54.29)	1		72.28 (60.56 – 86.27)	1	
	Baited	37.03 (30.92 – 44.34)	1.37 (1.08 – 1.74)	0.009	61.70 (51.09 – 74.53)	1.39 (1.10 – 1.77)	0.006	99.93 (84.49 – 118.20)	1.38 (1.11 – 1.72)	0.004
BFR vs VST	BFR (Control)	2.87 (1.71 – 4.79)	1		6.14 (4.23 – 8.92)	1		9.73 (7.34 – 12.92)	1	
	VST	9.99 (7.43 – 13.42	3.48	0.000	12.50 (9.22 – 16.94)	2.04 (1.53 – 2.73)	0.000	23.57 (19.25 – 28.84)	2.42 (1.91 – 3.10)	0.000

Since the three colour patterns used in this study had similar catch indices, we recommend the use of any of the three in the construction of VSTs for surveys whose objective is to identify the precise distribution limits and the main ecological niches of *G. m. morsitans* and *G. m. centralis*. For surveys whose objective is to establish the physiological age of the population using ovarian dissection, all-black and blue-black trap panels are recommended. In this study, the blue colour was chosen for subsequent experiments due to its potential for being used with imaging devices. Imaging devices could geo-reference a fly as soon as it lands on the trap panel and thus provide a precise location of capture. This feature may improve monitoring of low-density populations expected after vector control interventions.

Investigations on trap orientations indicated that there was no significant difference with the sticky panel traps oriented in-line or transverse to the horizontal axis of the vehicle for both *G. m. morsitans* and *G. m. centralis*. This finding may be attributed to the observed circulating behaviour of tsetse before they land on a visual bait (Green, 1994, 1986; Vale, 1982). The circulating behaviour of the following swarm may ensure that tsetse is equally available for capture no matter the orientation of the stick panel traps. In-line orientation was selected for use in subsequent experiments as it appeared to be more stable during sampling and non-sampling periods.

The catch indices of left and right panels were not significantly different for *G. m. morsitans*, even for separate analysis of morning and afternoon periods. However, left and right panel catch indices for *G. m. centralis* were significantly different for separate analysis of morning and afternoon periods with the panel facing the edge of the woodland, catching more tsetse than the panel facing the grassy dambo. The underlying cause of the observed contrasting response of the two subspecies was unclear since the circulating behaviour of tsetse before landing on a visual bait (Green, 1994; Vale, 1982), is expected to make flies equally available for capture on both panel traps, regardless of habitat structure. Further investigation into this observation is warranted as it may highlight behavioural

differences between *G. m. morsitans* and *G. m. centralis* that are yet to be established. The two-sided sticky trap panel was selected as the most effective design.

For *G. m. morsitans*, baiting with butanone and 1-octen-3-ol did not significantly increase the catch index of the VST. This result supports the work of Vale (Vale, 1974) who unequivocally demonstrated that tsetse were primarily attracted to mobile hosts largely in response to movement rather than odour. Tsetse close-range orientation towards a host has also been shown to be unaffected by synthetic odours (Torr, 1989) with carbon dioxide being the only host odour component that induces alighting response (Bursell, 1990; Warnes, 1995). Therefore, our results suggest that visual factors are more important than synthetic olfactory cues in the effectiveness of the VST to trap *G. m. morsitans*.

Baiting with butanone and 1-octen-3-ol was however observed to increase the catch index of the VST for *G. m. centralis*. This result appears to suggest that close-range orientation to a host and/or alighting response in *G. m. centralis*, is influenced by synthetic odours contrary to observations made in *G. m. morsitans* (Torr and Solano, 2010). Differences between the subspecies in response to a stimulus have been previously reported. Torr *et al.* (Torr et al., 2011) showed that very few *G. m. morsitans* were attracted to or landed on 0.25 × 0.25 m tiny targets relative to the 1 m^2 target. However, Byamungu *et al*. (Byamungu et al., 2018) observed that 0.5 m^2 targets were just as efficient as 1 m^2 target for *G. m. centralis*. It is unlikely that evolved differences in innate behaviour could account for the observed differences in response to odours, given the taxonomic classification of these two organisms as subspecies. More probable is the postulation by (Vale et al., 2014) that habitat geometry affects the effectiveness of odour attraction and the strength of the landing response among tsetse flies. Perhaps the differences between the geometry of the habitats of the two subspecies is sufficiently great to affect their responses to odours. Habitat effects are considered to cause the observed differences between savannah and riverine species in response to odour baits (Torr and Solano, 2010), but more needs to be done to assess what differences there might be between

subspecies of *G. morsitans*. Presently, we recommend the baiting of the VST with butanone and 1-octen-3-ol for sampling *G. m. centralis* but not for *G. m. morsitans*. However, if the identity of the subspecies in an area is unknown, baiting the VST is recommended.

For both *G. m. morsitans* and *G. m. centralis*, the VST was observed to have a higher catch index than the BFR, despite the former being operated at 10 km/hr and the latter at 5 km/hr. This result could be attributed to a combination of several factors. Firstly, the VST presented a larger moving target than that of the BFR. It has been previously demonstrated that larger moving objects are more attractive to tsetse than smaller ones (Byamungu et al., 2018; Colvin and Gibson, 1992; Vale, 1982). Secondly, a proportion of tsetse attracted to the BFR was repelled by human odour which has been previously shown to have a repellent effect on tsetse (Kuzoes and Schofield, 2005; Vale, 1974). As human operators were enclosed within the vehicle with closed windows, no such repellent effect could be present for the VST. Lack of repellency could also explain the higher female catch index of the VST as compared to the BFR since repellence to human odour is higher in female flies (Colvin and Gibson, 1992; Vale, 1974). Thirdly, BFR catches are influenced by the hand netting ability of operators (Leak et al., 2008), whilst the catch size of the VST is consistent within the effective range of the sticky film. Therefore, our results indicate that the VST is better suited for sampling both subspecies of *G. morsitans* than the BFR. However, we recommend further studies to establish the cost-effectiveness of the use of the VST in comparison with other traps.

In this study, the VST was operated at 10 km/hr following recommendations for vehicle electric nets (FAO, 1982). However, VST may be more effective in sampling tsetse at other speeds. Therefore, we recommend further studies to determine the most optimal operation speed for the VST. Nevertheless, given that flight speeds of up to 14.4 km/hr have been observed in the laboratory (Hargrove, 1975) and mean speeds of 22 km/hr recorded in the field for *G. m. morsitans* (Gibson and Brady, 1988), we suggest that the maximum operational speed of the VST should not exceed 20 km/hr. This speed should be efficient for sampling moderate to high-density populations of *G.*

morsitans with the lower speed of 10 km/hr being better suited to low-density populations. Intermittent stops at 1 km intervals ensured that most flies following the VST were captured. This practice is recommended to facilitate georeferencing of the catch.

A significant limitation of the VST is that sampling is largely restricted to motorable tracks. Thus, VST is likely to be used as a complementary sampling device to existing tools. The results showed that the VST had a higher sensitivity to male tsetse. This apparent bias may make it an ideal tool for monitoring flight ability, survival and competitiveness of sterile males released during the implementation of the sterile insect technique. Furthermore, since males copulate with multiple females, a vehicle-mounted panel target of a similar design could prove an effective mobile delivery system of entomopathogenic fungi for biological control of *G. morsitans*. Investigations on the suitability of using the VST as a personal protection device and control tool during game drives are further recommended.

We conclude that the VST constructed using an all-blue, all-black and 1:1 blue-black panel, oriented either in-line or transversely and baited with butanone and 1-octen-3-ol (*G. m. centralis*), is a more rapid and effective sampling tool for *G. morsitans* than BFR. This makes it an invaluable tool in planning and evaluating area-wide suppression/eradication campaigns. We recommend its use in sampling large geographic areas to facilitate *G. morsitans* population studies currently limited by the inadequate sampling of natural populations.

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Chapter 3: Spatial distribution of *Glossina morsitans* (Diptera: Glossinidae) in Zambia: a vehicle-mounted sticky trap survey and Maxent species distribution model

3.1 Overview

This chapter addresses the distribution of the tsetse flies in Zambia, directly answering the first specific objective and research question. Again, the tool developed in Chapter two was utilised in this chapter to analyse the spatial distribution limits of the tsetse populations.

The data collected using the trap enabled the researcher to explore how the distribution of these tsetse flies has changed over time and to assess population structuring components. This study has already been published as: Muyobela J, Pirk CWW, Yusuf AA, Sole CL (2023). Spatial distribution of *Glossina morsitans* (Diptera: Glossinidae) in Zambia: A vehicle-mounted sticky trap survey and Maxent species distribution model. PLoS Negl Trop Dis 17(7): e0011512.https://doi.org/10.1371/journal.pntd.0011512.

3.2 Introduction

Tsetse (Diptera: Glossinidae) occur in sub-Saharan Africa and are the only biological vectors of trypanosomes that cause human African trypanosomiasis (HAT or sleeping sickness) and animal African trypanosomiasis (AAT or nagana). Trypanosomiasis remains a debilitating and fatal disease if left untreated. In livestock, AAT causes annual losses of over 4 billion USD due to increased cattle and calf mortality, reduced calving rates, and reduction in milk and meat sales (Swallow, 1999). Diagnosis of HAT is difficult and treatments are often challenging to administer (Franco et al., 2017). Accurate knowledge of local tsetse spatial distribution patterns is essential to understanding trypanosomiasis epidemiology and transmission dynamics (Dicko et al., 2015) and is vital to the development of risk-based vector control strategies (Diall et al., 2017).

The distribution of tsetse is influenced by climate (Hargrove, 2001), vegetation (Van den Bossche et al., 2010), and host availability (Vreysen et al., 2013). Climate, particularly temperature, is an important driver of tsetse population dynamics affecting key aspects of tsetse ecology such as vector

survival, pupal development, fecundity, and density (Pagabeleguem et al., 2016). Global temperatures were reportedly 1.31ºC higher in 2017 from the last century (NCEI, 2022), and hence, there is a need to understand how this change has affected spatial distributions of tsetse. Vegetation is important for maintaining microclimatic environments that provide suitable conditions for adult resting sites and puparial development (Cecchi et al., 2008). In recent years, habitat fragmentation, defined as the breakup of native vegetation into smaller isolated fragments (Mweempwa et al., 2015), has led to a notable modification of tsetse habitat as a result of anthropogenic activities (Van den Bossche et al., 2010). Progressive clearing of natural vegetation for cultivation, the introduction of domestic animals, and the almost complete disappearance of large game animals have resulted in changes in tsetse distribution (Ducheyne et al., 2009), particularly among savannah group flies (Vreysen et al., 2013). These changes have important implications for disease epidemiology and need to be considered when developing area-specific evidence-based vector management strategies. Thus, regularly updating the distribution tsetse is essential.

Existing tsetse distribution maps in Zambia are more than 40 years old and were based on coarse spatial resolution data obtained from surveys done in the colonial era (Evison and Kathuria, 1982) and published by Ford and Katondo (Ford and Katondo, 1977). According to these maps, four species of *Glossina* occur in Zambia namely, *Glossina fuscipes martini* Zumpt, *G. brevipalpis* Newstead, *G. pallidipes* Austin, and *G. morsitans*. *Glossina morsitans* has the widest distribution covering an estimated 277,000 km^2 (or 38%) of the total land mass, with one subspecies, *G. m. morsitans* Westwood, occupying the hotter eastern part and the other subspecies, *G. m. centralis* Machado, occupying the cooler western and Northern part of the country (Evison and Kathuria, 1982) (Fig 3.1). The other three species exist within *G. morsitans* range but to a much lesser extent (Evison and Kathuria, 1982). Being an efficient trypanosome vector, *G. morsitans* is therefore the most economically important tsetse species in Zambia, and most research and control efforts are directed against it. Recent information on the distribution of this tsetse species is however lacking.

A significant hindrance to surveying large areas infested with savannah tsetse has been the high cost of deploying baited stationary traps (Bouyer et al., 2015; Krafsur, 2009; Kuzoes and Schofield, 2005). Area-wide tsetse surveys in large countries with a significant portion of their land mass infested with these tsetse are therefore not routinely undertaken (Leak et al., 2008). The recently developed vehicle-mounted stick trap (VST) (Muyobela et al., 2021) provides an alternative effective sampling device that has been shown to rapidly detect the presence of *G. morsitans*. The VST could provide a suitable method of implementing low-cost area-wide surveys for regular updating of *G. morsitans* distribution. However, it is limited to surveying motorable tracks (Muyobela et al., 2021) and its utility outside an experimental setting has not been demonstrated.

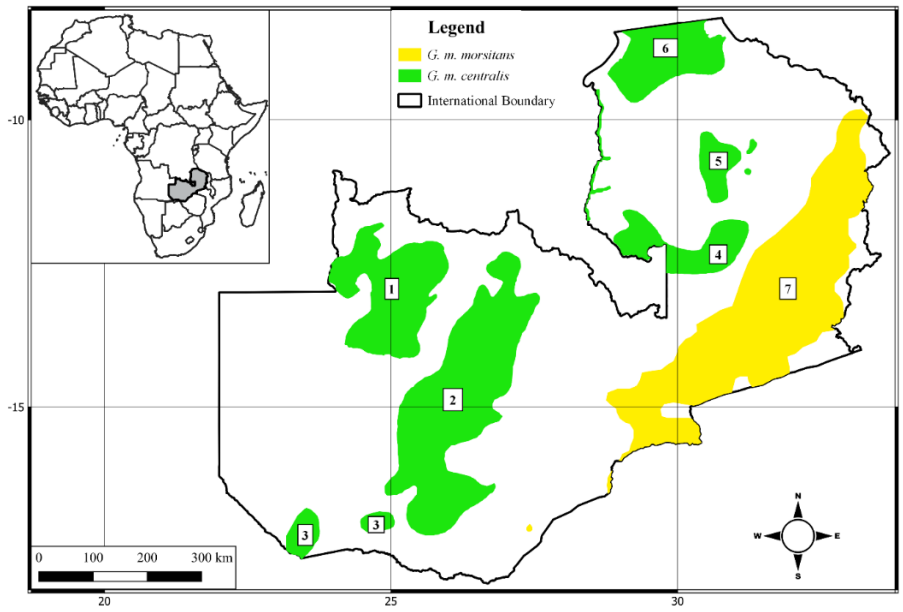

Fig 3.1. Historical distribution of the allopatric subspecies *G. m. morsitans* and *G. m. centralis* in seven distinct tsetse belts. Data on each subspecies in Evison and Kathuria (Evison and Kathuria, 1982). Tsetse belt 3 was eradicated using sequential aerial spray technique in two separate operations in 2009 and 2014. The base map layer was obtained from the Database of Global Administrative Area GADM (https://geodata.ucdavis.edu/gadm/gadm4.1/shp/gadm41_ZMB_shp.zip) and under the license https://gadm.org/license.html. The figure was created using QGISv3.0 (http://qgis.org/en/site/).

One strategy to mitigate the accessibility limitation of VST tsetse surveys is to model its occurrence records using species distribution models (SDMs). Ecological niche models (ENMs) estimate the relationship between species occurrences and various environmental variables to produce prediction distribution maps (Austin, 2007; Elith and Leathwick, 2009; Guisan and Thuiller, 2005). Several

statistical models have been used to predict species distributions (Guisan et al., 2017; Jarnevich et al., 2015), among which the maximum entropy model (Maxent) is effective for predicting species distribution locally and globally (Dicko et al., 2014; Elith et al., 2011; Phillips et al., 2006). From a Bayesian perspective, the principle of maximum entropy states that subject to known constraints, the probability distribution that best represents the data is the one with the greatest entropy, thus the one which best reproduces the data (Phillips and Dudík, 2008). Maxent aims to fit a penalized maximum likelihood model to presence-only species data that trades-off model fit and model complexity (Phillips and Dudík, 2008). Maxent has been used to predict current (Dicko et al., 2014) and future (Zhou et al., 2021) distributions of tsetse.

The objective of the present study was to demonstrate the effectiveness of utilizing the VST for area-wide surveys of *G. morsitans* and to use the occurrence records to locally predict its distribution under current environmental conditions using Maxent. The predicted distribution will provide the basis for developing evidence-based tsetse and trypanosomiasis control strategies in Zambia.

3.3 Materials and Methods

Study area.

The study was conducted in Zambia, between the longitudes 22 and 34 ˚E, and latitudes eight and 18 ˚S. Much of the country consists of a plateau averaging from 1067 to 1220 m in elevation. Vegetation on the plateau is dominated by Miombo woodlands interspaced with riverside dambos (grassy wetlands) (Wigg, 1949). Mopane woodland is the dominant vegetation type in the valleys. A tropical climate prevails that is characterized by unimodal rainfall ranging from 500 to 1400 mm (Muyobela et al., 2015). Rainfall is restricted to the period between November to April with temperature ranging from 14 to 30°C in the rainy season. May to July is cool and dry with temperature ranging from six to 26°C. August to October is hot and dry and temperature ranges from 17 to 35°C. Based on annual rainfall distribution, Zambia is divided into three agroecological zones namely Region I (less than 800 mm), Region II (800 to 1000 mm), and Region III (above 1000 mm) (Dittoh, 2010). *G. morsitans*

is known to occur in all three agroecological zones in eight distinct tsetse belts Fig 1 (Evison and Kathuria, 1982).

Sampling device and materials

A two-sided all-blue (blue polyester, ParmaNet, Vestergaard Frandsen, Denmark) VST with one-sided adhesive film (Rentokil FE45, Liverpool, UK) was constructed according to the method described by (Muyobela et al., 2021). Briefly, blue fabric was cut and fixed onto one side of two 5 mm × 1 m × 1.5 m plywood boards. To enumerate tsetse landing on trap panels, one-sided adhesive film was fastened above the cloth, using black duct tape. The trap was baited with butanone and 1-octen-3-ol which were dispensed at a rate of 150 mg/hr and 0.5 mg/hr respectively (Torr et al., 1997). The trap was mounted on the back of a white Land Cruiser pick-up with its longest side horizontal to the ground. Non-stick baking paper was used to cover the sticky surface of the trap when not in use.

Survey design and sampling.

Stratified random sampling was used to determine the presence and absence of *G. morsitans* in the seven tsetse belts (Fig 3.1), except for tsetse belt 3, whose population was eradicated by sequential aerial spraying with ultra-low volume deltamethrin. Each sampled tsetse belt was stratified according to agroecological region to ensure that the entire environmental space occupied by the belt was sampled. Sampling routes were then located using QGIS version 3.0 and randomly selected. Selected routes were then uploaded to QField for QGIS android application which aided navigation in the field. The VST was operated by 3 individuals.

The VST traversed selected transects at a maximum speed of 20 km/h (Muyobela et al., 2021) making interval stops at 1 km to identify, sex, enumerate, and geo-reference captured flies. A one-minute waiting period was undertaken at a stop to allow the trapping of trailing tsetse before milking of the trap. VST traversed most transects from at least 5 km away from the historical limit of a tsetse belt and continued sampling for at least 20 km after detecting tsetse. Where tsetse was not detected, sampling continued for at least 60 km into the tsetse belt, and the route was sampled twice. The total

distance surveyed was 692 and 1020 km for *G. m. centralis* and *G. m. morsitans* respectively. Sampling took place between September 2021 and August 2022. Actual sampling days consisted of 27 for *G. m. centralis* and 22 days for *G. m. morsitans*.

The results of a 1 km sampling interval were allocated to the mid coordinate of that km and were interpreted as a response from a 1 × 1 km area. Such an interpretation accounts for the daily displacement of *G. morsitans* which has been estimated to be between 167 m to 1.3 km (Williams et al., 1992) which plays a significant role in making the tsetse available for capture.

Presence - absence data

As described above, the VST survey involved continuous sampling along a transect. This sampling strategy is known to result in the loss of independence between samples located along the same transect (Elith and Leathwick, 2009), due to spatial autocorrelation. Geographic sampling bias can lead to environmental bias, resulting in an overrepresentation of environmental conditions associated with regions of higher sampling (Anderson and Gonzalez, 2011; Hijmans, 2012). Constructing an ecological niche model with such data may fit the environmental bias, in addition to the niche signal, thus hindering model interpretation and application (Wintle and Bardos, 2006).

Moran's I statistic was therefore used to assess the strength of spatial autocorrelation using the spdep package (Bivand and Wong, 2018) in R (R Core Development Team, 2015). As shown in Table 3.1, VST survey results for both *G. m. morsitans* and *G. m. centralis* were highly clustered (positive spatial autocorrelation). To reduce the effects of biased sampling while returning the signal of the species niche, spatial thinning of occurrence records for both subspecies based on nearest neighbour distance was done at 5 and 7 km using the spThin (Aiello-Lammens et al., 2015) package in R. Moran's I test was reperformed on the thinned datasets, for both full (presence and absence) and p (presence only) data (Table 3.1). Based on observed Moran I Statistic, 5 km presence-only data were selected for modelling.

Table 3.1: Spatial autocorrelation analysis

Subspecies	Data Type	No. of Records	Moran I Statistic	Expectation	Variance	Standard deviation	P-value
G. m. morsitans	VST raw full	1020	0.763	-0.0009	0.0002	50.699	$< 2.2\ e^{-16}$ *
	5 km thinned full	147	0.191	-0.0068	0.0013	5.505	$1.85\ e^{-8}$ *
	5 km thinned p	65	0.053	-0.0156	0.0028	1.288	0.10
	7 km thinned full	106	0.080	-0.0100	0.0017	2.149	0.02 *
	7 km thinned p	51	0.062	-0.0200	0.0033	0.454	0.32
G. m. centralis	VST raw full	692	0.567	-0.0014	0.0003	31.915	$< 2.2\ e^{-16}$ *
	5 km thinned full	106	0.182	-0.0095	0.0016	4.807	$7.64\ e^{-7}$ *
	5 km thinned p	35	0.027	-0.0294	0.0042	0.860	0.19
	7 km thinned full	79	0.057	-0.0182	0.0018	1.653	0.05
	7 km thinned p	23	0.047	-0.0454	0.0049	0.018	0.51

Full; full dataset with presence and absence records, P; dataset with presence-only records, *; statistically significant.

Environmental variables and processing

Climatic and environmental predictors were used to estimate the species' environmental relationship. Current climatic variables obtained were monthly minimum temperature, monthly maximum temperature, monthly average temperature, monthly precipitation, and 19 bioclimatic variables (bio1 – bio19) from WorldClim Global Climate Database version 2.1 (Fick and Hijmans, 2017). Environmental variables included Moderate Resolution Imaging Spectroradiometer (MODIS) composite time series leaf area index (LAI) (MOD152H), normalised difference vegetation index (NDVI) (MOD13Q1), land surface temperature day (LST) (MOD11A1) (Didan, 2015), and land cover type 1 (LC) (MCD12Q1) (Friedl and Sulla-Menashe, 2019) obtained from NASA EOSDIS Land Processes Distributed Active Archive Center (AppEEARS Team, 2022). The legend and class description of land cover type 1 (LC) (MCD12Q1) are given in Table 3.2. Data on human population density was obtained as Gridded Population of the World, Version 4 (GPWv4) from NASA's Socioeconomic Data and Applications Center (SEDAC) (Center for International Earth Science Information Network (CIESIN), 2018). Elevation data was obtained as Global 30 Arc-Second Elevation (GTOPO30) from the Earth Resources Observation and Science Center (Earth Resources Observation and Science Center/U.S. Geological Survey/U.S. Department of the Interior, 1997).

Table 3.2: International Geosphere-Biosphere Program (IGBP) legend and class descriptions of MODIS land cover type 1 (MCD12Q1)

Name	Value	Description
Evergreen Needleleaf Forests	1	Dominated by evergreen conifer trees (canopy > 2 m). Tree cover > 60%.
Evergreen Broadleaf Forests	2	Dominated by evergreen broadleaf and palmate trees (canopy > 2 m). Tree cover > 60%.
Deciduous Needleleaf Forests	3	Dominated by deciduous needleleaf (larch) trees (canopy > 2 m). Tree cover > 60%.
Deciduous Broadleaf Forests	4	Dominated by deciduous broadleaf trees (canopy > 2 m). Tree cover > 60%.
Mixed Forests	5	Dominated by neither deciduous nor evergreen (40-60% of each) tree type (canopy >2 m). Tree cover > 60%.
Closed Shrublands	6	Dominated by woody perennials (1-2 m height) > 60% cover.
Open Shrublands	7	Dominated by woody perennials (1-2 m height) 10-60% cover.
Woody Savannas	8	Tree cover, 30-60% (canopy > 2 m).
Savannas	9	Tree cover, 10-30% (canopy >2 m).
Grasslands	10	Dominated by herbaceous annuals (< 2 m).
Permanent Wetlands	11	Permanently inundated lands with 30-60% water cover and > 10% vegetated cover.
Croplands	12	At least 60% of the area is cultivated cropland.
Urban and Built-up Lands	13	At least 30% impervious surface area including building materials, asphalt, and vehicles.
Cropland/Natural Vegetation Mosaics	14	Mosaics of small-scale cultivation 40-60% with natural tree, shrub, or herbaceous vegetation.
Permanent Snow and Ice	15	At least 60% of the area is covered by snow and ice for at least 10 months of the year.
Barren	16	At least 60% of the area is non-vegetated barren (sand, rock, soil) areas with less than 10% vegetation.
Water Bodies	17	At least 60% of the area is covered by permanent water bodies.

For all monthly time series data, harmonic regression was performed using the TSA package (Kung-Sik and Ripley, 2020) in R. Seven coefficients for each variable were extracted from the annual time series. The first coefficient in the regression is the mean of the variable and each further coefficient contributes to explaining the complete series by determining the amplitude and phase of the period that are half the length of the preceding period (Estrada-Peña et al., 2014). To generate uniform data structures for modelling, climatic and environmental predictors were resampled using the nearest neighbour method to give a spatial resolution of 1 km, reprojected to WGS84 coordinate reference system and spatially masked to the extent of Zambia, using the raster package (Hijmans and van Etten, 2012) in R. All the environmental variables were converted into ASCII format.

Model calibration.

Pilot study

Environmental variable selection depends primarily on their restrictive effects on species distribution and spatial correlation (Zhang et al., 2019). In this study, variable classes, topographic, climatic, bioclimatic, and vegetation indices were run separately in Maxent software (Phillips et al., 2022) using default settings. Variables contributing 10% and 4% or more to model gain were pre-selected for *G. m. morsitans* and *G. m. centralis* respectively. Selected predictor classes were then combined and rerun in Maxent with the same criteria used to select variables for each subspecies. Land cover type and human population density were included in both models as they are known to be correlated with tsetse habitat (Cecchi et al., 2008; Ducheyne et al., 2009). Pearson correlation analysis was then conducted on selected variables to assess the risk of multicollinearity among variables. For *G. m. morsitans*, all pairwise correlation coefficients were less than 0.7, and hence no risk of multicollinearity was observed (Guisan et al., 2017). High correlation coefficient values (greater than 0.8) were however observed for some predictors of *G. m. centralis*. (Feng et al., 2019) showed that Maxent is robust to predictor collinearity in model training and that the strategy of excluding highly correlated variables has little impact because Maxent accounts for redundant variables. Therefore, all variables selected were included in the model due to their ability to improve model fit.

The ENMeval package (Muscarella et al., 2014) in R was used to test the sequential criterion of the lowest omission rate (OR) and the best area under the curve (AUC) to identify parameter settings providing the best model fit (Kass et al., 2018). Feature combination and regularization multiplier were confirmed through this process.

Formal Experiments

Occurrence data and selected environmental variables were uploaded into Maxent software. Various parameters were set, including ‘Create response curves’ and ‘Random seed’. Furthermore, 75% of the presence data was used to establish the model, and 25% was applied for testing, using bootstrap

sampling, 20 replicates, 5000 maximum iterations, and 10 percentile training presence threshold rule (Table 3.3).

Table 3.3: Maxent parameter settings

Option	Default Value	Setting Value
Randomly selected test set percentage	0	25
Regularization multiplier	1	0.5
Replicated run type	Crossvalidate	Bootstrap
Number of iterations repeated	1	20
Maximum number of repetitions Apply	500	5000
Apply threshold rules	None	10 percentile training presence
Features	Auto (hinge, product, quadratic, linear)	Auto (hinge, product, quadratic, linear)

The default feature setting of Maxent was chosen with a 0.5 regularization multiplier. Jackknife tests were used to measure variable contribution rates and importance. The log-log (cloglog) probability of presence maps produced were reclassified using the raster package in R and divided into four levels based on Jenks (Zhou et al., 2021): unsuitable area (0–0.097), marginally suitable area (0.097–0.323), moderately suitable area (0.323–0.603), and highly suitable area (0.603–1).

Model evaluation.

The training omission rate for both subspecies was close to the predicted omission rate which indicated that the models were well-established (Fig 3.2A and 3.2C). The receiver operating characteristic curve (ROC) obtained by plotting Sensitivity (true presence rate) on the y-axis against 1- Specificity (false presence rate) on the x-axis plotted across all available thresholds was used to evaluate the models (Phillips et al., 2006). The area under the curve (AUC) of the ROC, whose value ranges from 0.5 to 1, provides a measure of model accuracy (Fielding and Bell, 1997). A value of 0.5 indicates that a model performs no better than random. AUC values of 0.5 – 0.7 represent poor model performance, values of 0.7 – 0.9 are considered moderate, and values above 0.9 indicate excellent model performance (Phillips et al., 2006). The mean AUC values of ROC were observed to be 0.988 ± 0.002 and 0.982 ± 0.002 for *G. m. centralis* and *G. m. morsitans*, respectively. This indicated excellent Maxent model performance for both subspecies (Fig 3.2B and 3.2D).

3.4 Results

Survey Results

A total of 15,602 tsetse was captured during the survey (Table 3.4). The most abundant tsetse was *G. m. morsitans* (58%) followed by *G. m. centralis* (39%). *G. pallidipes* represented (2%) of the catch and was observed in *G. m. morsitans* range. *G. brevipalpis* was detected in *G. m. centralis* range in tsetse belt 6 (Fig 3.1). No *G. f. martini* tsetse were captured during the survey. *G. m. morsitans* was detected in Tsetse belt 7. *G. m. centralis* was only observed in tsetse belts 2, 4, and 6 (Fig 3.1). No tsetse was detected in tsetse belts 1 and 5 despite 123 and 92 km of road being surveyed respectively. Historical limits of all tsetse belts were observed to have changed by generally receding towards the centre of the distribution.

Table 3.4. Tsetse catches characteristics.

Species	Male	Female	Total
G. m. morsitans	6,713	2,378	9,091
G. m. centralis	4,264	1,919	6,183
G. pallidipes	173	144	318
G. brevipalpis	11	0	11
G. fuscipes martini	0	0	0
Total	**11,161**	**4,441**	**15,602**

Maxent Model

Environmental variable contribution

The variables selected for modelling are shown in Tables 3.5 and 3.6 for *G. m. morsitans* and *G. m. centralis* respectively. As indicated, 7 variables were sufficient to model the former while 8 were required to adequately describe the potential distribution of the latter subspecies. For *G. m. morsitans*, isothermality, minimum temperature of the coldest month, and annual precipitation were observed to contribute the most to Maxent model gain (Table 3.5). For *G. m. centralis*, land cover type, human population density, and precipitation of the driest quarter, contributed the most to the model (Table 3.6).

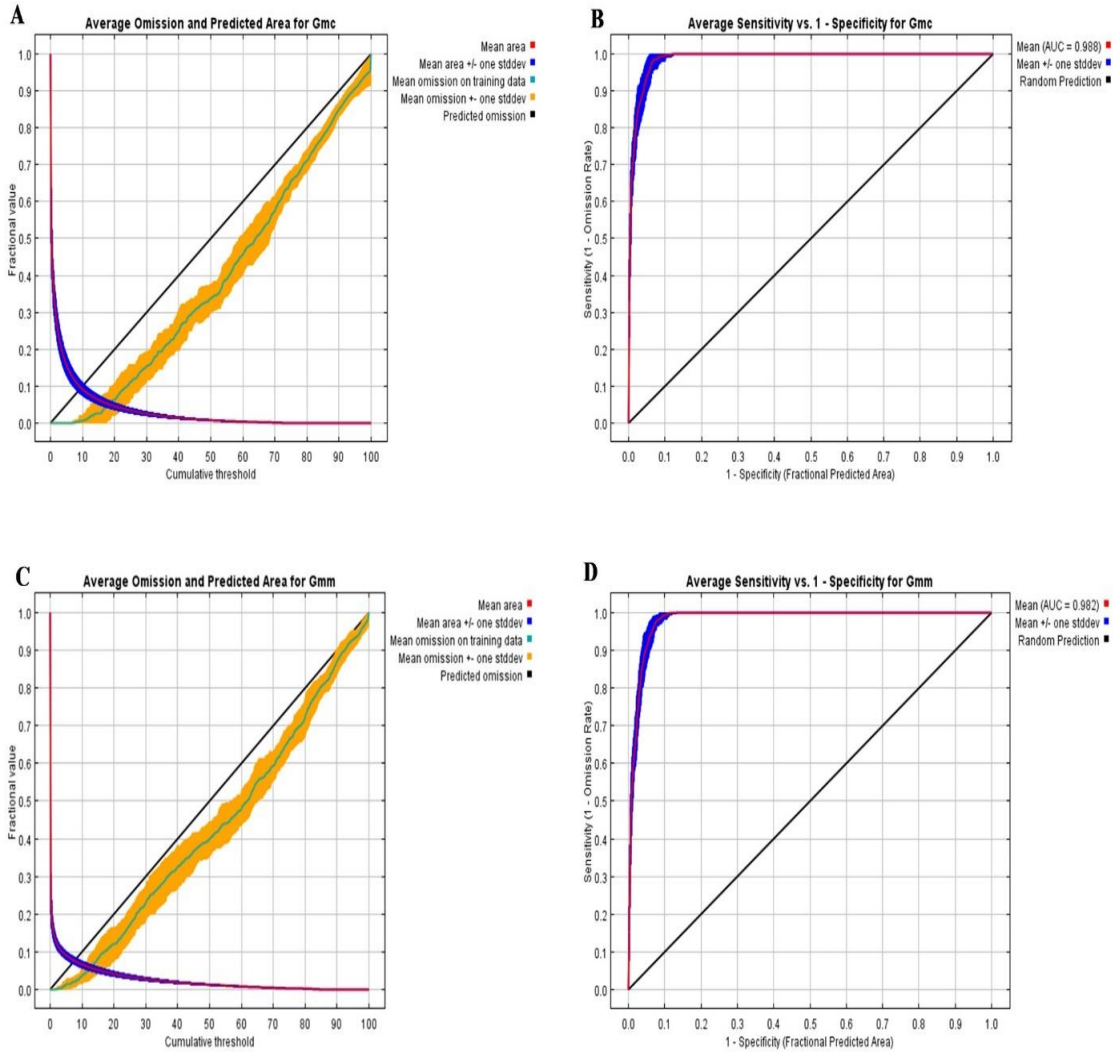

Fig 3.2. Validation charts of model performance. (A) *G. m. centralis* omission rate; (B) *G. m. centralis* operating characteristic curve; (C) *G. m. morsitans* omission rate; (D) *G. m. morsitans* operating characteristic curve

Table 3.5. Predictor variables for *G. m. morsitans* and their contribution rates

Abbreviation	Definition	Contribution Rate (%)
Bio3	Isothermality (%)	34.2
Bio6	Minimum Temperature of Coldest Month (ºC)	22.2
Bio12	Annual Precipitation (mm)	11.5
LSTD6	Land Surface Temperature (ºC) (Bi-Weekly Average)	11.2
Bio18	Precipitation of the Warmest Quarter	8.6
LC	Land Cover Type	8.0
H_popden	Human population density (number of persons per km^2)	3.9

Table 3.6. Predictor variables for *G. m. centralis* and their contribution rates

Abbreviation	Definition	Contribution Rate (%)
LC	Land Cover Type	31.1
H_popden	Human population density (number of persons per km^2)	16.0
Bio17	Precipitation of the Driest Quarter (mm)	13.1
Bio3	Isothermality (%)	11.0
Bio15	Precipitation Seasonality (%)	9.3
LSTD1	Land Surface Temperature (ºC) (Annual Average)	8.8
Bio8	Mean Temperature of the Wettest Quarter (ºC)	6.1
Vapr	Water Vapour Pressure (kPa)	4.6

Jackknife test for variable importance revealed that for *G. m. morsitans*, the environmental variable with the highest gain when used in isolation was isothermality (Fig 3.3A). This indicated that isothermality had the most useful modelling information when used by itself. Annual precipitation was observed to decrease model gain the most when omitted (Fig 3.3A). This showed that it had the most information that wasn't present in any of the other variables. For *G. m. centralis*, Jackknife test for variable importance revealed that land cover type had both the highest model gain when used in isolation and the highest decrease in gain when omitted (Fig 3.3B). Thus, it had the most useful information for modelling which was not present in any of the other variables.

Single-factor analysis was conducted on the three variables that contributed the most to Maxent model gain for both subspecies (Fig 3.4). According to Fig 3.4A and Table 3.2, *G. m. centralis* probabilities of occurrence were highest (100%) where more than 60% of the land cover was dominated by deciduous broadleaf trees having a canopy greater than two metres. Woody savannahs with tree cover ranging between 10 to 60% and a canopy of more than two metres were associated with moderate (60%) probabilities of occurrence for *G. m. centralis* (Fig 3.4A and Table 3.2). Increase in human population density from zero was associated with an abrupt decrease in *G. m. centralis* probabilities of occurrence (to less than 10%) (Fig 3.4B). A similar response was observed for *G. m. morsitans*. Low precipitation in the driest quarter (less than 3 mm) was observed to be associated with higher *G. m. centralis* probabilities of occurrence (Fig 3.4C). High probabilities of occurrence (100%) for *G.*

m. morsitans were observed to be associated with moderate isothermality values (50-52%) with values greater than 60% reducing occurrence probabilities to 0% (Fig 3.4D). *Glossina m. morsitans* had a unimodal association with minimum temperature of the coldest month and annual precipitation (Fig 3.4E and 3.4F). The highest probabilities of occurrence were observed at 11.9˚C and 850 mm for the minimum temperature of the coldest month (90%) and annual precipitation (92%), respectively. The highest probability of occurrence for *G. m. morsitans* was associated with woody savannahs with tree cover ranging between 10 to 60% having a canopy of more than two metres and being located near permanent water bodies.

Potential distribution

The predicted potentially suitable area for *G. m. centralis* and *G. m. morsitans* under current conditions are shown in Figs 3.5 and 3.6 respectively. About 80,863 km^2 was identified as suitable habitat for *G. m. centralis* within and around its historical distribution. A small area of *G. m. centralis* habitat was observed in *G. m. morsitans* historical range. The predicted distribution represented an estimated 71,970 km^2 (47%) reduction when compared to its historical distribution.

For *G. m. morsitans*, suitable conditions were predicted over 70,490 km^2 within and around its historical distribution (Fig 3.6). The predicted suitable area represented an estimated 29,081 km^2 (29%) reduction when compared to its historical distribution. The suitable area for *G. m. morsitans* was predicted as one tsetse belt with an isolated pocket in the southwest of the country.

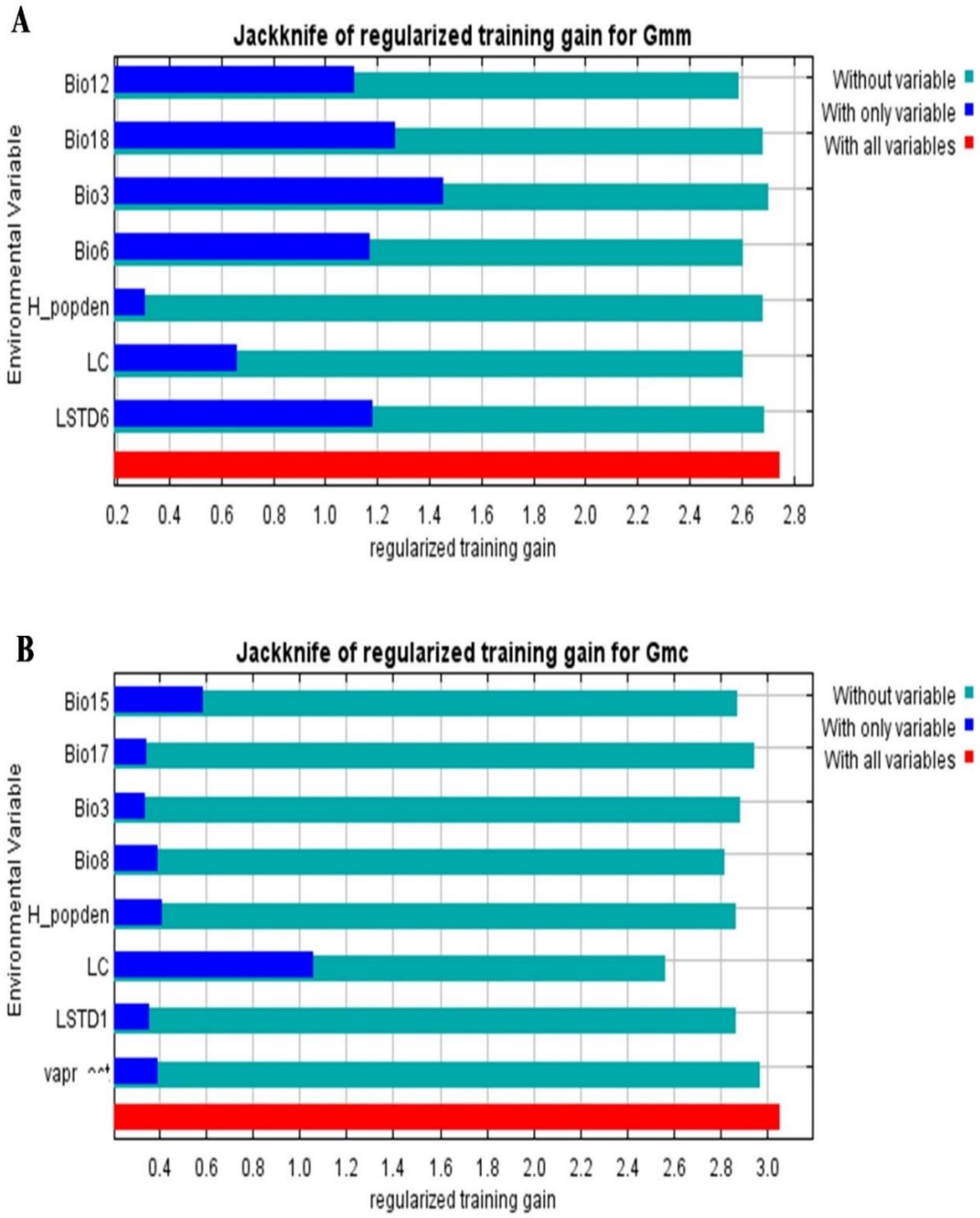

Fig 3.3. Jackknife tests for Maxent model variable importance. (A) *G. m. morsitans* model variable importance. (B) *G. m. centralis* model variable importance

3.5 Discussion

Our results reaffirm the conclusions of (Muyobela et al., 2021) that the VST is an effective tool for the rapid detection and sampling of *G. morsitans*. As demonstrated here, VST is capable of sampling large areas in a relatively short space of time with minimal labour compared to other sampling tools currently available. These attributes render VST an essential tool for the rapid generation of critical information on *G. morsitans* distribution required to facilitate the planning of more detailed surveys or control operations (Leak et al., 2008). Furthermore, our study showed that the tool could detect *G. pallidipes* and to a lesser extent *G. brevipalpis* despite being initially designed and optimised to sample *G. morsitans*. This result provides evidence that VST could be effective in sampling other savannah species of tsetse. We recommend studies to optimise VST design for sampling other economically important savannah tsetse.

Glossina f. martini was not captured during the survey. This may be attributed to the riverine nature of this tsetse such that its distribution is restricted to within a meter of the edges of water bodies (Evison and Kathuria, 1982). Consequently, the VST may not be an appropriate tool for sampling this tsetse fly as the banks of rivers and the shores of lake generally do not have motorable tracks to support mobile sampling.

An important limitation to the use of VST for tsetse surveys highlighted in this study was that species occurrence records obtained using this tool exhibit high positive spatial autocorrelation. Loss of independence among data causes parametric statistical testing procedures to give more significant results than what the data justifies, which is a serious problem for statistical and ecological interpretation (Dale and Fortin, 2002). We propose two approaches to ameliorate the effects of sampling bias of the VST based on the results of this study. Firstly, we recommend that sample transects should be set at a minimum distance of 5 km apart. This should nullify spatial autocorrelation between transects. Secondly, we recommend spatial thinning of occurrence records within a transect using the nearest neighbour distance as described by (Aiello-Lammens et al., 2015).

Random thinning at a 5 km radius was found to be sufficient to reduce spatial autocorrelation in our study, which is similar to the suggestion of (Zhou et al., 2021). The implication for VST survey design is that transects should be long enough (at least 40 km) to retain sufficient sample locations for further analysis.

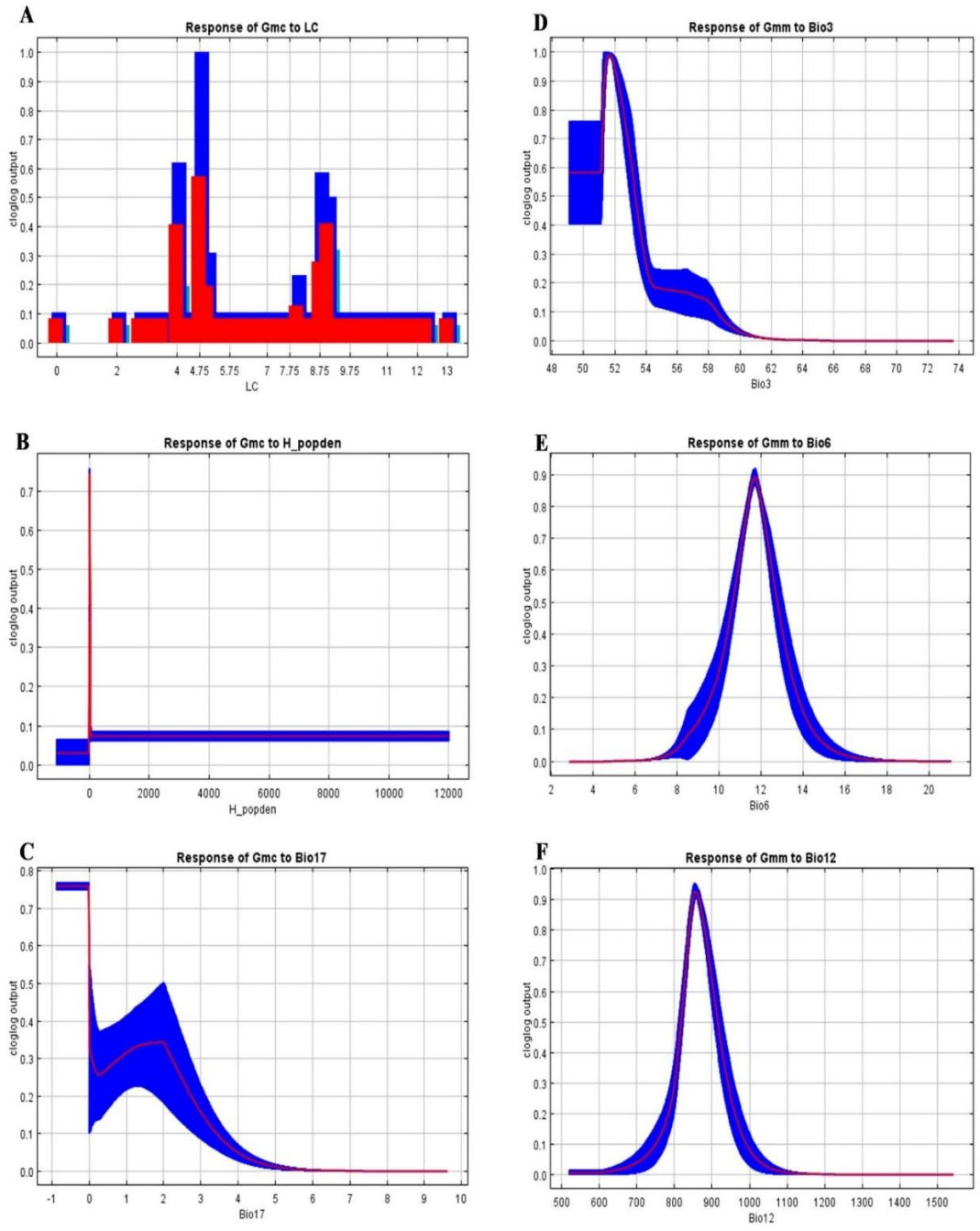

Fig 3.4. Response curves for dominant variables. (A) *G. m. centralis* response to land cover type (LC); (B) *G. m. centralis* response to precipitation seasonality (Bio15); (C) *G. m. centralis* response to precipitation of the driest quarter

(Bio17); (D) *G. m. morsitans* to isothermality (Bio3); (E) *G. m. morsitans* response to minimum temperature of the coldest month (Bio6); (F) *G. m. morsitans* response to annual precipitation (Bio12).

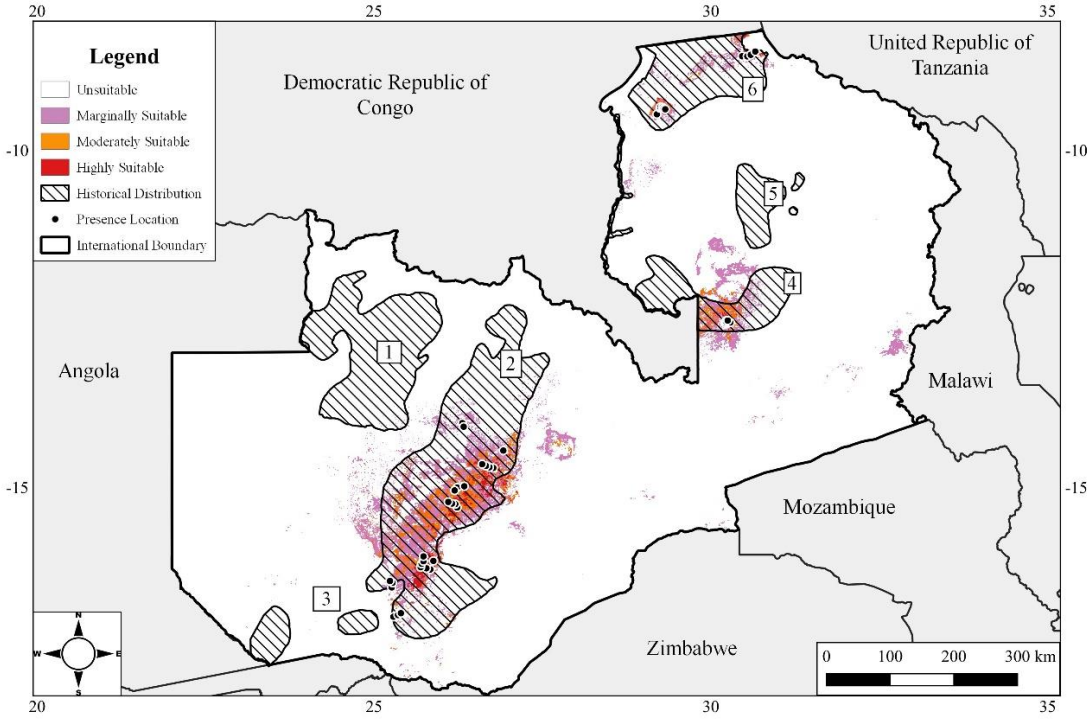

Fig 3.5. Historical distribution, presence locations, and potentially suitable area under current conditions of *G. m. centralis*. (Probability: unsuitable area 0–0.097; marginally suitable area 0.097–0.323; moderately suitable area 0.323–0.603; highly suitable area 0.603–1). The base map layer was obtained from the Database of Global Administrative Area GADM (https://geodata.ucdavis.edu/gadm/gadm4.1/shp/gadm41_ZMB_shp.zip) and under the license https://gadm.org/license.html. The figure was created using R.

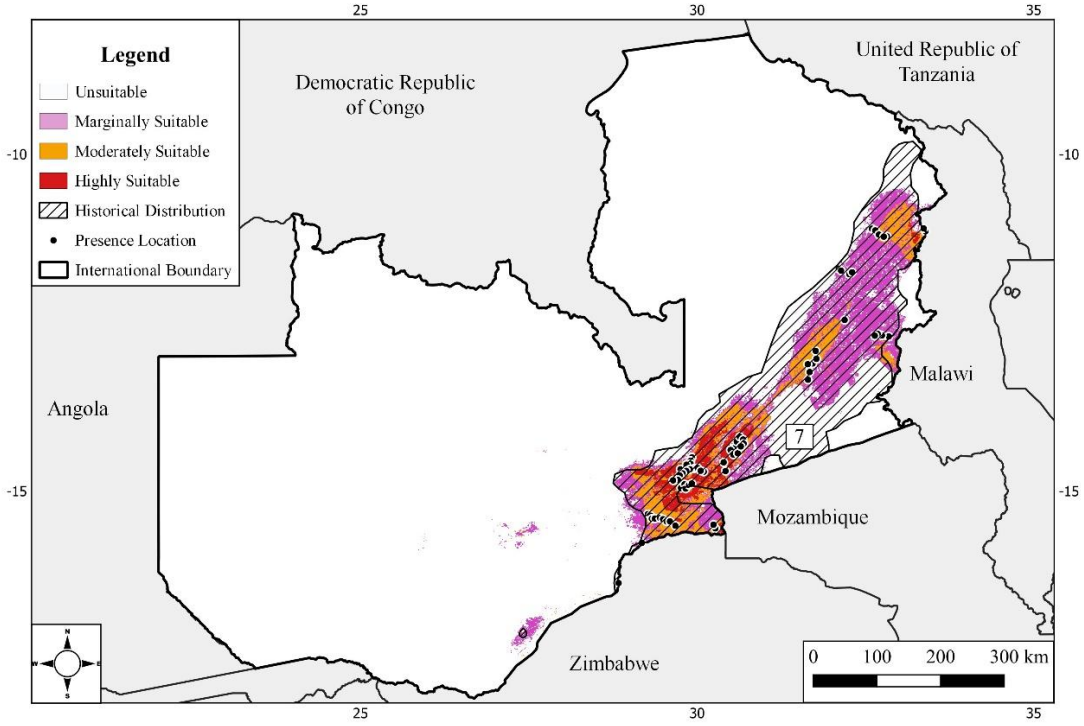

Fig 3.6. Historical distribution, presence locations, and potentially suitable area under current conditions of *G. m. morsitans*. (Probability: unsuitable area 0–0.097; marginally suitable area 0.097–0.323; moderately suitable area 0.323–0.603; highly suitable area 0.603–1). The base map layer was obtained from the Database of Global Administrative Area GADM (https://geodata.ucdavis.edu/gadm/gadm4.1/shp/gadm41_ZMB_shp.zip) and under the license https://gadm.org/license.html. The figure was created using R.

One of the primary objectives of conducting tsetse surveys is to determine the relative abundance or apparent density (AD) of species present in time and space (Kuzoes and Schofield, 2005). Tsetse apparent density is defined as the number of flies caught per unit effort and is calculated as the number of flies caught per trap per day for stationary traps or the number of flies per section of fly round section for fly round data (Leak et al., 2008). Clearly, the computation of AD depends on the sampling tool used. For the VST, our study interpreted catches in a 1 km sampling interval as a response from a 1× 1 km grid. The implication is that catches in the 1 km interval should be reported as the number of flies per km^2. To compute AD, we suggest accounting for the length of time the VST was operated in that km as it is part of the sampling effort. Thus, AD of the VST should be reported as the number of flies per km^2 per unit time.

Our results agree with the general notion that temperature (Zhou et al., 2021), precipitation (Nnko et al., 2021), and land cover type (Cecchi et al., 2008), are important factors in determining the distribution of *G. morsitans*. Isothermality was found a significant contributor to the models describing the habitats of both *G. m. morsitans* and *G. m. centralis*, similar to the findings of (Zhou et al., 2021). Isothermality quantifies how large the day-to-night temperatures oscillate relative to the summer-to-winter (annual) oscillations (O'Donnell and Ignizio, 2012). An isothermal value of 100 indicates that the diurnal temperature range is equivalent to the annual temperature range, while anything less than 100 indicates a smaller level of temperature variability within an average month relative to the year. Being poikilotherms, both low and high temperatures affect tsetse survival, negatively influencing rates of mortality and fat metabolism in adults and pupae, and rates of larviposition and pupal development (Hargrove and Vale, 2020; Lord et al., 2018). Our results suggest that *G. morsitans* prefers areas with stable temperatures similar to the findings of other researchers (Hargrove, 2001; Hargrove and Vale, 2020; Lord et al., 2018). Stable temperatures (25 – 27 ºC) provide optimal conditions for pupal fat accumulation and development. Pupal fat reserves are exhausted before they complete development when exposed to a constant temperature below 16 ºC,

while direct effects kill pupae before fat stores are exhausted at high temperatures (above 32 ºC) (Hargrove and Vale, 2020).

There is generally no record in the literature on the direct effects of rainfall on tsetse (Nnko et al., 2021). However, precipitation is thought to have two major indirect effects on tsetse survival; firstly, it maintains vegetation (tsetse habitat), and secondly, it causes local flooding which may drown pupae that are buried in loose soil causing depopulation (Challier, 1982). Moderate annual precipitation was associated with high *G. m. morsitans* probabilities of occurrence as this level of precipitation favours the growth of Mopane woodland (Makhado et al., 2014), the dominant vegetation type within this subspecies' range. Deciduous Miombo woodland, which losses foliage in the hot dry season, is the dominant vegetation type in *G. m. centralis* range (Frost, 1996). Low *G. m. centralis* probabilities of occurrence associated with an increase in precipitation of the driest quarter could be attributed to a reduction in leaf cover that serves to protect larviposition sites from direct rainfall and causes pupal mortality by drowning.

The correlation between land cover and the geographic distribution of tsetse is well established (Cecchi et al., 2008; DeVisser and Messina, 2009; Ford and Katondo, 1977). Land cover characteristics influence all major aspects of tsetse ecology such as the provision of suitable microclimatic conditions, the presence of suitable resting and larviposition sites, and the availability of wild and domestic hosts (Cecchi et al., 2008). For *G. m. morsitans*, high probabilities of occurrence were associated with riverine woody savannah that is known to be the dry season home of this subspecies (FAO, 1982a). Riverine woodland maintains suitable humidity conditions that prevent adult desiccation at high temperatures. High *G. m. centralis* probabilities of occurrence were associated with deciduous Miombo woodland which buffers microclimates within its range by maintaining the mean temperature of the coldest month at 16.9 ºC and mean temperature of the hottest month at 23.3 ºC (Frost, 1996). These temperatures are conducive to both pupal development and adult survival (Hargrove and Vale, 2020).

Our results further indicated that habitat suitability of *G. m. morsitans* and *G. m centralis* are determined by different environmental variables in agreement with the finding of (Robinson et al., 1997) and (Rogers and Robinson, 2004). This suggests that these allopatric subspecies have adapted to different environmental conditions that occur within their specific geographic ranges. Whether this adaptation is due to phenotypic plasticity (that ability of a genotype to produce different phenotypes when exposed to different environmental conditions (Pigliucci et al., 2006) or has a genetic basis is yet unclear (Patten and Remsen, 2017). The implication for ecological niche modelling of *G. morsitans* is that its subspecies should be modelled separately whether predicting current distributions or projecting in space and time.

The predicted potential distribution of *G. m. centralis* and *G. m. morsitans* under current environmental conditions was observed to have reduced by 47 and 29%, respectively. The reduction could be attributed to an increase in temperature and land cover change in areas that were previously deemed suitable. (Lord et al., 2018) showed that temperature increases of around 2 ºC between 1995 and 2017 could explain an estimated 90% reduction in tsetse abundance in the Zambezi Valley of Zimbabwe. Temperature-related climate change was also implicated in the spatial changes in tsetse distributions within this area (Longbottom et al., 2020). Similar temperature changes may be responsible for the observed reduction in *G. morsitans* distribution in this study as temperatures throughout the African continent have risen by 1.5 ºC since 1900 (Watson et al., 1997). Land cover changes due to the gradual clearing of natural vegetation for cultivation, the introduction of domestic animals, and the expansion of human settlements have led to a substantial reduction in and fragmentation of natural tsetse habitat (Van den Bossche, 2001). In Zambia, increased tsetse habitat fragmentation has been associated with a reduction in *G. morsitans* apparent density (Ducheyne et al., 2009). Habitat fragmentation reduces the ability of vegetation to buffer microclimates from climatic variation outside the tsetse habitat (Ewers and Banks-Leite, 2013) and results in climatic conditions that reduce the abundance of flies. Deforestation, on the other hand, results in the loss of

suitable resting and larviposition sites (FAO, 1982b) and causes changes in climatic variables important for tsetse development (Ewers and Banks-Leite, 2013). Similar reductions in the spatial distribution of tsetse have been reported in West Africa where there has been a general shift of the northern limit towards the south of the isohyets (Courtin et al., 2008). Furthermore, Courtin et al. (2010) reported that the northern limit of tsetse had shifted south wards by 25- to 150-km in Burkina Faso.

We conclude that the spatial distribution of *G. morsitans* in Zambia has reduced by an estimated 101,051 km^2. Tsetse densities are expected to be high where natural vegetation and wildlife are protected from anthropogenic influence, and suitable climatic conditions exist. Such areas are primarily located within and around national parks and game management areas. Biting intensity is therefore expected to be highest in these areas with increased risk of HAT transmission to tourists, wildlife scouts, and other individuals who utilize these areas for their economic survival. Further, AAT transmission is likely to be highest in cattle populations that graze at the interphase between open and protected lands. We, therefore, recommend that vector management strategies should be adjusted to account for the observed change in *G morsitans* distribution. We further conclude that VST is effective for sampling *G. morsitans* outside experimental settings and recommend its' incorporation as an additional tsetse survey tool.

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Chapter 4: Phenotypic divergence of *Glossina morsitans* (Diptera: Glossinidae) populations in Zambia: Application of landmark-based wing geometric morphometrics to discriminate population-level variation.

4.1 Overview

This chapter addresses phenotypic variation and population structure of the flies, addressing the second specific objective and research question. The same tool earlier developed and reported in Chapter two was deployed to study the phenotypic divergence of tsetse populations in Zambia and is already published in the following publication as: Muyobela J, Pirk CWW, Yusuf AA, Sole CL (2024) Phenotypic divergence of *Glossina morsitans* (Diptera: Glossinidae) populations in Zambia: Application of landmark-based wing geometric morphometrics to discriminate population-level variation. Ecol Evol. 2024;(September):1–16.

4.2 Introduction

Glossina morsitans (Diptera: Glossinidae) is a savannah tsetse species of the subgenus *Glossina* (*morsitans* group) whose distribution is restricted to savannah woodlands (Leak et al., 2008) and is correlated with that of wildlife (Vreysen et al., 2013). Three allopatric subspecies occur, namely, *G. m. submorsitans* Newstead, *G. m. centralis* Machado, and *G. m. morsitans* Westwood (Jordan, 1993), all of which are efficient vectors of trypanosomes (Kinetoplastida: Trypanosomatidae), which cause human and animal trypanosomiasis in sub-Saharan Africa (Rogers, 2000). The geographical distribution of *G. m. submorsitans* is from Western to Central Africa, while *G. m. centralis* and *G. m. morsitans* occur in Eastern, Central, and Southern Africa (Rogers and Robinson, 2004). In Zambia, *G. m. centralis* and *G. m. morsitans* are predicted to occupy 151,353 km^2 or 20% of the land mass (Muyobela et al., 2023).

In conformity with most insect species, the distribution of *G. morsitans* within its geographic range is generally discontinuous (Krafsur, 2009; Muyobela et al., 2023), being strategically arranged based on the availability of food sources, reproductive needs, dispersal capacity, and local environmental

conditions (Dujardin, 2008). The spatial arrangement of a species based on environmental heterogeneity can lead to divergent selection whereby local population demes evolve traits that provide an advantage under local environmental conditions regardless of the consequences for fitness in other habitats (Williams, 1966). In the presence of restricted gene flow (due to passive dispersal or active habitat selection), strong selection against genotypes adapted to other habitats, moderate selection against intermediate genotypes, little temporal variation in forces of selection, and small differences in habitat size and quality (e.g. resource availability), such population demes become locally adapted (Kawecki and Ebert, 2004). Local adaptation can give rise to population-level phenotypic variation that may result in the structuring of populations into biogeographical islands or subpopulations (Dujardin and Le Pont, 2004; Getahun et al., 2014; Mbewe et al., 2018). Where significant barriers to gene flow exist, these subpopulations become isolated and undergo rapid evolutionary changes in morphological traits due to founder effects and genetic drift (Ostwald et al., 2023). The identification of isolated tsetse populations has been deemed crucial for the successful and sustainable implementation of area-wide integrated vector management (AW-IVM) (Bouyer et al., 2010; Kgori et al., 2006), guiding the decision whether to undertake suppression or elimination campaigns (Bouyer et al., 2007).

A relatively low-cost approach for investigating tsetse population structure is the use of landmark-based geometric morphometrics (GM), defined as the statistical analysis of shape variation and its covariation with other variables (Rohlf and Bookstein, 2003). Unlike traditional morphometrics, GM is a powerful technique that captures the geometry of the morphological structure under study and retains this information throughout the analysis (Zelditch et al., 2004). The procedure is accomplished through the Procrustes paradigm (Adams et al., 2013) in which a set of two-dimensional landmark coordinates recording the relative positions of homologous anatomical points are obtained and then subjected to generalised Procrustes analysis (GPA) (Rohlf and Slice, 1990). This least-squares superimposition technique produces a set of shape variables whose geometric dissimilarity is

expressed as the Procrustes distance between the homologous points of two configurations (Zelditch et al., 2004) and whose pattern of variation can be visualised by graphical methods (Baken et al., 2021). An additional output of this analysis is centroid size (CS), defined as the square root of the summed squared distance of each landmark from the centroid of the form (Tatsuta et al., 2018). This isometric measure of size is used as an estimator of the global size of the form under study in GM studies (Dujardin, 2008).

Conspecific size variability within and among insect populations is generally known to be an environmentally induced and reversible character (Jirakanjanakit et al., 2007). In *G. morsitans*, size variability has been attributed to seasonal effects (Hargrove et al., 2019) with temperature being the major source of variation (Glasgow, 1961; Phelps and Clarke, 1974). High heritability values for insect size have however been reported (Lehmann et al., 2006) and the transgenerational effects of size among the *Glossina spp* have been demonstrated (Mbewe et al., 2018). Therefore, heritable size variation can be used to discriminate populations. Size-corrected or allometry-free shape is known to be a polygenic character and strong evidence of its genetic determinism has been provided (Klingenberg and Leamy, 2001; Patterson and Klingenberg, 2007). Allometry-free shape has also been shown to be a powerful discriminator of groups (Dujardin, 2008) and is, therefore, a very useful tool in taxonomic studies (Klingenberg, 2016).

The insect body part most subjected to GM studies is the wing (Tatsuta et al., 2018). This is due to several reasons. Firstly, insect wings are almost entirely two-dimensional structures, a fact that greatly reduces digitisation errors (Dujardin, 2008). Secondly, the arrangement and branching patterns of insect wing veins contain taxonomic information that has been used to construct classification schemes, infer phylogeny (Bybee et al., 2008), elucidate evolutionary patterns (Debat et al., 2003), and evaluate fluctuating asymmetry – deviations from perfect symmetry that indicate developmental noise (Klingenberg et al., 2001). Lastly, the geometric shape of insect wings has been shown to exhibit high environmental canalisation – the ability of a genotype's phenotype to remain relatively invariant

when exposed to different environments (Henry et al., 2010). These attributes, therefore, make the geometric shape of insect wings, a suitable phenotypic character to distinguish conspecific populations and species using GM (Dujardin, 2011). Insect wing shape is captured by placing homologous landmarks on the intersection of wing veins.

Geometric morphometrics has been used to study natural population variation in several insect species including the common fruit fly *Drosophila* (Diptera: Drosophilidae) (Gilchrist et al., 2000), honey bee *Apis* (Hymenoptera: Apidae) (Radloff and Hepburn, 2000), sand fly *Lutzomyia* (Diptera: Psychididae) (Dujardin and Le Pont, 2004), triatomine bug *Rhodnius* (Hemiptera: Reduviidae) (Villegas et al., 2002) and culicid mosquitoes *Culex*, *Aedes* and *Anopheles* (Diptera: Culicidae) (Virginio et al., 2015). Among the *Glossina* geometric morphometrics has been used to study phenetic variation in *G. palpalis gambiensis* (Bouyer et al., 2007; Solano et al., 1999), *G. p. palpalis* (Ebhodaghe et al., 2017; Kaba et al., 2012), *G. m. submorsitans* (Achukwi et al., 2013), *G. pallidipes* (Getahun et al., 2014), *G. austeni* (De Beer et al., 2019), *G. fuscipes fuscipes* (Mbewe et al., 2018), *G. tachinoides* (Mustapha et al., 2018) and *G. brevipalpis* (De Beer et al., 2019). However, phenotypic variation in natural populations of *G. m. centralis* and *G. m. morsitans* has not been investigated. Therefore, this study aimed to use landmark-based wing geometric morphometrics to investigate phenotypic variation and determine the level of population structuring in *G. m. centralis* and *G. m. morsitans* populations in Zambia.

4.3 Materials and methods

Study sites

The study was carried out in Zambia, between the longitudes 22 and 34 °E, and latitudes 8 and 18 °S. The two *G. morsitans* subspecies exhibit an allopatric distribution with *G. m. morsitans* occupying the hotter Eastern part and the other subspecies, *G. m. centralis*, occupying the cooler Western and Northern part of the country (Fig 4.1). The habitat of *G. m. centralis* is characterised by Miombo woodland interspaced with large dambos (grassy wetlands) with high annual rainfall (above 1000

mm) (Wigg, 1949). Mopane woodland is the dominant vegetation in the *G. m. morsitans* range with moderate to low annual rainfall (less than 800 mm). *Glossina m. centralis* was collected from four sites, namely Mumbwa South (KNP1) and Kasongo Busanga (KNP2) game management areas, and Kasanka (KSP) and Sumbu (SNP) national parks (Fig 4.1), while *G. m. morsitans* was captured in five sites: Mulangu (CMR and VNP) and Luano (LVA) game management areas, and South Luangwa (SLP) and Lower Zambezi (LZP) national parks (Fig 4.1).

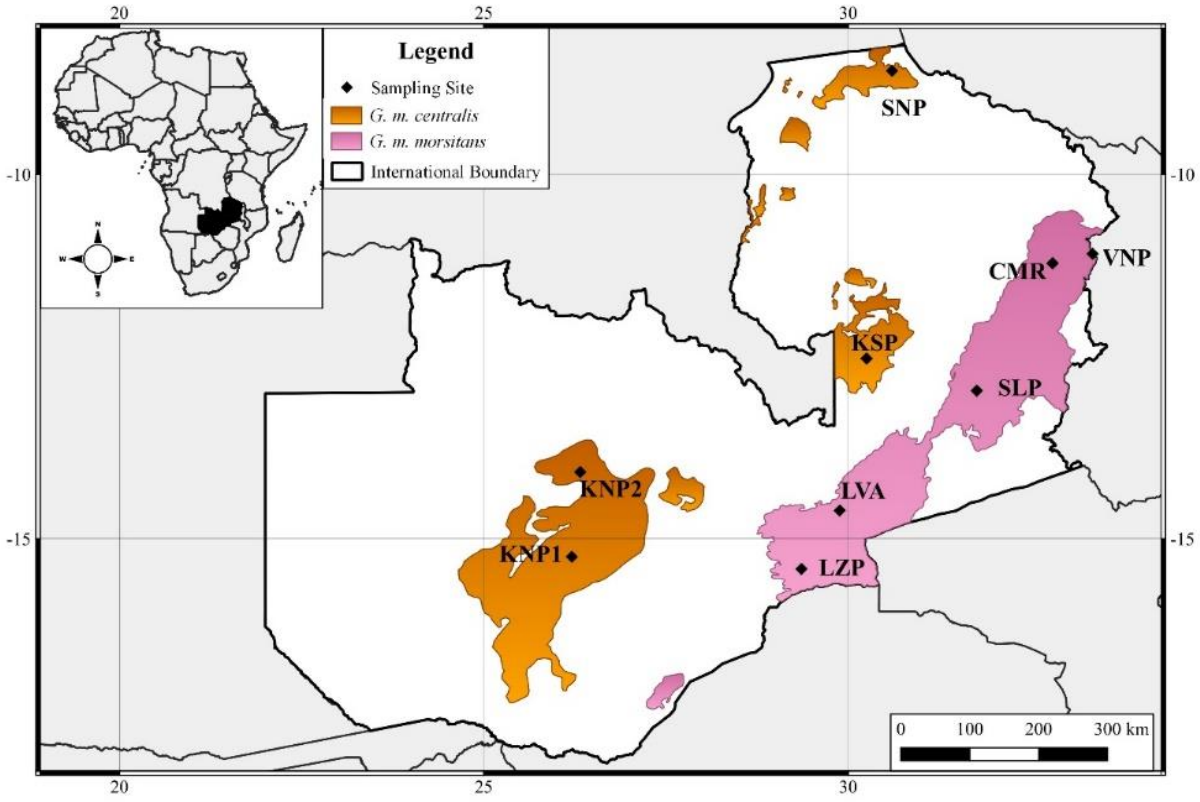

Fig 4.1. Distribution of *G. m. centralis* and *G. m. morsitans* in Zambia. Data on each subspecies in Muyobela et al. (2023). The base map layer was obtained from the Database of Global Administrative Area GADM (https://geodata.ucdavis.edu/gadm/gadm4.1/shp/gadm41_ZMB_shp.zip)
and under the license https://gadm.org/license.html. The figure was created using QGISv3.0 (http://qgis.org/en/site/).

Tsetse samples

The data used in this study form a subset of results of a cross-sectional tsetse survey conducted between September 2021 and August 2022 (Muyobela et al., 2023). The subset consists of flies captured in November 2021, chosen because this was the only month that recorded catches in all sample sites. The sampling was done using the vehicle-mounted sticky trap (VST) (Muyobela et al., 2021) baited with butanone and 1-octen-3-ol dispensed at a rate of 150 and 0.5 mg/hr respectively (Torr et al., 1997). Tsetse captured within a two-kilometre radius of a sample site were amalgamated from which 80 non-teneral (40 males and 40 females) flies with intact wings were selected. Subspecies identity was confirmed by dissecting male genitalia (hypopygium) as described by Leak et al. (2008). *Glossina m. morsitans* subspecies was identified by the presence of narrow median lobes on superior claspers of the hypopygium that had slightly divergent tips. The median lobes of *G. m. centralis* were relatively wider, with tips markedly divergent. A total of 720 (360 *G. m. centralis* and *G. m. morsitans*) were used in the study.

Wing measurements and Procrustes superimposition

The right wing of each fly was mounted on a glass slide and affixed with transparent sticky tape. The wings were then photographed using a Leica M165C stereomicroscope attached to a Leica camera (DMC-2900) (Leica Microsystems, Germany). The images were compiled using tpsUtil v1.79 (Rohlf, 2015) and digitised with tpsDig2 v2.32 (Rohlf, 2015). Twelve homologous landmarks defined as junctions of wing veins were identified and digitised (Fig 4.2A). To avoid individual bias, landmark digitisation was undertaken by the same person. To avoid operational bias during digitization, specimens were selected at random.

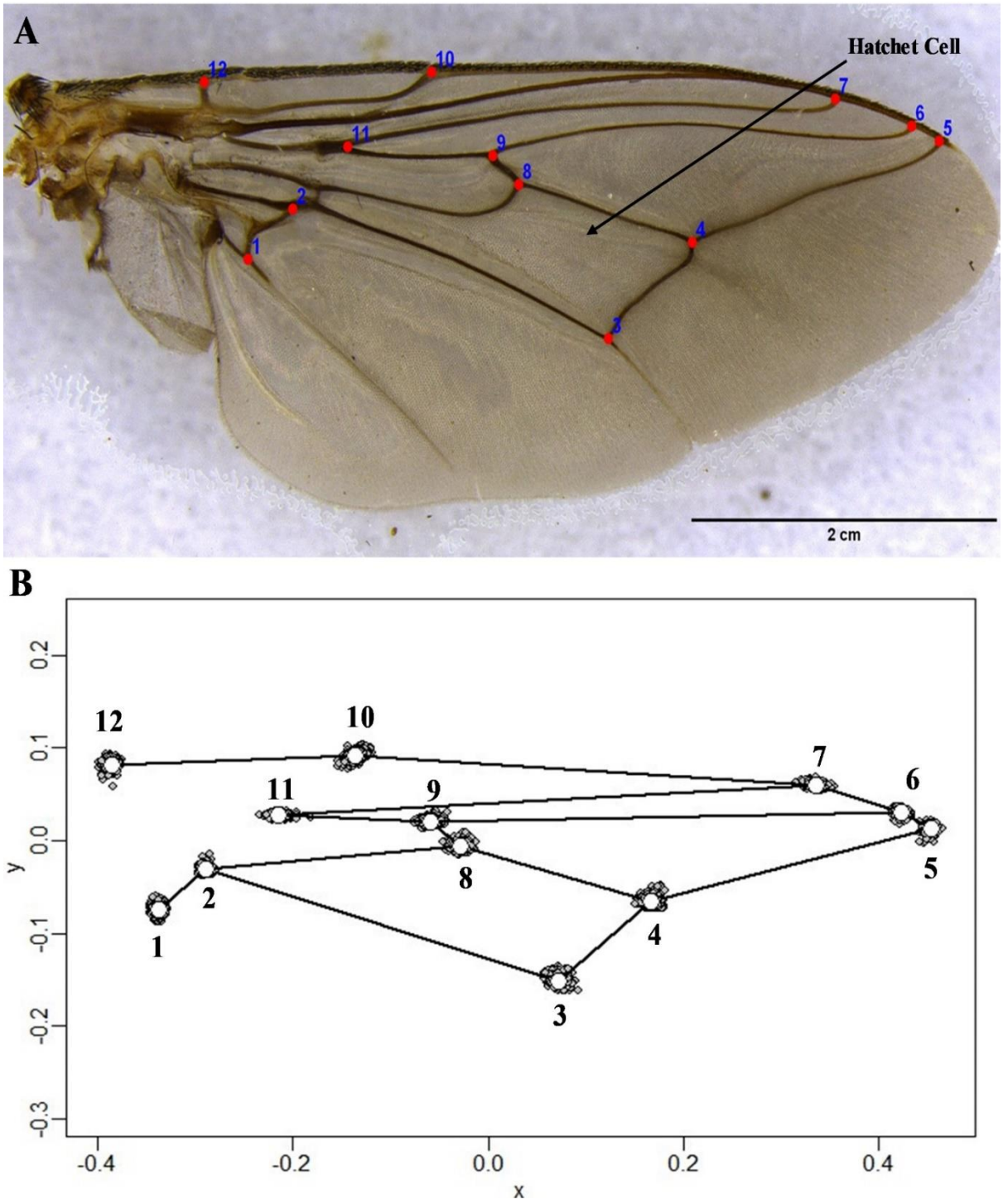

Fig 4.2. Landmark digitisation and general Procrustes analysis. (A) Image of the 12 landmarks and the order of landmark collection from the right wing of *G. morsitans*. (B) Scatter plot with wireframe links of landmark configurations of all 720 wings in the dataset after Procrustes superimposition. For each landmark, the white circle indicates the location of the landmark for the average shape and the grey dots indicate the locations for individual wings.

Procrustes superimposition of landmark configurations was performed using general Procrustes analysis (GPA) using Geomorph version 4.0 package (Baken et al., 2021) in R (R Core Development Team, 2015). The procedure translated all landmark configurations to a common location, scaled them to unit centroid size, and rotated them into an optimal least-squares alignment with an iteratively estimated mean reference form (Zelditch et al., 2004) so that the sum of squared distances between corresponding landmarks of each configuration and the mean configuration was minimized (Klingenberg, 2013). This analysis produced the Procrustes distances which measure shape dissimilarity as well as the centroid size (CS). A scatter plot of superimposed landmarks for all specimens is shown in Fig 4.2B.

Digitisation errors were identified by plotting the ordered Procrustes distance of aligned specimens from the mean shape (Sherratt, 2016) (Fig 4.3) using the Geomorph package in R. Specimens that have been digitised wrongly (for example, mixing up the order of landmarks) exhibit large variances and therefore fall outside the upper quartile range of the plot. As shown in Fig 4.3, the specimen Gmc_m_SU1_10_23 was observed to be furthest from the upper quartile range of the plot and was therefore identified as an outlier. This specimen was therefore omitted from further analysis.

The ability to reliably locate and digitise landmarks was determined by assessing the variance contribution of each landmark to the mean shape since landmark locations are not independent quantities but are relative to all other landmarks (Zelditch et al., 2004). This was done by sequentially computing the variation in landmark position around the mean shape, omitting one landmark each time the computation was made (Sheets, 2014). Omitting a landmark that is difficult to reliably digitise results in a decrease in variance around the mean, relative to the variation seen when other landmarks are omitted. This jackknife computation of variance was done in CoordGen8 (Sheets, 2014). As shown in Table 4.1, landmark 10 was found to be the most difficult to reliably locate and digitise. However, a histogram plot of variance density (Fig 4.4) showed that the variance of landmark

10 was part of a smooth distribution of variance around landmarks. Landmark 10 was therefore included in the study.

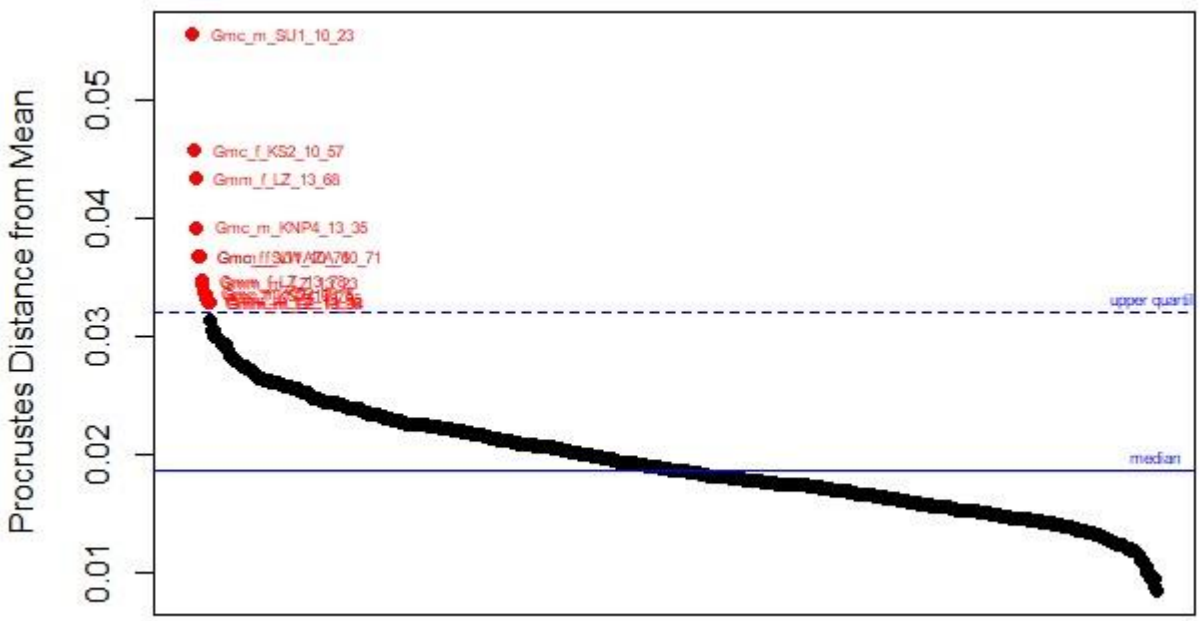

Fig 4.3. Procrustes distance of each specimen from the mean shape. The plot shows that specimen Gmc_m_SU1_10_23 had the largest distance from the mean shape and was therefore considered to be an outlier

Table 1. The variance around the mean shape as each landmark is omitted in turn. Low variance when a landmark is excluded indicates that the landmark contributes greatly to the total variance.

LM Omitted	Variance
10	0.000368
3	0.000389
11	0.000391
8	0.000394
9	0.000396
4	0.000404
1	0.00045
7	0.000452
2	0.000476
12	0.000476
6	0.000511
5	0.000524

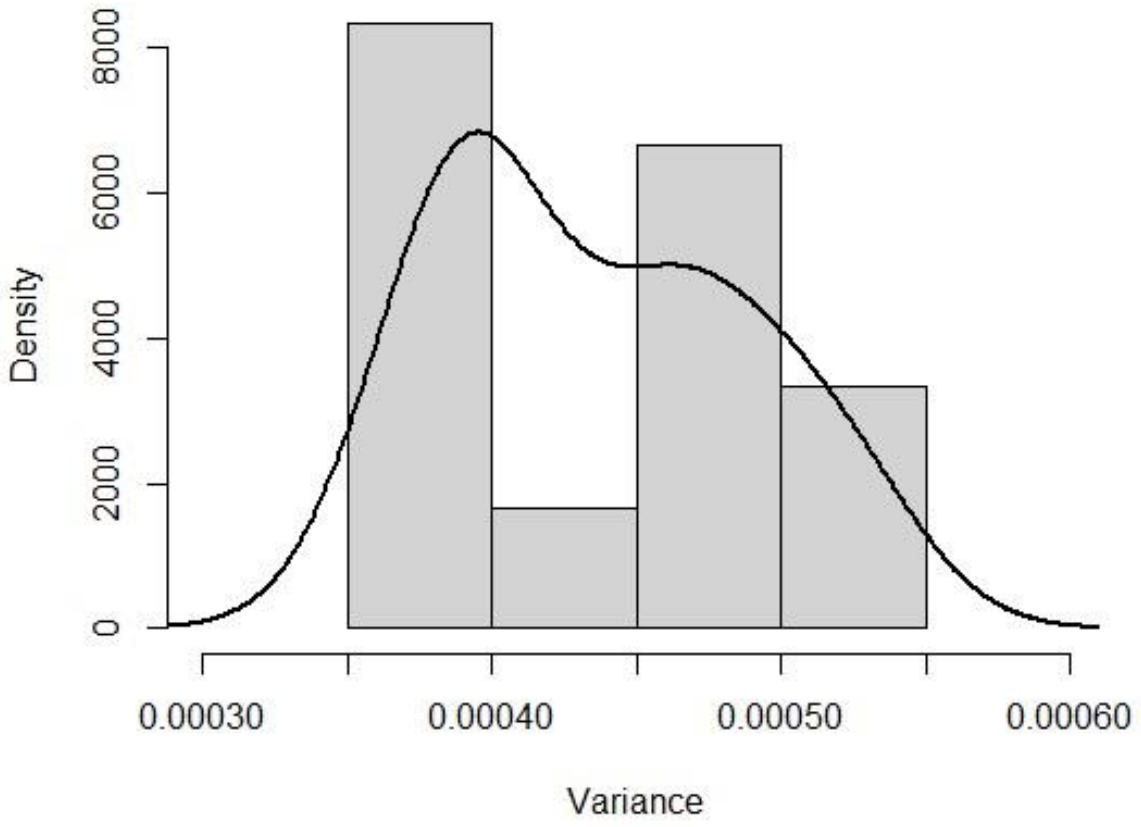

Fig 4. Distribution of landmark variance. The histogram indicates that all landmarks are part of the same distribution, and no outlier is present in the dataset.

Environmental data and processing

Elevation, annual temperature, isothermality, annual precipitation, land surface temperature, and vegetation cover are among the most important variables affecting the biology of *Glossina spp* (Challier, 1982; Muyobela et al., 2023; Nnko et al., 2021). Therefore, these variables were selected to assess the spatial environmental heterogeneity of sample sites and to estimate their effect on phenotypic variation. Annual temperature, isothermality, and annual precipitation data were obtained from WorldClim Global Climate Database version 2.1 (Fick and Hijmans, 2017). Moderate Resolution Imaging Spectroradiometer (MODIS) composite time series land surface temperature day (LST) (MOD11A1) (Didan, 2015) and percent tree cover based on the Vegetation Continuous Fields

(VCF) (MOD44B) (DiMiceli et al., 2015) were obtained from NASA's EOSDIS Land Processes Distributed Active Archive Center (AppEEARS Team, 2022). Elevation data was obtained as Global 30 Arc-Second Elevation (GTOPO30) from the Earth Resources Observation and Science Center (Earth Resources Observation and Science Center/U.S. Geological Survey/U.S. Department of the Interior, 1997).

Harmonic regression was performed on monthly time series LST data using the TSA package (Kung-Sik and Ripley, 2020) in R (R Core Development Team, 2015). The first coefficient in the regression, representing the mean of the variable, was selected for further analysis. Data values for all environmental variables at each sampling site were extracted using the Raster package (Hijmans and van Etten, 2012) in R.

Data analyses

Spatial autocorrelation analysis. Spatial autocorrelation is the positive or negative correlation of a variable with itself due to the spatial location of observations (Salima and de Bellefon, 2018). Residues of statistical models based on spatially autocorrelated data violate the key assumption of standard statistical tests, that residues are independent and identically distributed (Dormann et al., 2007). Violation of this assumption may bias parameter estimates and increase Type I error rates (falsely rejecting the null hypothesis of no effect). To ensure statistical independence of CS and shape variables, Global Spatial Autocorrelation Tests were conducted. For CS, a permutation Moran's I test was used to assess the strength of spatial autocorrelation using the spdep package (Bivand and Wong, 2018) in R for both *G. m. centralis* and *G. m. morsitans*. Mantel (Mantel, 1967) and Partial Mantel (Guillot and Rousset, 2013) tests were used to evaluate spatial autocorrelation of shape and environmental variables for both *G. m. centralis* and *G. m. morsitans* using the EcoGenetics package (Roser et al., 2017) in R.

Environmental characterisation of sample sites. Linear permutation models with 2000 iterations were used to test for the differences in elevation, annual temperature, isothermality, annual

precipitation, land surface temperature, and percent tree cover between *G. m. centralis* and *G. m. morsitans* sample sites using the Geomorph package in R. Principal components analysis (PCA) was used for the multivariate analysis of these environmental variables to identify the most important variables accounting for environmental variability between sample sites.

Centroid size analysis. Shapiro-Wilk normality test showed that both CS and log CS were not normally distributed ($P = 0.001$ for both variables). Therefore, permutation procedures were used to analyse CS. Linear permutation models with 2000 iterations were used to compare wing CS differences between *G. morsitans* males and females, *G. m. centralis,* and *G. m. morsitans* subspecies, males and females of each subspecies, as well as CS differences between sample locations within each subspecies range using the Geomorph package in R. The pairwise function was used for multiple group comparisons where CS was observed to be different between sample locations. Linear permutation models were further used to estimate the effect of elevation, annual temperature, isothermality, annual precipitation, land surface temperature, and percent tree cover on wing CS.

Allometric test and construction of allometry-free shape variables. To test whether there was significant covariance between wing shape and size (allometry), multivariate linear permutation regression of wing shape on CS was conducted using the Geomorph package in R. Hypothesis testing was accomplished using Goodall's F-test (Goodall, 1991), a statistical approach that partitions the variance of Procrustes distances rather than landmark coordinates. Goodall's F-statistic is the ratio of explained (between-group) and unexplained (within-group) components of shape variation (Klingenberg, 2016) and has been demonstrated to have higher statistical power than other approaches (Rohlf, 2000). Residues from this regression were then used to construct allometry-free shape variables that are recommended in taxonomic investigations (Klingenberg, 2009) and studies that define geographically constrained situations such as islands (Dujardin, 2011). The multivariate regression approach to remove the allometric component of shape variation offers a logical method as it partitions the variation in the dependent variables into predicted and residual components

(Klingenberg, 2016). The predicted component corresponds to allometric variation of shape, whereas the residual component encompasses non-allometric variation as residues are uncorrelated with CS.

Shape analysis. Redundancy analysis (RDA) (Zuur et al., 2007) was used to model allometry-free wing shape as a function of *G. morsitans* sex, subspecies, and geographic origin, using the vegan package (Oksanen et al., 2018) in R. The analysis consisted of the following steps. A multivariate linear permutation regression model was fitted to determine if *G. morsitans* allometry-free wing shape variation was significantly influenced by sex differences, subspecies identity, and the two-way interaction of these factors. The effect of geographic origin on allometry-free shape variation in both *G. m. centralis* and *G. m. morsitans* was evaluated using multivariate linear permutation regression models, accounting for sex differences and the two-way interaction between sex and geographic origin. Two PCAs were then performed on each regression model. A constrained PCA was applied to the fitted values of each regression model to summarise the variation in allometry-free wing shape data that could be explained by the explanatory variables. An unconstrained PCA was then applied to the residues of the regression to estimate the variation not explained by these constraining variables. The total percentage of allometry-free wing shape variation explained by sex and subspecies identity, and geographic origin within each subspecies range was estimated by the canonical R^2 bi-multivariate redundancy statistic (Miller and Farr, 1971) calculated as proposed by Peres-Neto et al. (2006) using the RVAideMemoire package (Hervé, 2023) in R. To test whether each variable explained a significant proportion of allometry-free wing shape variation, a permutation F-test based on the canonical R^2 (Legendre and Legendre, 2012) was used. Where differences between sample geographic origin were observed, multiple group comparisons were done using the RVAideMemoire package in R. Constrained PCA score plots were used to illustrate allometry-free wing shape cluster separation due to sex, subspecies, and sample geographic origin within each subspecies range. To estimate the amount of shape variation that could be attributed to environmental variability, allometry-free shape was regressed on elevation, annual temperature, isothermality, annual

precipitation, land surface temperature and percent tree cover with Goodall's F-test used for hypothesis testing. A Procrustes distance matrix, computed from the fitted values of a multivariate linear permutation regression of *G. morsitans* allometry-free shape variables on sex, subspecies and location, was used to build a neighbor-joining cladogram to illustrate divergence of wing shape of flies from different locations.

Isolation-by-distance test. Isolation-by-distance (IBD) hypothesis describes the pattern of population genetic variation that derives from spatially limited gene flow (Jensen et al., 2005) and is characterised by an increase in genetic or phenotypic differentiation among populations with increasing geographic distance (Van Strien et al., 2015). For IBD to occur, populations are assumed to be in gene-flow-drift equilibrium, experience no selection, and have dispersal rates that reduce with increasing geographic distance (Orsini et al., 2013). We evaluated whether allometry-free wing shape variation among sample locations was due to isolation-by-distance using the following procedures. Firstly, scatter plots were generated to visually assess the expected linear relationship between Procrustes and geographic distances under IBD for both *G. m. centralis* and *G. m. morsitans* populations. Secondly, Mantel-based correlogram analysis (Roser et al., 2017) was used to statistically test the hypothesis of IBD in both subspecies ranges using the EcoGenetics package in R.

Alpha was set at 0.05 for all statistically significant analyses (Pirk et al., 2013)

4.4 Results

Spatial autocorrelation

Centroid size data for both *G. m. centralis* (Moran's I Statistic = 0.078, *P = 0.220*) and *G. m. morsitans* (Moran's I Statistic = -0.270, *P = 0.595*) did not exhibit spatial autocorrelation. No spatial autocorrelation was observed among shape variables for both *G. m. centralis* (Mantel Statistic = -0.305, *P = 0.305*) and *G. m. morsitans* (Mantel Statistic = 0.089, *P = 0.344*). Environmental variables

did not induce any spatial dependency in *G. m. centralis* (Partial Mantel Statistic = -0.315, *P = 0.318*) and *G. m. morsitans* (Partial Mantel Statistic = 0.451, *P = 0.344*) shape variables.

Sample site characterisation

Elevation, isothermality, annual precipitation, and percent tree cover were significantly lower in *G. m. morsitans* than in *G. m. centralis* sampling sites (*P < 0.001*). Annual temperature was observed to be higher in *G. m. morsitans* than in *G. m. centralis* range (*P < 0.001*). Land surface temperature was higher in four of the five sampling sites of *G. m. morsitans* than in those for *G. m. centralis* (*P < 0.001*). The LZP sample site for *G. m. morsitans* was observed to have LST 4 °C lower than all other sampling sites. Within each subspecies range, environmental variables were observed to be significantly different between sample sites (*P < 0.001*). Elevation and annual precipitation were observed to be the environmental variables contributing most of the variation for Principal Component (PC) 1, whereas annual precipitation, vegetation continuous field (percent tree cover) and elevation contributed the most for PC2 (Fig 4.5). Principal Component 1 accounted for 91.62% of the variation between sites.

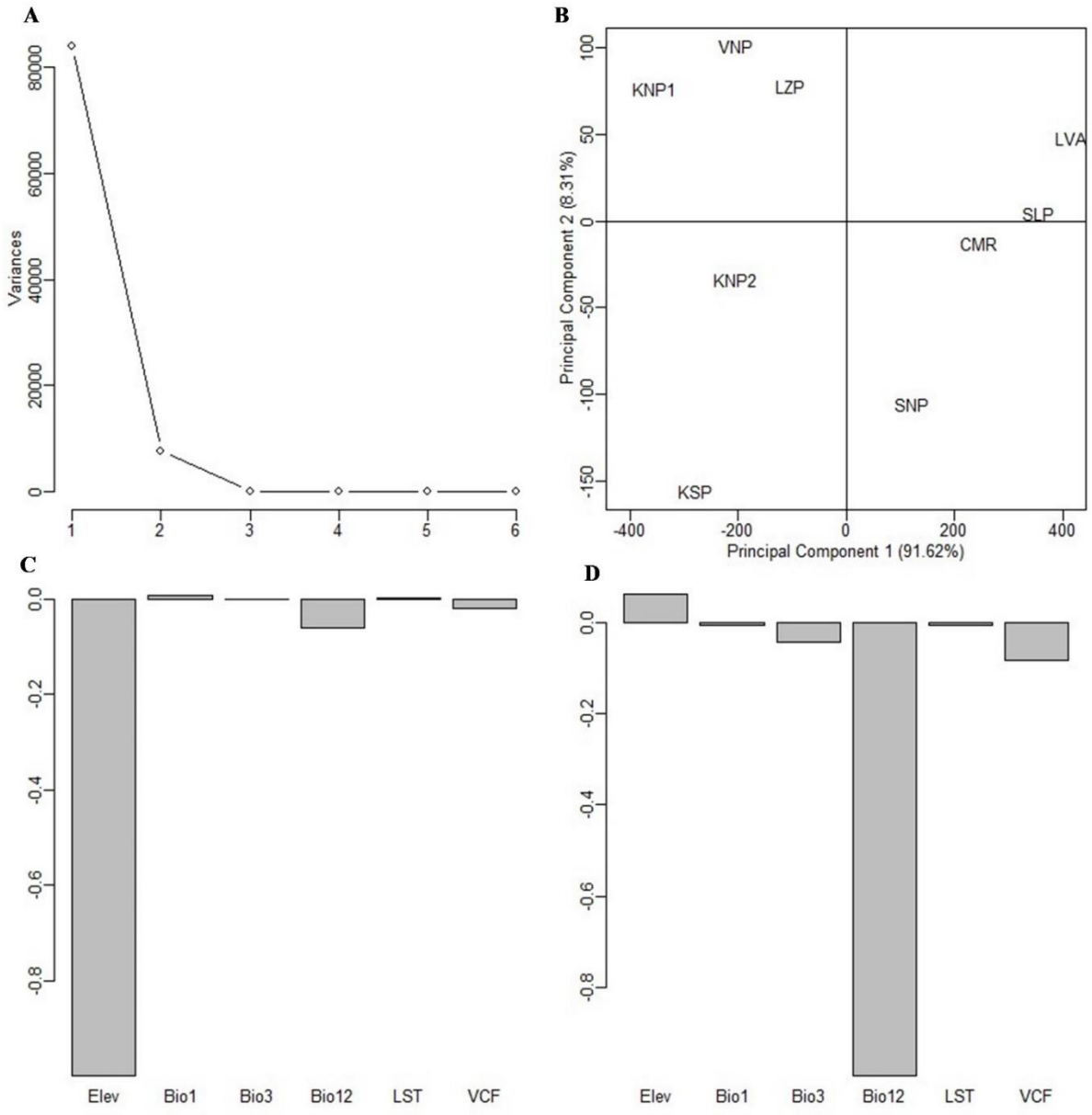

Fig 4.5. Principal components analysis of sample sites based on environmental variables. (A) Scree plot showing that most of the variance in the data set could be explained by the first two principal components PC1 and PC2. (B) Score plot indicating that PC1 and PC2 accounted for 91.62 and 8.31% of the variation among sites, respectively. (C) Vector loading plot showing that elevation and annual precipitation were the variables that contributed the highest variance to PC1. (D) Vector loading plot showing that annual precipitation, vegetation continuous field, and elevation contributed the highest variance to PC2. Abbreviations: Elev, Elevation; Bio1, Annual Temperature; Bio3, Isothermality; LST, Land Surface Temperature, VCF, Vegetation Continuous Fields indicating percent tree cover.

Centroid size comparison

Significant wing CS differences were observed between male and female *G. morsitans* flies, the two subspecies *G. m. centralis* and *G. m. morsitans*, among male and female flies within each subspecies and between sample locations within the two subspecies ranges (Table 4.2). Male flies were observed to have an absolute size nine percent smaller than females and *G. m. morsitans* was two percent smaller than *G. m. centralis*. At the subspecies level, male flies were observed to be ten and nine percent smaller for *G. m. centralis* and *G. m. morsitans*, respectively. Within the *G. m. centralis* range, flies from KNP1 and KNP 2 were observed to be three percent smaller than those from the KSP site ($P < 0.008$ and 0.013 respectively). In the *G. m. morsitans* range, flies from the LZP site were observed to be five percent larger than flies from all other sites ($P < 0.001$).

Table 4.2: Sex, subspecies, and location comparison of mean wing centroid size (CS) in *Glossina morsitans*.

Experiment	Treatment	Mean CS (Pixels)	Variance	Standard deviation (SD)	P-value
G. morsitans, male vs females	Female	356	196.80	14.03	0.001
	Male	323	89.37	9.45	
G. m. centralis vs *G. m. morsitans*, subspecies	*G. m. centralis*	344	424.70	20.61	0.001
	G. m. morsitans	336	383.41	19.58	
G. m. centralis, male vs females	Female	363	103.36	10.17	0.001
	Male	326	73.34	8.56	
G. m. morsitans, male vs females	Female	351	216.11	14.70	0.001
	Male	321	92.30	9.61	
G. m. centralis locations	KNP1	340	379.27	19.40	0.013
	KNP2	341	447.38	21.15	
	KSP	349	449.69	21.21	
	SNP	346	386.01	19.65	
G. m. morsitans locations	CMR	334	347.55	18.64	0.001
	LVA	332	372.86	19.31	
	LZP	349	527.02	22.96	
	SLP	333	124.10	11.14	
	VNP	333	356.63	18.88	

Elevation, annual temperature, annual precipitation, and land surface temperature were observed to have a significant effect on *G. morsitans* wing CS (Table 4.3). The coefficients of the regression model indicated that land surface temperature had the largest per-unit effect on CS whose net effect was a reduction in fly size.

Table 4.3: Effect of environmental variables on *G. morsitans* wing centroid size.

Variable	Coefficient	P-value
Elevation	0.0095	0.001
Annual Temperature	1.0223	0.009
Isothermality	-0.1928	0.280
Annual Precipitation	0.0462	0.001
Land Surface Temperature	-3.0115	0.001
Percent Tree Cover	0.16078	0.459
Total		

Allometry

The covariation of wing shape with CS was found to be significant (Goodall's F Statistic = 93.62, $P < 0.001$). Allometry was observed to account for an estimated 12% of shape variation in *G. morsitans*.

Allometry-free wing shape variation

Allometry-free wing shape variation in *G. morsitans* was observed to be significantly different due to sex ($P = 0.001$), subspecies identity ($P = 0.001$), and the two-way interaction between these factors ($P = 0.006$). Thus, the wing shape between male and female *G. morsitans* and between the subspecies *G. m. centralis* and *G. m. morsitans* was observed to be significantly different. Overall, sex and subspecies differences, as well as their interaction, accounted for 3.7% ($P = 0.001$) of the total allometry-free wing shape variation observed in *G. morsitans*. As shown in Fig 4.6A and 4.6B, the constrained principal components one and two accounted for 54.8 and 42.1% of this variation. The first and second constrained principal components we able to discriminate the centroid shape clusters of male and female *G. morsitans* and those of *G. m. centralis* and *G. m. morsitans*, respectively (Fig 4.6A and 4.6B).

Sex differences ($P = 0.002$) and geographic origin ($P = 0.001$) were observed to significantly influence allometry-free wing shape variation in *G. m. centralis*. However, the interaction between sex and geographic origin did not significantly affect allometry-free wing shape ($P = 0.099$). Therefore, the allometry-free wing shape in *G. m. centralis* was significantly different between males and females and between flies from different geographic locations. Overall, sex and location differences accounted for 10.3% ($P = 0.001$) of allometry-free wing shape variation in *G. m. centralis*.

The constrained principal components one and two explained 49.4 and 26.2% of this variation (Fig 4.6C and 4.6D). These constrained principal components did not discriminate *G. m. centralis* male and female wing shape clusters (Fig 4.6C). Pairwise comparisons of *G. m. centralis* allometry-free wing shape by geographic origin showed that the wing shape of flies from KSP and SNP sites were significantly different from those from KNP1 and KNP2 and each other (Table 4.4). Wing-shape of flies from KNP1 and KNP2 were not significantly different from each other (Table 4.4). The constrained principal components one and two discriminated *G. m. centralis* flies into 3 clusters (Fig 4.6D).

For *G. m. morsitans*, sex differences ($P = 0.001$), geographic origin ($P = 0.001$), and the interaction between these two factors ($P = 0.001$) were observed to significantly affect allometry-free shape variation. Therefore, size-adjusted wing shape in *G. m. morsitans* was significantly different between males and females and between flies from different geographic locations. Sex and location differences as well as the interaction of these two factors accounted for 18.9% ($P = 0.001$) of the total allometry-free shape variation in *G. m. morsitans*. An estimated 66.3% of this variation was explained by the constrained principal components one (36.0%) and two (30.3%) (Fig 4.6E and 4.6F). The constrained principal components one and two discriminated the wing shapes of male and female *G. m. morsitans* into two clusters (Fig 4.6E). Pairwise comparisons of *G. m. morsitans* allometry-free wing shape by geographic origin showed that only flies from SLP and LVA had similar sized-adjusted wing shapes (Table 4.4). The wing shape of flies from the other sites was significantly different (Table 4.4). Discrimination of *G. m. morsitans* size-adjusted wing shape from different sampling sites on the constrained principal components one and two is shown in Fig 4.6F. Flies from LZP were well separated from all other sites.

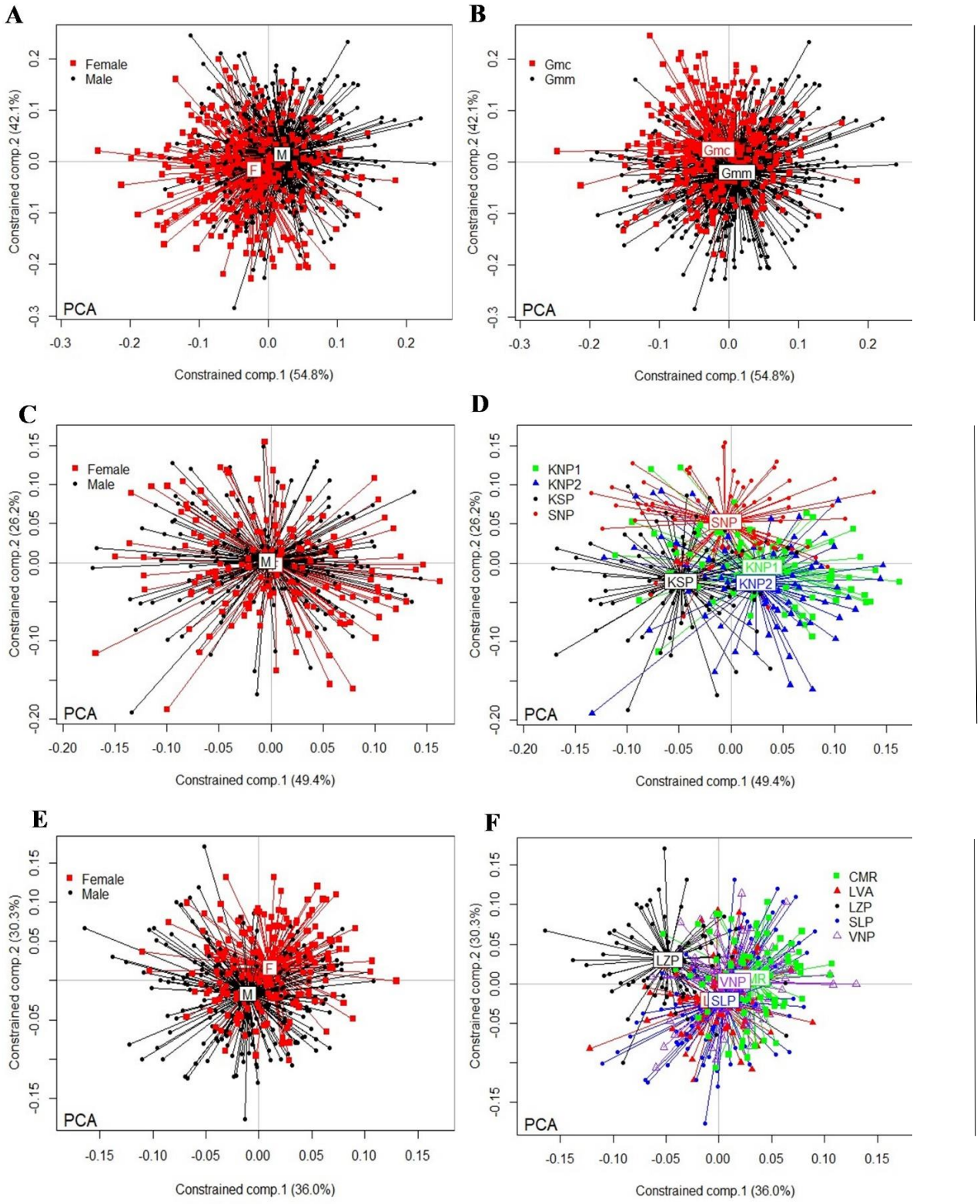

Fig 6. Redundancy Analysis (RDA) score plots from constrained Principal Component Analysis (PCA). (A) Score plot for the constrained PCA of allometry-free wing shape of male and female *G. morsitans*. (B) Score plot for the constrained PCA of allometry-free wing shape of *G. m. centralis* and *G. m. morsitans*. (C) Score plot for the constrained PCA of allometry-free wing shape of male and female *G. m. centralis*. (D) Score plot for the constrained PCA of allometry-free wing shape of *G. m. centralis* from different geographic locations. (E) Score plot for the constrained PCA of allometry-free wing shape of male and females *G. m. morsitans*. (F) Score plot for the constrained PCA of allometry-free wing shape of *G. m. morsitans* from different geographic locations.

Table 4.4. Pairwise comparison of allometry-free wing shape of *G. morsitans* from different locations

		KNP1	KNP2	KSP	
G. m. centralis	KNP2	0.051	-	-	
	KSP	0.001	0.001	-	
	SNP	0.001	0.001	0.001	

		CMR	LVA	LZP	SLP
G. m. morsitans	LVA	0.001	-	-	-
	LZP	0.001	0.001	-	-
	SLP	0.001	0.584	0.001	-
	VNP	0.029	0.001	0.001	0.016

Size-adjusted wing shape of *G. morsitans* was observed to be significantly associated with elevation, annual temperature, isothermality, annual precipitation, land surface temperature, and percent tree cover (Table 4.5). Collectively, these variables accounted for 10.7% of the observed variation in wing shape at the species level. Land surface temperature, annual precipitation, and isothermality contributed the most to this environmental variation (Table 4.5).

Table 5. Effect of environmental variables on *G. morsitans* wing shape.

Variable	F-value	P-value	Percent Explained
Elevation	5.600	0.001	0.71
Annual Temperature	13.734	0.001	0.81
Isothermality	17.656	0.001	2.38
Annual Precipitation	4.951	0.001	2.33
Land Surface Temperature	17.965	0.001	3.51
Percent Tree Cover	12.185	0.001	0.96
Total			**10.70**

The neighbor-joining cladogram derived from the analysis of Procrustes distances indicated divergence of *G. morsitans* wing shape based on subspecies and geographic origin (Fig 4.7). The ancestral shape was observed among *G. m. morsitans* flies caught from the SLP and VNP sites. The wing shape of *G. m. centralis* appears to have diverged from that of *G. m. morsitans* caught from the CMR site (Fig 4.7). In *G. m. centralis*, flies from KNP1 and KNP2 were shown to be closely related while flies from KSP and SNP were divergent from this group and each other. For *G. m. morsitans*, flies from SLP and VNP were closely related while those from LVA, LZP, and CMR were divergent from this group and each other.

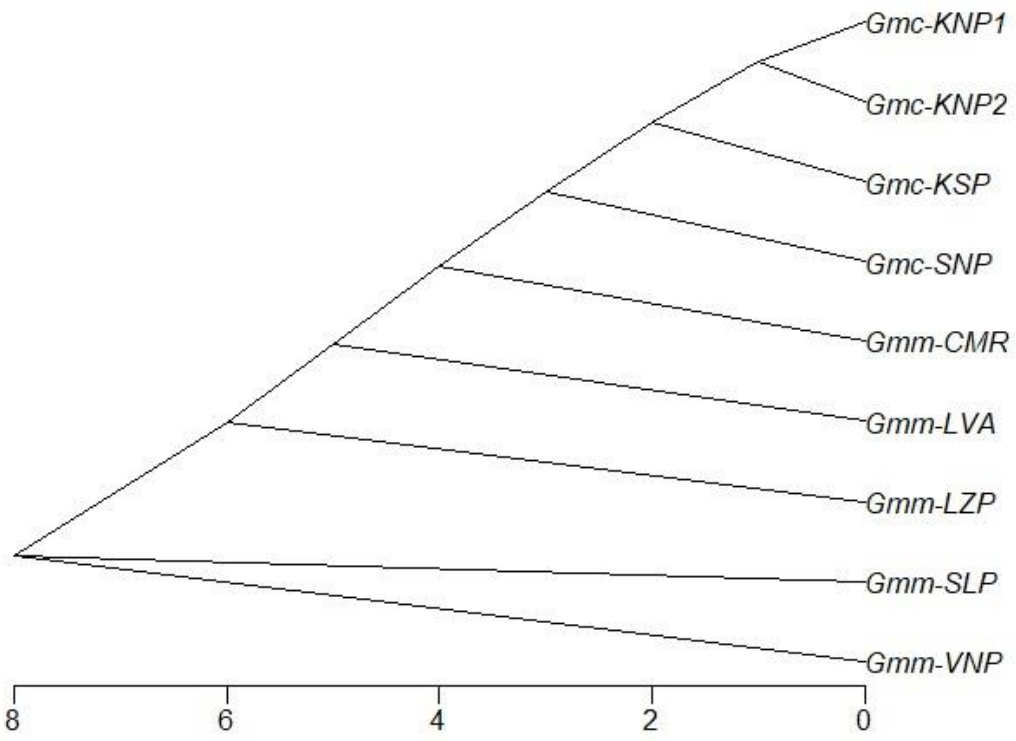

Fig 7. Cladogram of *G. morsitans* based on wing shape Procrustes distances. The figure indicates the divergence of *G. morsitans* wing shape based on subspecies and geographic origin.

Isolation-by-distance

Scatter plots of Procrustes distance versus geographic distance suggested a linear relationship between the two variables for *G. m. centralis* (Fig 4.8A) but not for *G. m. morsitans* (Fig 4.8C). As shown in Table 4.6 and Figures 4.8B and 4.8D, all distance lags between sampling points did not show positive spatial autocorrelation. Therefore, the hypothesis that Procrustes distance increases with geographic distance in *G. m. centralis* and *G. m. morsitans* was rejected.

Table 4.6: Mantel correlogram analysis results for isolation-by-distance tests.

Subspecies	Breaks of Distance Lag (m)	Mean Distance (m)	Observed Mantel Statistic	Expected Mantel Statistic	P-Value	Cardinal
G. m. centralis	0 – 219831	129194	-0.6591	0.4130	0.20	1
	219831 – 439663	438686	0.4130	0.4130	0.61	1
	439663 – 659494	495076	0.6948	0.0269	1.00	2
	659494 – 879325	767470	-0.2537	-0.2537	1.00	1
G. m. morsitans	0 – 129422	83710	0.1229	0.0600	0.63	2
	129422 – 258844	224917	0.0997	0.0504	1.00	1
	258844 – 388267	306886	-0.4177	-0.0151	0.60	3
	388267 – 517689	492314	0.0504	0.0997	1.00	1
	517689 – 647111	595850	0.2121	0.0643	1.00	3

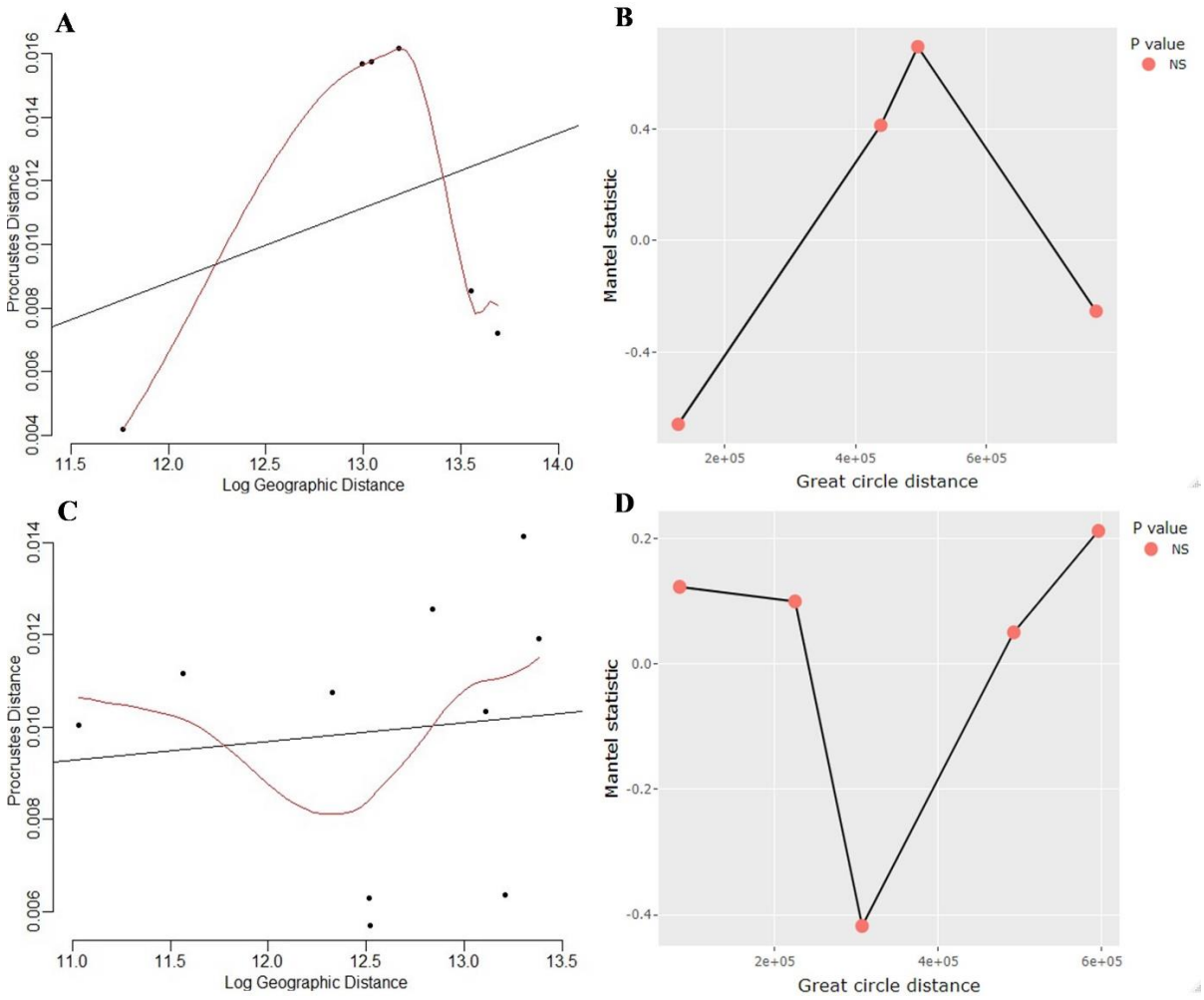

Fig 4.8. Isolation-by-distance plots. (A) Scatter plot of Procrustes distance vs. geographic distance for *G. m. centralis*. The plot suggests an increase in Procrustes distance with geographic distance. (B) Mantel correlogram of Procrustes and geographic distance for *G. m. centralis*. This plot indicates that Procrustes distance was uncorrelated with geographic distance. (C) Scatter plot of Procrustes distance vs. geographic distance for *G. m. morsitans*. The plot suggests no linear relationship between Procrustes and geographic distance. (D) Mantel correlogram of Procrustes and geographic distance for *G. m. morsitans*. This plot indicates that Procrustes distance was uncorrelated with geographic distance.

4.5 Discussion

A geometric morphometrics framework was employed to elucidate the intraspecific phenotypic variability of the two subspecies of *G. morsitans* that occur in Zambia. Population-level variability in centroid size (CS) and wing morphology can serve as a useful proxy for assessing the extent of divergence between conspecific populations (Ostwald et al., 2023) and may further provide preliminary data for the diagnosis of isolated populations (Dujardin, 2008). This information has important implications for the area-wide integrated vector management (AW-IVM) of *G. morsitans* in Zambia and further provides insights into the population differentiation status in its entire

geographical range. Broadly, these results provide evidence for microevolutionary change in both CS and wing morphology in *G. m. centralis* and *G. m. morsitans* populations in Zambia.

The results are consistent with the long-held observation that size sexual dimorphism is well established in tsetse as female *G. morsitans* were found to be larger than male flies. The estimated CS difference between the two sexes (nine percent) was similar to that reported by Hargrove et al. (2019), who found the wings of female *G. morsitans* to be eight percent longer than those of males. This observation provides further evidence that size studies based on wing measurements as described by Hargrove et al. (2019) and CS generated by geometric morphometric analysis, produce comparable results. Therefore, both measures are reliable estimators of mean wing size in *Glossina spp*.

This study has demonstrated that the mean wing size of *G. m. centralis* is larger than *G. m. morsitans*. It has been suggested that the size of tsetse is largely dependent on the nutritional state (Bursell, 1966) and temperature (Hargrove, 2001) experienced by the female. High temperatures exceeding 32°C result in tsetse entering cooler dark refuges such as rot holes in trees and antbear holes in the ground (Vale, 1971), a behaviour that reduces their metabolic rate but also reduces feeding opportunities (Lord et al., 2018). As such, female tsetse have reduced fat levels and produce progressively smaller pupae as temperature increases (English et al., 2016). Hargrove et al. (2018) showed that small pupae have lower fat reserves which results in the emergence of smaller-sized adults. Thus, the smaller fly size of *G. m. morsitans* may be an adaptation to its occupation of a hotter environment than that of *G. m. centralis* as reported by Evison and Kathuria (1982) and Muyobela et al. (2023) and reaffirmed by our results. Location differences in mean wing size were observed in both subspecies' ranges and temperature is again implicated as the major source of fly size variation.

It is postulated that the observed environmentally driven fly size variation between the two subspecies may be explained by the hypotheses of phenotypic plasticity and genetic assimilation (Dujardin, 2011). Phenotypic plasticity is defined as the occurrence of phenotypic variation of a single genotype interacting with different environments (Pigliucci et al., 2006). The observed within species

differences in fly size are probably adaptive to the different ecotopes where *G. morsitans* occurs, with plastic responses facilitating the enlargement of its ecological range. Consequently, phenotypic plasticity may have aided *G. morsitans* to survive in both warm (*G. m. morsitans*) and cooler (*G. m. centralis*) environments within its range, by providing both small and large-sized flies upon which natural selection has acted. It is conceivable that selection has resulted in fly size being genetically determined at the subspecies level through the process of genetic assimilation (Flatt, 2005), and has now become a heritable trait. Heritability for insect size has been demonstrated in *Anopheles* mosquitoes (Lehmann et al., 2006) and its transgenerational effects were shown in *G. f. fuscipes* (Mbewe et al., 2018).

Although fly size differences within the subspecies *G. m. morsitans* are known to occur (Bursell, 1966) and are reported in this study, it is unlikely that these within subspecies differences are heritable. This is because temperature variability within a subspecies range is expected to be less variable than across the subspecies range. Therefore, other factors that affect size variability such as host availability, the nutritional state of females, ovarian age, and capture month and year (Hargrove et al., 2019) are likely to be more important. Since these factors are highly variable within the subspecies range, they consequently do not exert selection in any specific direction. Fly size change driven by these factors is therefore unlikely to result in heritable change (Jirakanjanakit et al., 2007). As such, size is expected to be a poor discriminator of *G. morsitans* subspecies population structure. The results showed that allometry and environmental variability accounted for 11.6 and 10.7% of shape variation in *G. morsitans*. As such, we estimate that 77.7% of wing shape variation could be attributed to genetic effects, a finding in support of the suggestion by Patterson and Klingenberg (2007) that shape exhibits high genetic determinism. The low contribution of environmental variability to allometry-free wing shape variation suggests that *G. morsitans* wing shape exhibits high environmental canalization, in agreement with results from other Diptera such as sand flies (Dujardin and Le Pont, 2004) and mosquitoes (Henry et al., 2010).

The wing shape in *G. morsitans* was observed to vary according to sex, subspecies, and geographic origin. The detection of allometric-free shape sexual dimorphism indicates that the phenotypic expression of wing shape in this tsetse is sex-specific. Shape sexual dimorphism has been reported in other Dipteran families such as Drosophilidae (Gilchrist et al., 2000) and Culicinae (Virginio et al., 2015). Gilchrist et al. (2000) suggest that the sex regulation of shape in the Diptera represents a developmental constraint during morphogenesis rather than adaptive change. Tsetse biology appears to support this view as female flies reproduce by adenotrophic viviparity (Vreysen et al., 2013) which may present a different aerial dynamic challenge to pregnant females which are heavier compared to males, hence the need for female wings to be designed differently. Evidence of strong genetic determinism of wing-shape sexual dimorphism in the Diptera has been presented by Cowley et al. (1986).

Subspecies wing shape variation in *G. morsitans* may be an adaptive trait as *G. m. centralis* and *G. m. morsitans* occur in different habitats with different aerodynamic conditions due to temperature differences. Temperature is known to significantly affect aerodynamic lift (Liu et al., 2015). As air temperature increases, its density decreases leading to a decrease in the amount of lift generated by the wings. Therefore, selection may be acting on the wing phenotypes of the two subspecies differently as *G. m. centralis* occupies a cooler environment than *G. m. morsitans*, thereby producing wing shapes aerodynamically suitable for their specific environments. Ray et al. (2016) showed that selective pressure resulting in large and small changes in the wing shape of *Drosophila* can lead to significant changes in key flight performance metrics, leading to improved manoeuvrability and agility.

Significant wing shape variation was also observed within the subspecies ranges of both *G. m. centralis* and *G. m. morsitans.* Since shape is known to be the output of polygenic genes (Patterson and Klingenberg, 2007), within subspecies shape variation may be due to local adaptation or random genetic drift. Within the *G. m. centralis* range, random genetic drift is perhaps the primary cause of

the observed population structuring given that the KNP, KSP, and SNP populations are physically separated by large areas of unsuitable habitat (Muyobela et al., 2023) (Fig 4.1). Under such a spatial arrangement of populations, it is highly unlikely that gene flow will occur between these populations, and genetic drift is expected to quickly generate wing shape changes. Several field studies have implicated genetic drift as a source of shape variation among geographic isolates of conspecific populations (Camara et al., 2006; Dujardin, 2011; Henry et al., 2010; Kaba et al., 2012). Shape change due to genetic drift has also been demonstrated in the laboratory (Jirakanjanakit et al., 2007).

In the *G. m. morsitans* range, physical separation between sample locations does not occur (Fig 1). The observed population structuring at these locations could therefore be primarily due to local adaptation to the different environmental conditions between sample sites. A key prerequisite to local adaptation is restricted gene flow among population demes (Kawecki and Ebert, 2004). Limited gene flow within the *G. m. morsitans* range may be attributed to high habitat fidelity as the interchange of individuals between contiguous parts of the general population of this tsetse is reportedly limited (Bursell, 1966). Rapid adaptation of wing shape to different environmental conditions has also been observed in *Drosophila melanogaster* (Önder and Aksoy, 2022).

The results show that *G. m. centralis* and *G. m. morsitans* populations in Zambia are highly structured and exhibit significant morphological divergence. This observation suggests that the implementation of tsetse population management technologies that target an entire isolated population may be technically feasible. However, to categorically designate populations as isolated, it is essential to estimate the number of migrants per generation or the levels of gene flow between them (Bouyer et al., 2007), and methods using morphometric variation are not suited for these tasks (Dujardin, 2008). Therefore, the results presented in this study only provide preliminary information justifying further investigation using molecular techniques to conclusively identify genetically isolated populations (Dujardin, 2008). This is particularly crucial in the *G. m. morsitans* range where physical separation of sample locations was not apparent. It should be noted however, that some authors have suggested

that results from geometric morphometric studies are comparable to those of molecular studies using microsatellite markers (Bouyer et al., 2010, 2007; Solano et al., 1999).

It is concluded that *G. morsitans* populations in Zambia exhibit significant population-level variation in body size and allometry-free wing shape. This variation suggests high levels of population structuring that may be indicative of population isolation. Molecular studies to estimate the levels of gene flow between these populations and determine their levels of genetic isolation will be able to shed even more light on *G. morsitans* population structure in Zambia and possibly identify its underlying drivers.

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Chapter 5: General discussion

This study aimed at investigating the current distribution and phenotypic population structure of *G. m. morsitans* and *G. m. centralis* in Zambia to inform effective vector management strategies in an area-wide integrated vector management (AW-IVM) framework. To achieve these objectives, it was necessary to develop the novel vehicle-mounted sticky trap (VST) to surmount the challenges associated with efficient cost-effective sampling of *G. morsitans* over large geographic areas (Leak et al., 2008). To this end, VST was demonstrated to be much more effective than the black screen fly round (BRF), the standard mobile sampling tool for *G. morsitans* (Muyobela et al., 2021). Furthermore, the study showed that VST was capable of sampling large areas in a relatively short space of time with minimal labour compared to other sampling tools currently available (Muyobela et al., 2023). These attributes allowed the generation of large amounts of *G. m. centralis* and *G. m. morsitans* occurrence data from all tsetse belts in Zambia which facilitated species distribution modelling to update the current distribution of the two subspecies in the country using Maxent. Maxent species distribution model showed that the spatial distribution of *G. m. centralis* and *G. m. morsitans* in Zambia has reduced by an estimated 101,051 km^2 (Muyobela et al., 2023). This observation was largely attributed to changes in climate and a reduction in suitable habitat due to anthropogenic led habitat fragmentation. The ability to generate large samples for both *G. m. centralis* and *G. m. morsitans* within a month across all tsetse belts, enabled the population-level analysis of morphometric variables that were not confounding for seasonal effects (Muyobela et al., 2024). Landmark-based wing geometric morphometrics analysis of these variables showed that the populations of both *G. m. centralis* and *G. m. morsitans* exhibited significant population-level variation in body size and allometry-free wing shape. The main drivers of this variations were attributed to random genetic drift in *G. m. centralis* and local adaptation to environmental conditions in *G. m. morsitans* populations.

5.1 Interpretation of tsetse catches from mobile trapping devices.

The current computation of relative abundance of tsetse catches using mobile trapping devices only accounts for distance covered as sampling effort (Ducheyne et al., 2009; Leak et al., 2008). This however does not consider daily tsetse displacement rates and the variability in exposure time of the sampling tool. Chapter Three demonstrated for the first time how these variables can be incorporated in the computation of tsetse apparent density from mobile device catches hence making comparisons more accurate. Accurate comparison of tsetse apparent density of different tsetse infested areas is important in assessing the risk of trypanosomiasis transmission (Takken and Knols, 2010) and facilitates the identification of priority areas for vector control interventions (Diall et al., 2017).

5.2 Modelling the distribution of *G. morsitans* subspecies

A recent Maxent species distribution modelling study based on current climatic conditions, over-predicted the habitat suitability of *G. morsitans* (Zhou et al., 2021). This over prediction may be due to non-separation of subspecies occurrence records and the use of coarse spatial resolution during modelling. Chapter Three clearly demonstrates that the sub-species environmental response of *G. m. centralis* and *G. m. morsitans* is different which implies that each subspecies must be modelled separately. Robinson et al. (1997) and, Rogers and Robinson (2004) used this approach to model the distribution *G. morsitans* subspecies but did not provide justification for their decision. To the best of our knowledge, the data presented in Chapter Three represents the first detailed account of the different species environmental response of *G. m. centralis* and *G. m. morsitans* especially in Zambia.

5.3 Fly Size differences among *G. morsitans* subspecies.

Intraspecific size variability in *G. morsitans* species is well known and has been demonstrated in several studies (Glasgow, 1961; Hargrove et al., 2019; Phelps and Clarke, 1974). However, the data in Chapter Four represents the first report of heritable fly size differences among the subspecies of *G. morsitans*. This information may be of practical value in operations that utilise insecticide treated targets as it has been suggested that targets selectively kill larger flies within the populations and that

such selection effect might explain the claimed rarity of reports of the successful elimination of tsetse populations using targets alone (Mbewe et al., 2018). As such, it is expected that targets may be more effective in the control of the larger *G. m. centralis* subspecies than *G. m. morsitans* given that the former was observed to have a larger fly size than the latter. Larger flies are known to disperse further than smaller ones, which increases their probability to encounter targets (Hargrove et al., 2019). It is therefore expected that targets are more effective in controlling *G. m. centralis* than *G. m. morsitans.* For the smaller sized *G. m. morsitans* targets should be used in combination with other techniques such as sequential aerial spraying (Kgori et al., 2006) and sterile insect technique (Vreysen et al., 2000) that do not require the tsetse to encounter a mortality inducing device.

5.4 Significant *G. morsitans* phenotypic population structuring

Highly differentiated populations and restricted gene flow among *G. morsitans* populations have been reported using molecular markers at macro-geographic scales in Eastern and Southern Africa (Krafsur, 2009). The data in Chapter Four suggests that *G. morsitans* population structuring is also apparent at micro-geographic scale, within and among tsetse belts. This finding is likely to improve the planning and implementation of control operations as intervention areas may be more accurately delimited and thus better protected from tsetse reinvasion. Since random genetic drift appears to be the primary cause of the observed population structure, Chapter Four reinforces the notion that tsetse are largely local insects (Glasgow, 1961; Krafsur, 2009; Krafsur and Maudlin, 2018) despite the ecological observation that they have significant capacity of dispersal (Williams et al., 1992).

5.5 Vehicle-mounted sticky trap (VST)

Vale (1974), showed that mobile baits were more effective in sampling *G. m. morsitans* than stationary baits. However, mobile sampling devices developed since then have been either too expensive to use for routine sampling (vehicle-mounted electric targeted - VET) or produce samples that are highly dependent on the varying ability of operators to catch tsetse with hand nets (black-screen fly rounds - BFR) (Leak et al., 2008). As demonstrated in Chapter Two, the novel VST is more

effective in catching both male and female *G. m. centralis* and *G. m. morsitans* flies compared to the BFR despite covering the same distance in a much shorter time and is independent of operator skill and ability. Chapter Three further showed that the VST can be used to rapidly survey large areas to detect *G. m. centralis* and *G. m. morsitans* using very little labour which points to its ability to aid the implementation of cost-effective surveys. This characteristic means that for the first time, large areas infested with *G. m. centralis* and *G. m. morsitans* can be effectively sampled at a fraction of the cost because of the significant reduction in manpower and equipment required to conduct surveys. As such, VST has the potential to facilitate the regular updating of *G. m. centralis* and *G. m. morsitans* spatial and temporal distribution, apparent density and vector parasite infection rate which may aid consistent estimation of the intensity of trypanosomiasis disease transmission and risk, over large areas. Furthermore, VST sampling may facilitate cost-effective regular assessment of the variation in trypanosomiasis disease risk due to changes in temperature, humidity, rainfall, and habitat quality. This information is likely to lead to improved understanding of trypanosomiasis epidemiology and transmission dynamics (Dicko et al., 2015) which is crucial for development of risk-based vector control strategies (Diall et al., 2017).

A significant limitation of the use VST is that sampling is largely restricted to motorable tracks (Muyobela et al., 2021). This means that VST sampling is inherently biased to areas and seasons that permit motorable access. While this limitation can be mitigated by careful planning of survey routes and modelling occurrence records (Muyobela et al., 2023) the inherent sampling bias of the VST cannot be fully overcome. As such, it is recommended that it be used in combination with other sampling tools especially when the objective of the survey is to delimit a tsetse population for control purposes.

5.6 Future directions

Improvement of VST design

Despite the reported effectiveness of the VST as demonstrated in Chapter Two, more research is required to establish whether its efficiency can be further improved. An important area of further investigation is the testing of different colour designs to increase the visual response of *G. morsitans* to the trap panels. Santer et al. (2019) reported that a new violet polyester cloth caught 1.5 – 1.6 times more female *G. m. morsitans* than the typical phthalogen blue polyester on stationary targets. As such, comparing of the violet polyester cloth with the colour designs used in Chapter Two may yield a more effective VST. Furthermore, the experiments conducted in Chapter Two did not investigate the effect of different trap panel sizes on VST catch. Larger objects are more attractive to tsetse than smaller ones (Colvin and Gibson, 1992). However, Byamungu et al. (2018) reported that landing rates for *G. m. centralis* were higher on 1 and 0.5 m^2 square and horizontal oblong stationary targets than on pyramidal traps. Therefore, it is possible that these and other panel sizes may be as effective as the 1.5 m^2 used in Chapter Two to construct the VST. The ability to use smaller sized trap panel in VST construction implies reduced trap construction costs.

Further work on species distribution modelling of G. m. centralis and G. m. morsitans

A major limitation to the use of Maxent in modelling species distributions is that the algorithm is susceptible to sample selection bias (Guisan et al., 2017) as only presence records are used in the modelling procedure (Phillips and Dudík, 2008). This means that if presence-absence data are both available and reliable, the Maxent model does not utilise all the information available in the data. Brotons et al. (2004) indicated that presence-absence models generally work better and Elith et al. (2006) demonstrated that the approaches based on boosting (boosted regression trees) or bagging (random forests) tend to offer higher predictive performance. In this study, most of the absence points were unreliable as sampling started five km away from tsetse belts which is within the dispersal capacity range of *G. morsitans* (Williams et al., 1992). It is therefore recommended that a sampling

regime aimed at producing reliable presence and absence point be undertaken and the resulting occurrence records be modelled using boosting or bagging procedures. Alternatively, an ensemble modelling approach, based on the idea of multi-model inference, were predictions from several models such as Maxent, boosted regression trees and random forests, may be used to derive average predictions across models (Guisan et al., 2017), can be used. This is likely to improve the predicted habitat suitability of *G. m. centralis* and *G. m. morsitans* presented in this study.

Data obtained during the tsetse survey

A huge amount of occurrence tsetse data were collected during the study. This data will be included in the Zambia Geospatial Database of African Animal Trypanosomiasis and its Vectors (Atlas) that is currently being developed by the Tsetse and Trypanosomiasis Control Unit Zambia. Once completed, this database with be published. The tsetse samples collected will be used to investigate cuticular hydrocarbon variation, population genetic analysis and the tsetse infection rate in *G. morsitans* subspecies in Zambia.

Chemotaxonomy as a prospective tool for delineating G. morsitans populations

The findings in Chapter Four of significant phenotypic population structuring within and among *G. m. centralis* and *G. m. morsitans* populations suggests that other phenotypic or physiological markers may be used to investigate population level variation in these subspecies. Physiological traits such as the composition of cuticular hydrocarbons (CHC) which insects use to protect themselves from desiccation can be used. Cuticular hydrocarbons can be inherited genetically or acquired from the environment. Stennett and Etges (1997) illustrated that when insect populations are isolated, cuticular hydrocarbons can rapidly diverge due to differences in genetic makeup, diet and/or temperature, thus leading to reproductive isolation. Drijfhout et al. (2010) suggested that cuticular hydrocarbons may be better indicators of recent speciation events and reproductive isolation than morphological characters which require more time to accumulate changes after speciation. Thus, the analysis of cuticular hydrocarbon within and among *G. morsitans* populations may serve as an invaluable tool in

investigating population structure as they represent discrete, constant and heritable characters which are under direct selection (Bagnères and Wicker-Thomas, 2010). Therefore, the analysis of cuticular hydrocarbon variation in *G. m. centralis* and *G. m. morsitans* populations may reveal higher levels of population structuring than that presented in Chapter Four.

Population genetic analysis and genetic friction mapping

As indicated above, extensive investigations of tsetse fly population genetic structure over the past two decades have been conducted (Krafsur, 2009). However, few studies have provided a fine-scale spatial resolution of *G. morsitans* population genetic structure (Krafsur and Maudlin, 2018). The findings presented in Chapter Four are indicative of limited gene flow within and among *G. m. centralis* and *G. m. morsitans* populations in Zambia and therefore the use of molecular markers to investigate the degree of isolation is Justified. Furthermore, population genetic analysis may facilitate the production of genetic friction maps that utilise landscape resistance (friction) to tsetse genetic flow to identify natural barriers that isolate populations (Bouyer et al., 2015). The production of friction maps will greatly improve the planning and implementation of area-wide vector management by facilitating the sequential targeting of isolated populations.

5.7 Concluding remarks

In conclusion, this study demonstrated that the VST is a rapid and effective sampling tool for *G. morsitans* and recommended its use in sampling large geographic areas infested with tsetse. Its ability to generate large samples within a short space of time makes it an invaluable tool in studies that require samples to be drawn from a large area and within the same generation. It further shows that the spatial distribution of *G. morsitans* in Zambia has reduced and that tsetse densities are expected to be highest where natural vegetation and wildlife are protected from anthropogenic influence, and suitable climatic conditions exist. This information has important implications for trypanosomiasis risk and therefore vector management strategies should be adjusted to account for the observed change in *G morsitans* distribution. Finally, it demonstrated that *G. morsitans* populations in Zambia

exhibit significant phenotypic population-level variation which may be indicative of population isolation. As such, area-wide suppression and/or eradication campaigns can be sustainably implemented. Further studies based on cuticular hydrocarbons and molecular markers are however necessary to elucidate the degree of population isolation.

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