Abstract

Anopheles mosquitoes present a major public health challenge in sub-Saharan Africa, notably as vectors of malaria killing over 600,000 people annually. In parts of the east and southern Africa regions, one species in the *Anopheles funestus* group, *An. funestus* has established itself as an exceptionally dominant vector in some areas and is currently responsible for 9 out of every 10 malaria transmission events. Despite this apparent public health importance, research on this vector has historically lagged behind those of other malaria vectors in the *An. gambiae* complex, leaving many aspects of its population biology uncertain. For instance, the genetic diversity of this vector across its ecological range in Africa is poorly understood making it difficult to decipher true population structure, patterns of gene flow, and signatures of selection. These are crucial for understanding the distribution of insecticide resistance genes, how vector populations are structured in space for targeted interventions and devising sustainable insecticide-based vector control approaches. Additionally, information on this vector's resistance status and behavioural data is very limited across Africa, partly due to difficulties with laboratory colonization and the unresolved aspects of its aquatic ecology. Attempts at controlling this vector require deliberate efforts to understand these aspects that might impact their response to vector control interventions.

This thesis presents the first large-scale genomic survey and population biology study in *An. funestus* populations in Tanzania. My overarching aim was to provide a comprehensive understanding of the population biology, insecticide resistance, and population genetic structure of the major malaria vector *An. funestus* in mainland Tanzania. To achieve this, I began by conducting a wide-scale population sampling in 11 locations in Tanzania, purposively selected to encompass variations in vector ecology and climatic conditions. These samples were individually whole genome sequenced and analysed to elucidate the population genetic diversity and gene flow patterns of *An. funestus* across Tanzania. This analysis demonstrated the existence of two genetically differentiated *An. funestus* populations; the inland high-altitude populations, and coastal low-altitude populations, potentially separated by an area of unsuitable climatic conditions, geographically coincident with the eastern arm of the Great Rift Valley. These genetic populations showed demographic differences and divergence, likely due to differentiation at insecticide resistance genes reflecting diverse historical and contemporary

dynamics. The genomic analysis also serendipitously led to the first discovery of knockdown resistance (*kdr*) in *An. funestus* mosquitoes. The *kdr* mutations were found at high frequency only in one location, the Morogoro region. One of the mutations, *kdr* L976F was strongly associated with survivorship to exposure to DDT insecticide, while no clear association was noted with a pyrethroid insecticide (deltamethrin). An urgent follow-up study is required to monitor the evolution of *kdr* and determine whether *kdr* confers resistance phenotypes to other widely used pyrethroids, such as permethrin, and alpha-cypermethrin, as well as other insecticide families.

Alongside this genomic survey, I characterised the different Tanzanian *An. funestus* populations by measuring their behaviour, ecology and level of resistance to insecticides. This analysis revealed widespread pyrethroid resistance, but susceptibility was restored with PBO preexposure. The insecticide resistance genotype patterns indicated a north-south gene flow restriction. Additionally, while the biting behaviour of *An. funestus*, level of anthropophily, and age structure were similar across the genetic populations, differences were noted in the prevalence of *Plasmodium* infections, mosquito sizes, and fecundity between the inland and coastal genetic populations. Further research is needed to determine whether these phenotypic and demographic differences are a consequence of, or contribute to, the observed genetic separation between the inland and coastal populations of *An. funestus*.

Taken together, this thesis provides a deeper understanding of the population biology and genetic diversity of *An. funestus* in Tanzania. The whole genome sequence dataset developed in this thesis, publicly available at the European Nucleotide Archive (study number PRJEB2141), represents the largest data on a disease vector in Tanzania to date and provides a crucial resource in the continued monitoring of the vector. The population diversity disconnectedness revealed here should be considered when implementing insecticide resistance management and the future rollout of novel genetic-based vector control approaches. It is important for future research to examine the epidemiological relevance of this discontinuity in gene flow and whether these populations have different malaria transmission abilities.

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Acknowledgement

First, I would like to say a huge thank you to my supervisors Dr. Francesco Baldini, Prof. Fredros Okumu, Prof. Heather Fergusson at the University of Glasgow, and Dr. David Weetman from the Liverpool School of Tropical Medicine. The support you have given me throughout my PhD journey has been incredible - I couldn't have asked for a better supervisory team! A special thank you goes to Dr. Francesco Baldini and Prof. Fredros Okumu. Thank you for being there, for the support, being patient with me, explaining and clarifying concepts, for the many enlightening conversations and discussions, and pushing me to reach my potential.
I would also like to thank Dr Tristan P.W. Dennis – thank you for all the support and guidance, especially when I was learning genomic analysis skills, but most importantly for being a true friend.
I also want to thank my annual review assessors Prof. Collette Britton and Dr Roz Laing for their guidance and contribution towards the development of this research over the four years. Thank you to all my colleagues at the Ifakara Health Institute (Environmental Health and Ecological Sciences (EHES) Department) and the University of Glasgow (Vector group). I would also like to extend my deepest gratitude to Dr. Alistair Miles, Dr. Chris Clarkson, and Dr. Anastasia Hernandez Koutoucheva from the Genomic Surveillance Unit, Wellcome Sanger Institute. Thank you to our funding partner, the Bill and Melinda Gates Foundation, for providing financial support to the project and funding my PhD. None of this would have been possible without your support. A huge thank you to the local communities in Tanzania that welcomed us to their home to enable us to collect the samples used in this thesis.
To the friends I made along the way in Scotland, thank you for making this amazing country feel like home. Thank you, Antonia – for being a great friend, for all the encouragement and for offering a listening ear whenever I needed to vent out my frustrations.
Thank you to my parents Julius and Cerine Odero for always believing in me. You are all amazing parents, and I wouldn't have been where I am today without your support, guidance, and inspiration.
On a deeply personal note, I would like truly to thank my partner Lynn and daughter Angie. I'm so grateful to both of you for being my rocks throughout this

long PhD journey. Your love and support, especially when I had to be away over extended periods, meant the world to me, and made this journey so much easier. I would like to dedicate this thesis to my grandfather, Ker Stephen Othoo Deya, who sadly passed on in the final year of my PhD.

Author's Declaration

I, Joel Ouma Odero, declare that all the work presented in this thesis is my work, written in my own words and all the sources used are fully acknowledged and cited. Any collaborations are clearly stated.

Definitions/Abbreviations

AFG – *An. funestus* group

WHO – World Health Organization

IRS – Indoor residual spraying

ITN – Insecticide-treated net

PCR – Polymerase chain reaction

RFLP – Restriction fragment length polymorphism

WGS – whole-genome sequencing

NHEJ - nonhomologous end-joining

HDR - homology-directed repair

PBO - piperonal-butoxide

GSTe2 – Glutathione s-transferase epsilon 2

CDC – Centres for Disease Control and Prevention

SV – structural variant

CYP450 – Cytochrome P450 gene family

Kdr – knockdown resistance

IR – Insecticide resistance

Vgsc – Voltage-gated sodium channel

1 General Introduction

The twentieth century has witnessed considerable progress in malaria control across Africa evidenced by a 37% reduction in malaria incidences and a 60% decrease in mortality between 2000 – 2015 (Cibulskis et al., 2016, WHO, 2015b). However, progress has stagnated since around 2015; with only a ~3% incidence drop observed since 2015, with countries such as Nigeria, Uganda, and Papua New Guinea observing an increase in malaria burden (WHO, 2023f). Malaria remains actively transmitted in over 90 countries and territories across the globe. In 2023 the World Health Organization (WHO) estimated over a quarter billion reported malaria cases and over 600,000 malaria deaths, with over 95% of the burden occurring in sub-Saharan Africa (SSA) (WHO, 2023f).

The control of malaria is primarily through prompt treatment of clinical malaria cases using artemisinin-based combination therapy (ACT), insecticide-treated bed nets (ITNs), indoor spraying of houses with residual insecticides (IRS), and more recently inoculation of children with preventative malaria vaccines; RTS,S/AS01(Mosquirix) and R21/Matrix-M (WHO, 2023c). Estimates indicate that these control interventions, excluding vaccines, have prevented ~663 million clinical malaria cases, mostly by ITNs (Bhatt et al., 2015). The stalling of malaria gains and the increasing biological threats of parasite and vector resistance across malaria countries led to a call for action to ensure backtracking progress.

1.1 Global agenda for malaria control

In response to the stalling of progress in malaria control, the WHO developed an ambitious plan, *The Global Technical Strategy for Malaria* (*GTS*) 2015 – 2030, ratified by the member countries and multilateral partners committing to a 90% reduction in malaria mortality and cases by 2030 (WHO, 2015a). The strategy had three pillars: universal access to malaria prevention, diagnosis, and treatment, accelerating efforts towards eliminating and attaining malaria-free status, and transforming malaria surveillance into a core intervention, envisioning '*a world free of malaria*'. Implementing this strategy resulted in 10 countries, Algeria, Belize, Cabo Verde, China, El Salvador, Iran, Azerbaijan, Sri Lanka, Tajikistan, and Malaysia, achieving transmission interruption status within the first 5 years.

Transmission interruption status is a pre-elimination status accorded when previously malaria-endemic countries record zero local malaria transmission over a defined period. Of note are two sub-Saharan African countries amongst the 10, Algeria and Cabo Verde, which have recently been declared malaria-free (WHO, 2023a).

However, the malaria transmission landscape greatly evolved over this period with increasing malaria burden, worsening biological threats (parasite and vector resistance), extreme weather events such as flooding and heatwaves, shrinking funding, and the potential impact of the COVID-19 pandemic necessitating a strategy update in 2021 to reflect these dynamics, but with similar pillars and milestones (WHO, 2021a). In efforts to further accelerate the progress towards achieving the *GTS* 2030 targets, the WHO Global Malaria Programme (GMP) recently unveiled a 2024 – 2030 operational strategy (WHO, 2024a). The strategy is anchored around developing and disseminating up-to-date and relevant norms and standards, shaping the research agenda and accelerating the development, introduction, and adoption of new malaria control products, tracking global malaria trends, providing strong technical leadership, and providing tailored support for countries.

The new malaria vector control interventions under consideration by the GMP include attractive targeted sugar baits (ATSB), eave tubes, and updating guidelines for larval source management (LSM). With only five years to 2030, it remains to be seen if the *GTS* target of a 90% global reduction in malaria burden can be attained.

1.2 The human malaria parasites

Malaria is a parasitic infection transmitted when a female *Anopheles* mosquito carrying infective stages of the *Plasmodium* parasites bites a human while acquiring a blood meal. Initially described by Charles Louis Alphonse Laveran in the 1880s (Cox, 2010), *Plasmodium* is an obligate unicellular eukaryote parasite capable of infecting a variety of vertebrate hosts including humans, birds, and reptiles. The human infective parasites in the *Plasmodium* genus are *Plasmodium malariae, P. vivax, P. knowlesi, P. ovale* and *P. falciparum*. *Plasmodium*

falciparum is the most dominant malaria parasite in Africa and accounts for most cases and deaths (Snow, 2015).

The life cycle of *Plasmodium* malaria occurs between two hosts, humans and the mosquito vector (Figure **1-1**). The human stages include the pre-erythrocytes, the asexual erythrocyte, and the gametocyte. This stage begins when a female *Plasmodium* mosquito transfers *P. falciparum* sporozoites into the human bloodstream in the process of blood feeding. The sporozoites travel to the liver infecting the hepatocyte cells. In the liver, the sporozoites undergo asexual reproduction resulting in thousands of merozoite parasites. The merozoites are released into the blood circulation and begin a cycle of erythrocyte invasion, replication, erythrocyte rupture, and schizonts releasing merozoite-causing symptoms in humans. For *P. vivax* and *P. ovale*, a few schizonts remain dormant in the hepatocytes and cause relapse in malaria days or sometimes years following an infection. Some merozoites develop into male and female gametocytes ingested by mosquitoes to begin the mosquito stage infection. In the mosquito, they undergo a sporogony cycle where the gametocytes form into zygotes and eventually into ookinetes that invade the mosquito midgut walls and grow into oocytes. Mature oocytes rupture to release infective sporozoites that move to the mosquito's salivary glands. The mosquito carrying infective sporozoites transfers them to a healthy human during blood feeding to perpetuate the cycle.

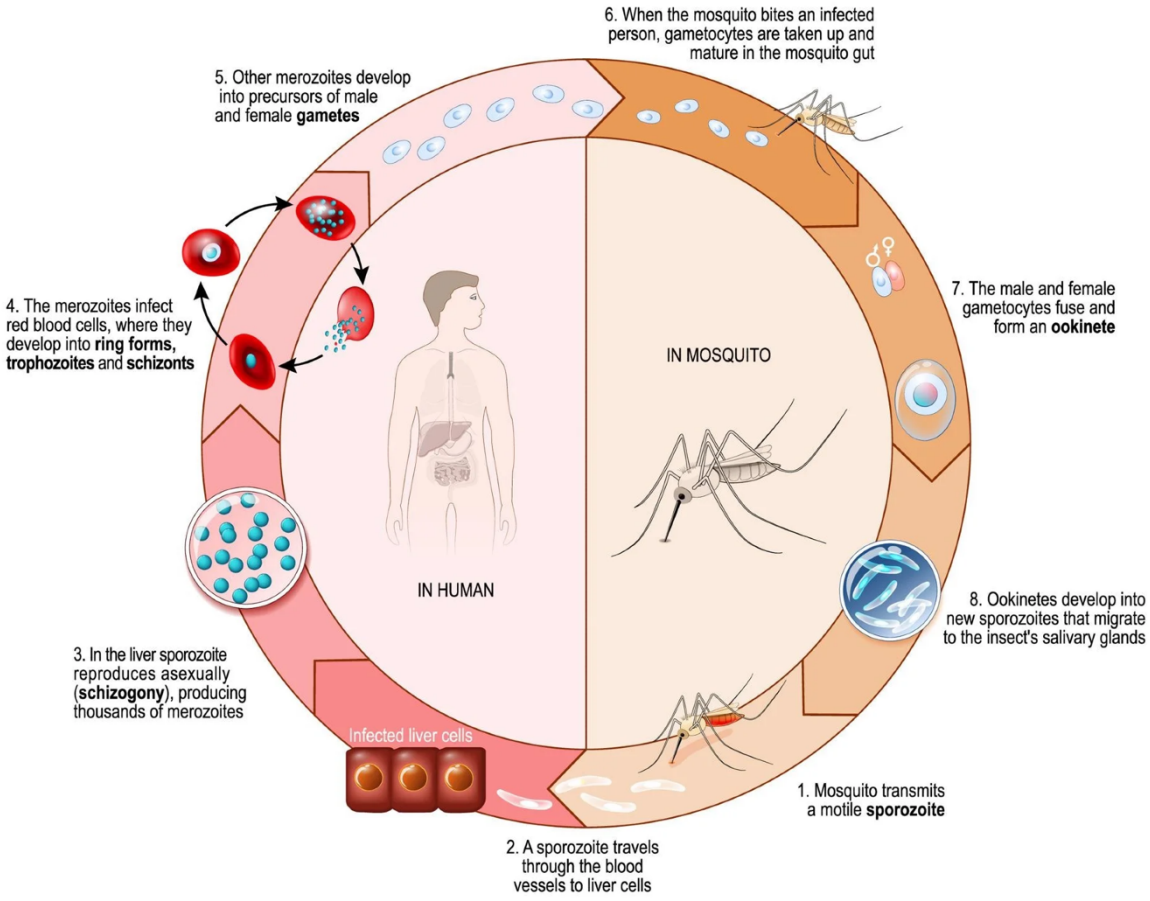


Figure 1-1: The *Plasmodium* malaria life cycle. Image credit: Designua/Shutterstock (https://www.shutterstock.com/image-vector/life-cycle-malaria-parasite-vector-diagram-1435662671)

1.3 The mosquito life cycle

Mosquitoes (Order Diptera, Family Culicidae) are among the oldest insects believed to have evolved alongside humans around the earth for over 200 million years. There are over 3,000 mosquito species but only a handful can transmit human diseases, mostly in the *Anopheles* genus. Mosquitoes of the *Anopheles* genus have a wide geographical distribution and are historically known to transmit several diseases of medical importance including malaria, lymphatic filariasis, and arboviruses. However, all mosquitoes require an aquatic habitat to lay their eggs and undergo the same life cycle.

The life cycle of a mosquito begins when a gravid mosquito lays her eggs in an aquatic habitat (**Figure 1-2**). Mosquitoes usually lay between 50 – 200 eggs per oviposition cycle. *Anopheles* mosquito's typical aquatic habitats are characterized

by freshwater bodies ranging from temporary puddles and hoof prints to permanent shallow river streams (Nambunga et al., 2020, Minakawa et al., 2012). The eggs take 2-3 days to hatch into larvae that develop through four instar stages and eventually into pupae after ~10 days. At the pupae stage, morphological features can help separate males from female juveniles. After about 3 days, the pupae morph into an adult mosquito.

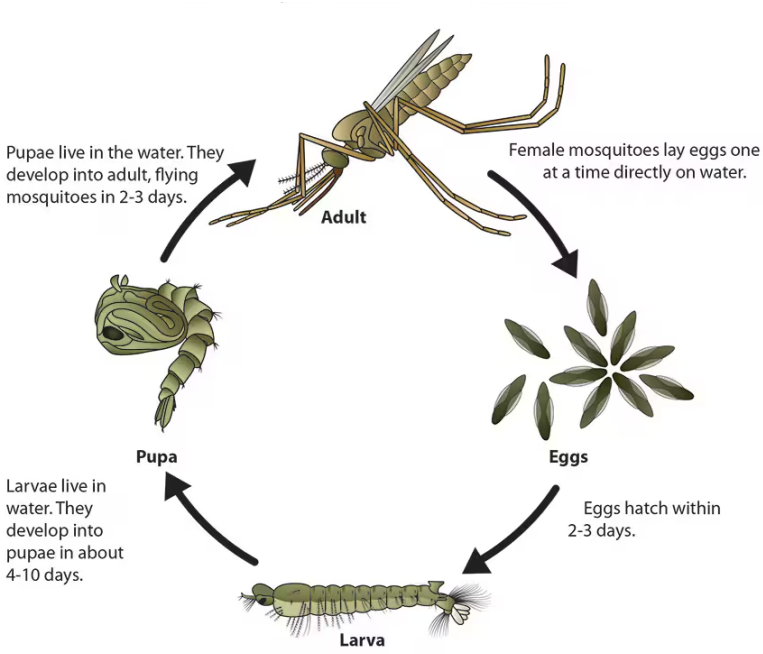


Figure 1-2: The life cycle of an *Anopheles* mosquito. Image credit: Centres for Disease Control and Prevention (https://www.cdc.gov/mosquitoes/about/life-cycle-of-anopheles-mosquitoes.html)

1.4 African malaria vectors of the *Anopheles* genus

Globally, over 40 *Anopheline* mosquito species and complexes can transmit the *Plasmodium* parasite (**Figure 1-3)** (Sinka et al., 2012). *Anopheles gambiae* complex and *Anopheles funestus* group are the dominant vector species in Africa sustaining malaria transmission (Sinka et al., 2012). Within each group/complex, sibling species are often morphologically similar and can only be distinguished using molecular methods such as polymerase chain reaction (PCR) (Koekemoer et al., 2002b, Scott et al., 1993). The *Anopheles gambiae* complex members include *An. gambiae, An. coluzzi, An. arabiensis, An. quadriannulatus, An. melas, An. merus, An. bwambae* and *An. amharicus* (Davidson, 1964). The *An. funestus* group of mosquitoes comprises at least 11 African sibling species namely, *An. funestus* sensu stricto, *An. funestus-like, An. parensis, An. vaneedeni, An. aruni, An.*

confusus, An. leesoni, An. longipalpis, An. rivulorum, An. rivulorum-like, An. brucei, and *An. fuscivenosus* (Coetzee and Koekemoer, 2013).

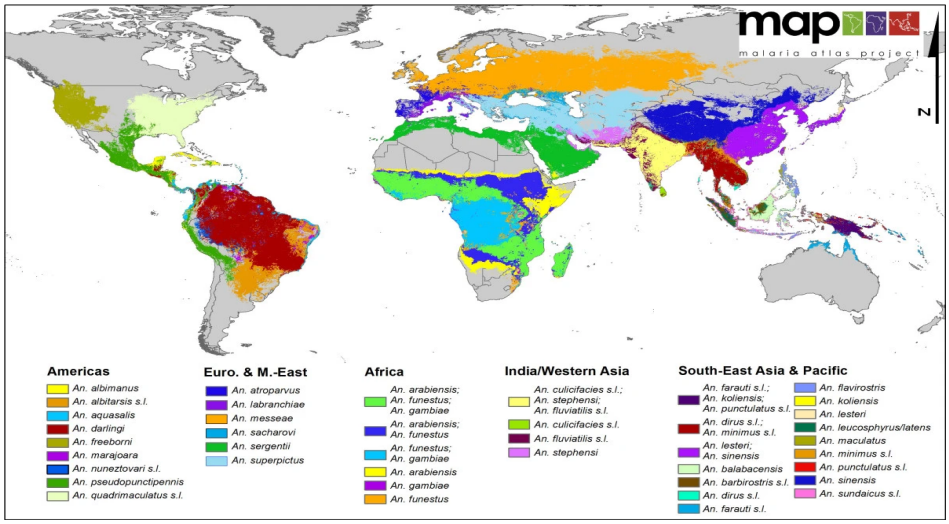


Figure 1-3: The global distribution of malaria mosquito vectors. Map has been adopted from Sinka *et.al.*(Sinka et al., 2012).

1.5 Ecology and behaviour of African *Anopheles* malaria vectors and implication for control

Different *Anopheles* species exhibit unique behavioural and physiological characteristics that determine responses to control interventions. For instance, *An. gambiae, An. coluzzii,* and *An. funestus* s.s predominantly prefer blood-feeding on humans (anthropophilic) and resting indoors (endophilic) (Akogbeto et al., 2018, Kabbale et al., 2013), whereas *An. arabiensis* is a more generalist vector, biting humans but also readily feeding on domestic animals (zoophilic) and resting both outdoors (exophilic) and indoors (Tirados et al., 2006). However, some evidence indicates that *An. funestus* s.s is shifting resting and feeding behaviour to the outdoors (Kreppel et al., 2020) and sometimes during the daytime (Sougoufara et al., 2014) in some settings as an adaptation to indoor interventions. Such innate or evolving behaviour differences between vector species mean that vector control interventions can have differential effects depending on local vector ecology.

1.6 Malaria control

1.6.1 Malaria parasite control and case management

A key element in malaria parasite control is diagnosis – by using rapid diagnostic tests to detect *P. falciparum* histidine-rich protein 2 (*pfhrp2*) and infection confirmation by microscopic examination of peripheral blood smear. Confirmed and uncomplicated *P. falciparum* malaria infections are treated by artemisinin-based combination therapy (ACTs). However, in areas where *P. vivax* or *P. ovale* are widespread, it is recommended for adults and non-pregnant persons, not glucose-6-phosphate dehydrogenase (G6PD) deficient to receive a 14-day dose of primaquine to prevent relapses in malaria. The WHO recommends treating severe malaria with artemether injections followed by ACTs.
Preventative treatments against *P. falciparum* malaria include intermittent preventive treatment of pregnant women and infants, seasonal chemoprevention for children under 5 years, and malaria prevention for short-term travellers to malaria transmission zones.

The main biological threats to malaria parasite control are genetic evolution and the spread of the *P. falciparum* histidine-rich protein 2 (*pfhrp2*) to avoid detections with RDTs (Feleke et al., 2021) and the increasing parasite resistance to ACTs (Dondorp et al., 2010), which are the first-line drugs for treating uncomplicated *P. falciparum* malaria in Africa.

1.6.2 Malaria vaccines

Vaccines have been a crucial arsenal in the control and eradication of many infectious diseases around the globe. With the challenges faced by current first-line antimalaria tools (Ranson and Lissenden, 2016a, Dondorp et al., 2010), an effective, durable, and affordable malaria vaccine would be a welcomed development. The process of developing malaria vaccines against the *Plasmodium* parasite is challenging mainly due to the complexities of its lifecycle (human and mosquito) and evolving protein mutations on the parasite (Arama and Troye-Blomberg, 2014). Decades of research have gone into overcoming these challenges to bring malaria vaccines to the market.

The WHO 2021 approved the first malaria vaccine, RTS,S/AS01 (Mosquirix) for wider public health use (WHO, 2021c). RTS,S/AS01 is based on the circumsporozoite protein (CSP) of the *P. falciparum* 3D7 clone (RTS, 2015). The vaccine underwent clinical trials in countries with moderate to high *P. falciparum* malaria reducing clinical malaria by 36% in 5 -17 months children in a 4-year follow-up (RTS, 2015). In 2023 the WHO pre-qualified the second malaria vaccine, R21/Matrix-M (WHO, 2023d) and recommended it for wider usage (WHO, 2024b). The R21/Matrix-M vaccine contains a higher proportion of the malaria antigen (CSP) compared to RTS,S making it more effective. The R21/Matrix-M showed a higher efficacy of up to 77% in reducing clinical malaria in 5 -36-month-old children (Datoo et al., 2021). But vaccines alone cannot drive malaria to elimination. Recent studies are showing that a novel approach of combining RTS,S/AS01 vaccine with seasonal malaria chemoprevention (SMC) increases malaria protection in children (Dicko et al., 2024). Even as malaria vaccines are rolled out in Africa, there are impending challenges to vaccine production capacity and long-term funding to ensure these lifesaving products reach the communities in need.

1.6.3 Malaria vector control

Before the 2000 millennium, vector control campaigns by environmental management achieved tremendous success in controlling and eliminating malaria and other diseases in multiple countries. In Malaysia, Sir Malcolm Watson led vector control initiatives that involved an initial careful understanding of local vector ecology followed by marsh drainage, mosquito habitat modification, and forest clearing around people's housing to control *An. umbrosus* and *An. maculatus* and leading to eventual malaria elimination (Field and Reid, 1956). A similar approach was used with house screening and the distribution of the antimalarial drug quinine to eliminate malaria and the Italian vector *An. labranchiae* (Majori, 2012). Similar environmental management strategies by drainage clearing, habitat oiling, house screening, quinine prophylaxis, and use of untreated bed nets led to malaria suppression in the Zambian copper belt between 1920 -1949 (Freund, 1986).

The beginning of the 20th century witnessed a shift in malaria vector control strategies with concerted global lobbying and multisectoral partnerships to

increase access to control strategies with high public health impact in Africa. These have mainly been through mass distribution of insecticide-treated bed nets (ITNs), case management, and spraying house walls with residual insecticides (IRS). The WHO recommends universal ITN coverage of 80% in terms of access and usage with at least one net per every two persons at risk (Koenker et al., 2018). There are now over 2 billion nets distributed in Africa, albeit with stark variabilities in access and usage across the continent (Bertozzi-Villa et al., 2021). About 46 countries have IRS as part of their control programs but with focal deployment mostly in high and year-round malaria transmission areas (Tangena et al., 2020). Together, these two vector control strategies have had a tremendous impact on the malaria burden with a ~70% reduction in malaria mortality and over 600 million cases averted (Bhatt et al., 2015).

1.7 Insecticide resistance - an evolving threat to malaria vector control

The stalling gains in malaria control have been attributed to the widespread evolution of insecticide resistance in mosquito vector populations (Ranson and Lissenden, 2016a). The WHO classifies increasing vector resistance as a biological threat to malaria control and elimination (WHO, 2022a). Insecticide resistance is the selection of a heritable characteristic in an insect vector population that results in the repeated failure of an insecticide product to provide the intended level of control when used as recommended (Nauen, 2007). The overreliance on insecticide-based ITNs and IRS for malaria control have led to unprecedented incidences of vectors evolving resistance to the insecticides (Ranson and Lissenden, 2016a, Ranson et al., 2011). Countries monitor for resistance in mosquitoes by exposing them to a discriminating insecticide dose in a bioassay (WHO, 2022b). Across Africa, outcomes from such bioassays have shown a concerning trend of increasing pyrethroid resistance phenotypes in malaria vectors between 2000 - 2015 (Hancock et al., 2020). Five insecticide classes are recommended to control adult *Anopheles* vectors: pyrethroids, carbamates, organophosphates, organochlorines, and neonicotinoids. Major malaria vectors are now resistant to at least one insecticide class recommended for vector control (Ranson and Lissenden, 2016a, Hemingway et al., 2016). The threat of insecticide resistance has spurred intense interest in using proactive insecticide resistance management (IRM) to deploy vector control strategies. Control programs are now

integrating measures to monitor phenotypic insecticide susceptibility and underlying resistance mechanisms to select appropriate insecticide products to manage resistance.

Establishing the direct epidemiological link between insecticide resistance and increased community malaria can be challenging. A WHO-coordinated study in five countries from Africa and Asia found no evidence that insecticide resistance led to increased malaria incidence (Kleinschmidt et al., 2018b). However, recent evidence shows that changes in vector control insecticides in areas with high resistance significantly reduce malaria incidence. For instance, combining the pyrethroid insecticide with a synergist, piperonyl butoxide (PBO), enhances the effectiveness of the pyrethroid making it better at killing mosquitoes and abating blood-feeding when compared to pyrethroid-only ITNs (Gleave et al., 2021). Combining pyrethroids with a pyrrole (chlorfenapyr) which targets the energy production system in the mosquito (oxidative phosphorylation), also shows similar superiority compared to standard ITNs (Mosha et al., 2024, Accrombessi et al., 2023a). In response to the increasing vector resistance, the WHO has recently updated its guidelines recommending the mass deployment of chlorfenapyr-pyrethroid and PBO-pyrethroid ITNs in areas of high pyrethroid resistance (WHO, 2023e).

1.8 Insecticide resistance surveillance

Vector resistance surveillance is a core strategy important for making data-driven resistance management decisions. Routine physiological resistance monitoring to determine phenotypic resistance is conducted following either the WHO or Centre for Disease Control and Prevention (CDC) bottle assays; in which mosquitoes are exposed to standard concentrations of an insecticide and either mortality or knock-down measured after a specific time (WHO, 2022b). When resistance is confirmed, molecular assays can be used to establish the underlying mechanisms which may include metabolic detoxification enzymes, target site mutations, cuticular thickening which reduces insecticide penetration, or behavioral avoidance to reduce exposure to insecticides (Liu, 2015). Details of these resistance mechanisms including their spatial distribution across Africa are discussed in chapter II of this thesis.

Integration of genomic surveillance to proactive insecticide resistance management for the deployment of vector control strategies is increasingly apparent (Hancock et al., 2024). Genomic surveillance in vectors includes the use of genomic data for the identification of known resistance alleles in vector populations (Donnelly et al., 2016), tracking the change in frequencies in resistance alleles over time and pre-post-interventions (Lynd et al., 2023), the identification of new mutations using signatures of genomic selection on putative resistance alleles (Vontas et al., 2018), as well as genome-wide association studies linking mutations with resistance phenotypes (Lucas et al., 2023). Genomic surveillance has led to the discovery of new resistance mechanisms in the form of copy number variants (Lucas et al., 2019, Weetman et al., 2018) in addition to previously unknown polymorphisms in the *An. funestus*, *An. gambiae* and *An. coluzzii* voltage-gated sodium channel (*Vgsc*) gene (Odero et al., 2024a, Clarkson et al., 2021).

1.9 New tools and vector control approaches

With the core vector control tools nearing their limits, it is important to develop alternative complementing tools and technologies that can help drive malaria to elimination. Such tools include attractive targeted sugar baits (Müller et al., 2010), systemic insecticides and endectocides (Khaligh et al., 2021), and sterile insect techniques (Alphey et al., 2010). This can also include the genetic manipulation of vectors for disease control (Bier, 2021). Genetic manipulation of vectors involves deliberately releasing individuals with a desirable genetic trait to introduce this trait into the extant population via mating (Nolan, 2021). The most widely used technology to genetically manipulate mosquito vectors is gene drive (Bier, 2021). Gene drive refers to the ability of one or more genetic elements to be inherited at a greater rate than that predicted by Mendelian genetics, leading to a rapid increase in their frequency within a population. This can be either through population suppression by targeting genes responsible for vector lifespan, mortality, and reproduction or through population replacement by targeting genes promoting vector resistance to pathogens or deleting genes that facilitate pathogen infection (Nolan, 2021, Bier, 2021). With the invention of CRISPR-Cas9 technology (Doudna and Charpentier, 2014), genetic manipulation by gene drive in *Anopheles* mosquitos has rapidly advanced albeit with a disproportionate focus

on *An. gambiae*, with the assumption that resultant genetic constructs can be easily introgressed in related sibling species *An. arabiensis* and *An. coluzzii* (Nolan, 2021). However, a holistic gene drive approach targeting the increasingly important malaria vector *An. funestus* is needed. Gene drive is a promising high-impact tool that could potentially drive malaria to elimination, however, multiple gaps especially in ethics and community engagement should be bridged before any field deployments (James et al., 2018, Knols et al., 2007).

1.10 Vector population genetic structure

Malaria vectors *An. gambiae, An. coluzzii, An. arabiensis* and *An. funestus* has a wide geographical range encompassing varying ecologies and landscapes (Tene Fossog et al., 2015). Understanding population structure is important for predicting the spread of beneficial genes in populations such as insecticide resistance genes or gene drive constructs. Early African-wide microsatellites analysis on *An. gambiae* found two populations; a northwest composed of samples from West Africa, the DRC, and Kenya, and a southeastern cluster of mostly southern African samples (Lehmann et al., 2003). A recent analysis spanning similar vector ranges of *An. gambiae* and *An. coluzzii* found a similar clustering pattern using whole genome sequence analysis (Anopheles gambiae Genomes, 2020, Anopheles gambiae Genomes et al., 2017). *Anopheles funestus* is continentally structured into eastern, western, and central African genetic populations (Michel et al., 2005). Genetic diversity studies in southern African countries have revealed finer-scale variations within the population clusters. Barnes *et al* analyzed samples from Malawi, Zambia, and Mozambique and found strong north-south segregation within mosquitos from Malawi and Zambia, indicating high levels of gene flow (Barnes et al., 2017a). However, within Malawi, they also found high F_{ST} values between southern populations when compared to those in the north, indicating the presence of a gene flow barrier (Barnes et al., 2017a). Analysis of additional *An. funestus* samples from Uganda and Zimbabwe later corroborated the same diversity observed in the southern African region (Kaddumukasa et al., 2020). In Kenya, *An. funestus* collected from the western part of the country were found to be genetically distinct from coastal populations (Ogola et al., 2019). These studies implicate large geographical feature as a

possible barrier to gene flow between populations. Genetic structuring has also been observed within and between island *An. funestus* populations in Comoros and Madagascar (Ayala et al., 2006). Ayala *et al* found samples from the two islands to cluster separately but also observed in-country structure within Madagascar, which might be driven by landscape features and isolation by distance. As *An. funestus* undergoes speciation across the continent, the role of these observed diversities should be investigated on how they influence vector adaptation, dispersal, and potential vectorial capacity.

1.11 The Structure of this Thesis

In this thesis, I focused on providing a comprehensive understanding of the genetic diversity and gene flow patterns of *Anopheles funestus* populations across Tanzania. The population genomic analysis serendipitously detected knockdown resistance (*kdr*) in *An. funestus*, which apart from showing the potential linkages with organic pollutants also revealed the impact of environmental pollution on insect evolution. Further, the analysis also revealed two distinct *An. funestus* population with varied demographic differences and divergence. Alongside the genetic analysis, I also investigated the insecticide resistance profile of these vectors to public health insecticides and genotyped them at known resistance markers. In addition to establishing a baseline chart of insecticide resistance in Tanzania, this analysis was also important in understanding recent gene flow patterns. Finally, I conducted a supplementary investigation to explore ecological differences in these mosquitoes.

1.11.1 Chapter II: A Review of the Advances in the Genetic Characterization of the Malaria vector, *Anopheles funestus*, and Implications for improved surveillance and Control

In this introductory chapter, I begin by reviewing the recent molecular advances in the genetic characterization of *An. funestus* across Africa. I review a selection of these advances and their potential applications along four key research themes relevant to the biology and control of *An. funestus* in Africa, as follows: i) taxonomic and systematic characterization of the vector, its sibling species, and their roles in malaria transmission in different settings; ii) improvements in insecticide resistance profiling and associated technologies; iii) improved

understanding of the genetic structure and gene flow patterns between and within population clusters of the vector species, and iv) potential applications of genetic technologies for surveillance and control of the species. The review provides a basis for identifying key research gaps and opportunities for an R&D agenda relevant to the surveillance and control of *An. funestus* and malaria transmission.

A version of this chapter was published in *Malaria Journal* in August 2023.

Odero, J.O., Nambunga, I.H., Wangrawa, D.W. *et al*. Advances in the genetic characterization of the malaria vector, *Anopheles funestus,* and implications for improved surveillance and control. *Malar J* 22, 230 (2023). https://doi.org/10.1186/s12936-023-04662-8

My contributions to this publication were: Designing the manuscript framework and scope, writing the first and subsequent drafts of the manuscript, and reviewing and approving the final version of the manuscript.

1.11.2 Chapter III: Genetic Markers Associated with the Widespread Insecticide Resistance in Malaria Vector *Anopheles funestus* Populations Across Tanzania

In this chapter, I comprehensively analyse the insecticide resistance phenotypes and genotypes *An. funestus* mosquitoes in nine regions representing different eco-epidemiological settings in Tanzania. The chapter aims to understand the potential responsiveness of these mosquitoes to public health insecticides, generate insights for more effective strategies for malaria control, and establish a baseline chart of insecticide resistance in Tanzania's *An. funestus*. Additionally, this analysis is crucial in having a fundamental understanding of recent gene flow patterns in Tanzania. I screened the mosquitoes for five major genes and structural variants that confer metabolic resistance and conducted standard susceptibility tests against pyrethroids, carbamates, organophosphates, and organochlorides. This chapter represents the first large-scale survey of the insecticide resistance patterns in *An. funestus*, which despite being a dominant vector in the country, remains far less investigated compared to other malaria vectors.

A version of this chapter was published in *Parasites Vectors* in May 2024.

Odero, J.O., Nambunga, I.H., Masalu, J.P. *et al*. Genetic markers associated with the widespread insecticide resistance in malaria vector *Anopheles funestus* populations across Tanzania. *Parasites Vectors* 17, 230 (2024). https://doi.org/10.1186/s13071-024-06315-4

My contributions to this publication were: Performing field experiments and sample collections, conducting laboratory assays, analyzed the data and writing initial and subsequent drafts of the manuscript, developing the interactive map for visualizing resistance phenotypes, and reviewing and approving the final version of the manuscript.

1.11.3 Chapter IV: Discovery of knock-down resistance in the major African malaria vector *Anopheles funestus*.

In this chapter, I report the findings of phenotypic and genomic surveillance done in Tanzania to understand the evolution and spread of insecticide resistance in *Anopheles funestus* - the dominant malaria vector in Eastern and Southern Africa. I report the first discovery of knock-down resistance (*kdr*) mutations in *An. funestus*. I discover that, in Tanzanian *An. funestus*, this mutation confers resistance to DDT, but not deltamethrin, despite a complete ban on DDT use for agriculture and vector control in Tanzania since 2008. I conclude the chapter by suggesting environmental contamination from extensive DDT stockpiles, or unofficial agricultural use of DDT, as plausible causes of the *kdr* evolution.

A version of this chapter was published in *Molecular Ecology* in October 2024.

Odero JO, Dennis TPW, Polo B, Nwezeobi J, Boddé M, Nagi SC, Hernandez-Koutoucheva A, Nambunga IH, Bwanary H, Mkandawile G, Govella NJ, Kaindoa EW, Ferguson HM, Ochomo E, Clarkson CS, Miles A, Lawniczak MKN, Weetman D, Baldini F, Okumu FO. Discovery of Knock-Down Resistance in the Major African Malaria

Vector Anopheles funestus. Mol Ecol. 2024 Oct 7:e17542. doi: 10.1111/mec.17542. Epub ahead of print. PMID: 39374937.

My contributions to this chapter were: Field collection of mosquito samples, laboratory analysis, data acquisition and management, and preparing samples for whole genome sequencing, data analysis and generation figures and tables, and wrote initial and subsequent drafts of the chapter.

1.11.4 Chapter V: Genomic Analysis Reveals Distinct Genetic Populations of The Malaria Vector *Anopheles funestus* in Tanzania

In this chapter, I use whole genome sequence data from Chapter IV to investigate the population genetic structure of *An. funestus* populations in Tanzania. I compare genetic diversity within these populations and quantify genetic differentiation between them. I also perform haplotype clustering and genome-wide selection scans to detect signatures of recent positive selection. I conclude the chapter by hypothesizing that the observed population clustering could be driven by potential genetic discontinuities such as topography, ecology and behavioral differences, or major geographical features such as the Great Rift Valley.

My contributions to this chapter were: Field collection of mosquito samples, laboratory analysis, data acquisition and management, and preparing samples for whole genome sequencing, data analysis and generation figures and tables, wrote initial and subsequent drafts of the chapter.

1.11.5 Chapter VI: Ecological and Behavioral Correlates of The Genetic Variations in *Anopheles funestus* Populations Across Mainland Tanzania

During a large-scale field study on genetic variations in *Anopheles funestus* populations across Tanzania (Chapter V), I conducted a supplementary investigation to explore ecological differences in these mosquitoes. The primary

objective was to examine variations in key vector bionomics, as well as phenotypic and transmission traits that might be linked to the observed genetic differences between inland populations and those from coastal, lower-altitude regions. While the biting behaviour of *An. funestus* (primarily indoors), the level of anthropophily, and age structure were similar across populations, differences were noted in malaria parasite infections, mosquito sizes, and fecundity between the inland and coastal genetic populations. Further research is needed to determine whether these phenotypic and demographic differences are a consequence of, or contribute to, the observed genetic separation between inland and coastal populations.

My contributions to this chapter were: Field collection of mosquito samples, laboratory analysis, data acquisition and management, data analysis and generation figures and tables, wrote initial and subsequent drafts of the chapter.

1.11.6 Chapter VII: General Discussions

In this final chapter, I put into a wider perspective the main findings from this thesis. I also look into the future on the potential pragmatic applications of the thesis findings on the control of *An. funestus* in Tanzania and beyond.

2 Advances in the Genetic Characterization of the Malaria Vector, *Anopheles funestus,* and Implications for Improved Surveillance and Control

2.1 Abstract

Anopheles mosquitoes present a major public health challenge in sub-Saharan Africa, notably, vectors of malaria kill 600,000 people annually. In parts of the east and southern Africa region, one species in the *An. funestus* group, *Anopheles funestus,* has established itself as an exceptionally dominant vector in some areas, it is responsible for more than 90% of all malaria transmission events. However, compared to other malaria vectors, the species is far less studied, partly due to difficulties in laboratory colonization and the unresolved aspects of its taxonomy and systematics. Control of *An. funestus* is also increasingly difficult because it has developed widespread resistance to public health insecticides. Fortunately, recent advances in molecular techniques are enabling greater insights into species identity, gene flow patterns, population structure, and the spread of resistance in mosquitoes. Here, we review these advances and their potential applications with a focus on four research themes relevant to the biology and control of *An. funestus* in Africa, namely: i) the taxonomic characterization of different vector species within the *An. funestus* group and their role in malaria transmission; ii) insecticide resistance profile; iii) population genetic diversity and gene flow, and iv) applications of genetic technologies for surveillance and control. The research gaps and opportunities identified in this review will provide a basis for improving the surveillance and control of *An. funestus* and malaria transmission in Africa.

2.2 Introduction

Malaria transmission is driven by female *Anopheles* mosquitoes. In Africa, the four major malaria vectors are *Anopheles gambiae, Anopheles coluzzii, Anopheles arabiensis,* and *Anopheles funestus* s.s. Different *Anopheles* species exhibit contrasting behaviours and physiological responses to interventions such as insecticide-treated nets (ITNs) and indoor residual spraying (IRS), which are the core WHO-recommended vector control methods (WHO, 2023c). For instance, *An. gambiae, An. coluzzii,* and *An. funestus* s.s predominantly prefer blood-feeding on humans (anthropophilic) and resting indoors (endophilic) (Akogbeto et al., 2018, Kabbale et al., 2013), whereas *An. arabiensis* is a more generalist vector, biting humans but also readily feeding on domestic animals (zoophilic) and resting both outdoors (exophilic) and indoors (Tirados et al., 2006). However, some evidence indicates that *An. funestus* s.s is shifting resting and feeding behaviour to the outdoors (Kreppel et al., 2020) and sometimes during daytime in some settings as an adaptation to indoor interventions (Sougoufara et al., 2014). Models predict such behavioural shifts coupled with vector resistance could have devastating public health outcomes (Sherrard-Smith et al., 2019). Additionally, such innate or evolving differences in behaviors between vector species mean that interventions can have differential effects depending on local vector ecology.

Anopheles funestus s.s., hereafter referred to as *An. funestus*, is emerging as one of the most important malaria vectors and is increasingly dominant even in areas where it co-occurs with the other prominent vector species. For instance, in certain parts of Tanzania, *An. funestus* contributes consistently more than 90% of the yearly entomological inoculation rate (EIR) even in areas where it is far less abundant than *An. arabiensis* (Matowo et al., 2021, Kakilla et al., 2020, Kaindoa et al., 2017, Lwetoijera et al., 2014). A similar trend of vectorial dominance has been observed in Cameroon where the vector contributes over 70% EIR in certain villages (Djamouko-Djonkam et al., 2020, Cohuet et al., 2004), in Burkina Faso where it seasonally dominates transmission (Soma et al., 2020), and in Malawi (Riveron et al., 2015), Zambia (Stevenson et al., 2016), and Kenya (McCann et al., 2014b) where it is established as the primary malaria vector. Given the observed competence of *An. funestus* in the East and Southern Africa and in parts of Central and West Africa, it might be reasonable to generate a continent-wide comparative

assessment on the importance of *An. funestus* in malaria transmission in different settings.

An. funestus belongs to the *An. funestus* group of mosquitoes, hereafter referred to as AFG, which has at least 10 other African sibling species, namely: *An. funestus-like, An. parensis, An. vaneedeni, An. aruni, An. confusus, An. leesoni, An. longipalpis, An. rivulorum, An. rivulorum-like, An. brucei,* and *An. fuscivenosus* (Coetzee, 2020). Some of these species are morphologically indistinguishable at the adult stage, though experienced entomologists can distinguish some species at the aquatic stage using identification keys (Coetzee, 2020). Collectively, the AFG mosquitoes have a wide geographical distribution across sub-Saharan Africa (Figure **2-1**). *An. funestus, An. leesoni* and *An. rivulorum* occur in most tropical Africa, while *An. parensis, An. confusus,* and *An. aruni* are localized to eastern African countries. *Anopheles vaneedeni, An. parensis*, *An. fuscivenosus, An. funestus*-like and *An. longipalpis* are native to southern African countries while *An. rivulorum-like* and *An. brucei* are also frequent in West and Central Africa (Dia et al., 2013, Sinka et al., 2012, Mouatcho et al., 2018). Within the AFG, *An. funestus* is considered the main malaria vector across Africa due to its preferentially anthropophilic nature (Takken and Verhulst, 2013) and having the highest malaria infection rates (Kaindoa et al., 2017) However, other members in this group have also been shown to be naturally infected by malaria parasites. For instance, *An. rivulorum* were observed to be active early in the night, outdoors, and were found to be carrying *Plasmodium falciparum* in Tanzania and Kenya (Kinya et al., 2022, Wilkes et al., 1996). Similar observations have been made on *An. longipalpis* in Kenya (Ogola et al., 2018), *An. vaneedeni* in South Africa (Burke et al., 2017), and *An. parensis* in Uganda (Mulamba et al., 2014a) and South Africa (Burke et al., 2019) where they are considered to contribute to residual malaria transmission. While *An. funestus* is likely the most important vector in this group in most settings, others may play a role as secondary vectors. Thus, it is important to ensure accurate and robust methodologies to distinguish members of this group as required to assess their relative contribution to residual transmission.

In many countries, malaria vector control is also increasingly difficult because of widespread resistance to public health insecticides (Ranson and Lissenden,

2016b). Compared to other malaria vectors, *An. funestus* populations are increasingly surviving diagnostic doses of pyrethroids in bioassays (Pinda et al., 2020). Experimental work has shown that the increasing resistance in this vector significantly reduces the effectiveness of ITNs (Mugenzi et al., 2019). Despite the elusiveness of quantifying the impact of vector resistance on malaria epidemiology at the community level (Kleinschmidt et al., 2018a), recent data from an area with highly resistant *An. funestus* as the primary vector (Matowo et al., 2023, Matowo et al., 2021) indicates the superiority of dual active ITNs compared to standard ITNs in reducing malaria cases, suggesting a key role of resistant *An. funestus* in malaria transmission (Mosha et al., 2022). As physiological resistance continues to spread in Africa, it is important to understand its underlying mechanisms to inform more effective control strategies. Since the spread of resistance genes in mosquito populations is influenced by gene flow (Caprio and Tabashnik, 1992), population genetics using whole genome sequencing provides unique insights into the structure of malaria vectors, as recently shown in *An. gambiae* (Anopheles gambiae Genomes et al., 2017). This approach also opens avenues for discovering new resistance mechanisms (Lucas et al., 2019) and could generate essential information for the development and deployment of genetic control tools such as gene drives (Hammond et al., 2021).

Previous reviews have outlined recent advances in characterizing chromosomal inversion and the development of PCR molecular diagnostic assays for *An. funestus* (Coetzee and Fontenille, 2004b), and the advances in insecticide resistance profiling and population genetics of *An. funestus* (Coetzee and Koekemoer, 2013). However, despite a significant increase in research on these topics over the last ten years, there has not been a recent review publication of current knowledge on the molecular basis of insecticide resistance, gene flow patterns, and fine-scale population structuring of *An. funestus*. This is warranted as advances in molecular techniques in the last decade can enable greater insights into the real identity of vector species and how the resistance genotypes are spreading within and between mosquito population clusters. There have also been recent improvements in the *An. funestus* reference genome (Ayala et al., 2022a, Ghurye et al., 2019), which can provide newer insights into the genetic basis of the mosquitoes' behavioural and physiological attributes.

Here, we review a selection of these advances and their potential applications along four key research themes relevant to the biology and control of *An. funestus* in Africa, as follows: i) taxonomic and systematic characterization of the vector, its sibling species, and their roles in malaria transmission in different settings; ii) improvements in insecticide resistance profiling and associated technologies; iii) improved understanding of the genetic structure and gene flow patterns between and within population clusters of the vector species, and iv) potential applications of genetic technologies for surveillance and control of the species. The review provides a basis for identifying key research gaps and opportunities for an R&D agenda relevant to the surveillance and control of *An. funestus* and malaria transmission.

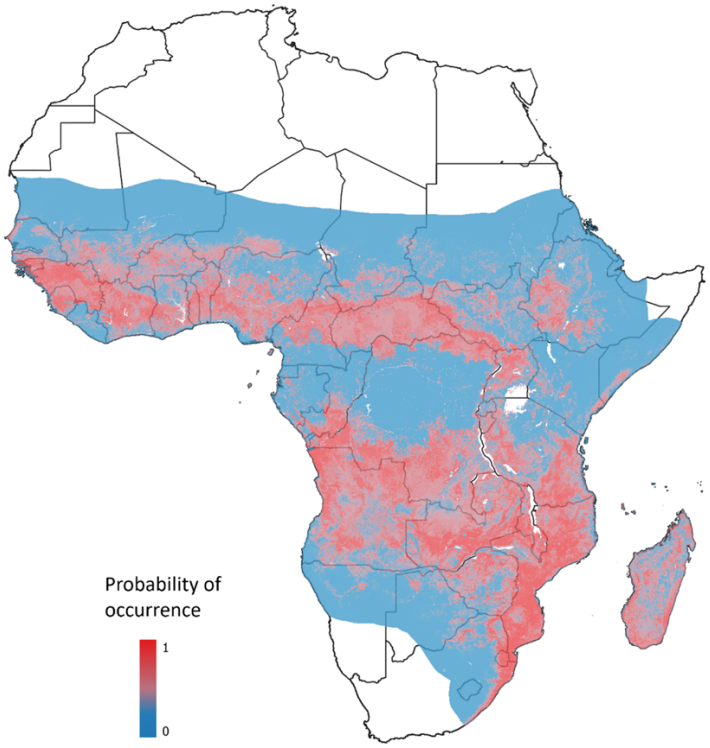


Figure 2-1: The distribution of the *Anopheles funestus* group in Africa. The red areas indicate countries with the confirmed presence of at least one member in the group while the blue area indicates areas where the species is not yet recorded. The map was created in R software (v 4.2.1) and QGIS (v 3.18) using raster data sourced from Malaria Atlas Project (https://malariaatlas.org/).

2.3 Molecular approaches to identify members of the *An. funestus* group

Morphological keys for the identification of members of the AFG and other African *Anopheles* mosquitoes were established between 1968 (MT and Maureen, 1987) and recently updated in 1987 (MT and Maureen, 1987) and more recently in 2020

with an improved description of many *Anopheles* species complexes and groups (Coetzee, 2020). This method of identification requires well-trained taxonomists, and either field collection of aquatic stages that are reared to the adult stage, which takes between 2-4 weeks, or trap collection of adults. Even when samples suitable for morphological identification are available, some members of the AFG are challenging to distinguish taxonomically at either stage, necessitating the adoption of molecular methods (**Table 2-1**).

Before 1990, *Anopheles* identification was primarily done by a combination of morphological characterization (MT and Maureen, 1987) and cytogenetic analysis of chromosomal inversions (Green and Hunt, 1980, Mahon et al., 1976, Hunt, 1973). Cytogenic analysis is however a tedious process, stage and sex-specific, and requires semi-gravid females (Hunt, 1973). Efforts to develop molecular diagnostics for the *An. funestus* group began in the 1990s with a polymerase chain reaction (PCR) coupled with restriction fragment length polymorphism (PCR-RFLP) targeting the 28S ribosomal gene to distinguish between *An. funestus* and *An. vaneedeni* (Koekemoer et al., 1998). This was later expanded to a single-strand conformation polymorphism (SSCP) assay, targeting the same gene, to distinguish *An. funestus*, *An. vaneedeni*, *An. rivulorum*, and *An. leesoni* (Koekemoer et al., 1999). Overlap in DNA banding patterns between the renatured and denatured single strands necessitated the development of a further assay, targeting ribosomal internal transcribed spacer 2 (ITS2), to distinguish the two medically important vectors, *An. funestus* and *An. rivulorum* (Hackett et al., 2000). This eventually laid the ground for the development of the AFG multiplex PCR assay, which is a cocktail PCR assay that can distinguish five members (*An. funestus, An. rivulorum, An. vaneedeni, An. leesoni*, and *An. parensis*) of the AFG in a single run (Koekemoer et al., 2002a). However, an initial challenge observed with the AFG multiplex PCR assay, was that its performance was not generalizable to some parts of Africa, especially Central and West Africa where it could not readily identify *An. rivulorum* (Cohuet et al., 2003). Further analysis revealed the presence of *An. rivulorum*-like as a separate species (Cohuet et al., 2003), which was later also confirmed in South Africa (Mouatcho et al., 2018). Similarly in Malawi, the AFG multiplex PCR assay failed to identify samples morphologically characterized into the AFG but were later designated as *An. funestus*-like by using cytogenesis and DNA-based methods (Spillings et al., 2009). Following these

discoveries, the AFG multiplex PCR assay has since been expanded to include species-specific primers for *An. funestus*-like and *An. rivolurum*-like.

Given several challenges in AFG species identification, it is key to combine different approaches, such as morphological and molecular data that could be used together with geographical location data to infer the species. For example, although *An. leesoni* is closely related to the Asian vector *An. minimus*, it is considered a separate species based on its geographical separation. Similarly, and indeed an often-neglected challenge is the inclusion of non-AFG samples in a diagnostic PCR assay, which may erroneously identify them as a member of the AFG (Erlank et al., 2018). For example, a member of the *An. marshalli* group or *An. gambiae* complex mistakenly included in the AFG multiplex PCR assay would be erroneously identified as *An. leesoni* (Erlank et al., 2018)). Therefore, careful sample handling and morphological identification are key to improving this analysis. Accurate species identification is also critical for epidemiological studies, for example, the malaria parasite was recently reported from *An. longipalpis* C, considered not to be a malaria vector (Kent et al., 2006), and a molecularly unidentified cryptic species within the AFG (Kinya et al., 2022); similarly to findings in cryptic species within the *An. gambiae* complex (Anopheles gambiae Genomes, 2020). These new findings highlight the need to acknowledge mosquito species whose identity is not fully resolved as potential vectors and the need for field estimates of malaria transmission to incorporate the *Anopheles* species found in an area more broadly. Lastly, the employment of vector genomic surveillance, even on a small scale, should be established as part of routine national vector monitoring programs. Country-level investments are therefore necessary to enhance training for control programs to better integrate taxonomy and molecular techniques to improve species identification and incrimination.

Table 2-1: Molecular diagnostic approaches to distinguish members of the *An. funestus* group.

Diagnostic method	Approach	Members distinguished	Advantages	Limitations	Reference
PCR-RFLP-1	PCR primers developed from *An. funestus* D3 region of 28S ribosomal gene amplified and digested using restriction endonuclease *HpaII*	*An. funestus* and *An. vaneedeni*	Ability to distinguish two morphologically similar species.	Limited to only two members of the *An. funestus* group. Minimal sequence variation between the two species poses the challenge of specificity when other members are added to the assay. Post PCR processing	(Koekemoer et al., 1998)
Single-strand conformation polymorphism (SSCP) PCR	PCR primers developed from *An. funestus* D3 region of 28S ribosomal gene amplified and separated based on DNA conformation on SSCP gels	*An. funestus*, *An. vaneedeni*, *An. rivulorum*, and *An. leesoni*	Ability to distinguish four members of the *An. funestus* group. Can distinguish east from West African *An. funestus*	Overlap on the denatured single strand (DSS) banding patterns for *An. funestus* and *An. vaneedeni* Post PCR processing	(Koekemoer et al., 1999)
Internal transcribed spacer 2 (ITS2)	PCR amplification using internal transcribed spacer 2 (ITS2) region primers	*An. funestus* and *An. rivulorum*	Differentiating two sympatric vectors in the *An. funestus* group	Limited to only two members within the group Post PCR processing	(Hackett et al., 2000)
ITS2 Cocktail PCR		*An. funestus, An. rivulorum, An. vaneedeni, An. leesoni, An. parensis, and An. rivulorum-like*	Simultaneous identification of six members within the group in a single PCR run	Post PCR processing	(Cohuet et al., 2003, Koekemoer et al., 2002b)
PCR-RFLP-2	PCR amplification using AFG multiplex PCR assay followed by *Eco*RI RFLP digestion	*An. funestus, An. funestus*-like, *An. parensis, Anopheles rivulorum, An. vaneedeni, An. leesoni, and An. longipalpis*-type C	Identification of *An. longipalpis*-type C from the other members of the AFG	Post PCR processing and requirement of enzyme digestion	(Choi et al., 2010)

2.4 Population genetics of *An. funestus*

Understanding mosquito populations enables the assessment of the feasibility and potential impact of genetic control approaches such as gene drives for disease control. It also provides a basis for monitoring the spread of genetic traits such as insecticide-resistance alleles. Molecular techniques ranging from chromosomal inversions, mitochondrial DNA analysis, restriction fragment length polymorphisms (RFLP), microsatellite genotyping, and whole-genome sequencing (WGS) have been employed to provide insights into patterns of *An. funestus* population interactions and structuring (Weedall et al., 2020, Koekemoer et al., 2006, Michel et al., 2005, Costantini et al., 1999). We highlight below how these techniques have been useful in advancing studies in *An. funestus* population genetics.

2.4.1 Chromosomal inversions

Chromosomal inversions in mosquitoes are important drivers of local adaptation to varying environmental factors (Mugenzi et al., 2019, Kirkpatrick and Barton, 2006). This is important for *An. funestus* that has a wide geographical range across Africa (Figure **2-1**) and high levels of chromosomal polymorphisms (Coetzee and Fontenille, 2004a). The technique for studying inversions was developed in the 1970s and follows a process where the ovaries of half-gravid females are squashed, stained, and observed under a phase contrast microscope to reveal the polytene chromosomes (Hunt, 1973), which are then scored using a chromosome map developed by Sharakhov *et al* (Sharakhov et al., 2004).

Constantini *et al* first identified two chromosomal forms of *An. funestus* in Burkina Faso, namely Kiribina and Folonzo, based on 3Ra, 3Rb, and 2Ra inversions (Guelbeogo et al., 2014, Costantini et al., 1999). The Kiribina form is distinguished by an inversion in the 2R and 3R chromosomes, while Folonzo has inversions 3Ra and 3Rb (Costantini et al., 1999). Both ecotypes are sympatric, highly anthropophilic, and contribute significantly to malaria transmission in their localities (Guelbeogo et al., 2014). In Cameroon, Folonzo was found to occupy the equatorial zones of the country while Kiribina occupied the dry savannah regions (Cohuet et al., 2005). Similarly, Ayala *et al* found differentiation in chromosomal inversions in *An. funestus* collected from different ecologies in the same country (Ayala et al., 2011) with the inversions causing a reduction in nucleotide diversity

resulting in overall low genetic differentiation among the ecotypes (Kamdem et al., 2017). In Angola, five inversions were detected, 2Ra, 2Rh, 3Ra, 3Rb, and 3La, with only samples from central Angola (Huambo province) designated as Folonzo chromosomal forms (Boccolini et al., 2005). Taken together, these studies suggest an important association between chromosomal inversions and adaptation to diverse ecological conditions, which might be contributing to the spatial and temporal extension of malaria transmission (Fontenille and Simard, 2004).

Cytogenic karyotyping is still largely based on a tedious technique that requires highly skilled personnel and cannot be used on mosquito life stages other than gravid females, thus constraining the scalability of its use. Hence, a genotyping assay has recently been developed that utilizes tag single nucleotide polymorphisms (tag SNP) on the 3Ra, 3Rb, and 2Ra inversions to distinguish different *An. funestus* ecotypes (Lukindu et al., 2020). This new method has a high agreement with the traditional cytogenic karyotyping method and can be deployed on any mosquito life stage, making it more convenient for high throughput cytogenic studies. However, this method has some limitations as current tag-SNPs are suitable only for distinguishing between the two West-African ecotypes of *An. funestus*, Folonzo and Kiribina. Additional innovation will be necessary to develop high-throughput techniques that can be used for broader analyses of chromosomal inversions across the continent; especially in an era where genomic sequence data are increasingly available. This will enable investigation of how these inversions might influence vector dispersal and adaptability in the face of climate change.

2.4.2 Mitochondrial DNA analysis

Analysis of mitochondrial DNA (mtDNA) is useful in reconstructing mosquito phylogenetic relationships, molecular evolution, and understanding their population history, i.e. time of taxon divergence (Krzywinski et al., 2006). For example, Michel *et al* analyzed partial mitochondrial genes, NADH-ubiquinone oxidoreductase chain 5 protein (ND5) and Cytochrome c oxidase I (COI), from 11 countries across Africa, showing that *An. funestus* is genetically grouped into Eastern, Western, and Central populations, and detected two lineages I and II (Michel et al., 2005), defined as a group of mosquitoes that are ancestrally

connected by using maternally inherited mitochondrial genes. Whilst lineage I was widespread across sub-Saharan Africa, lineage II was found restricted to mosquitoes from Tanzania, Madagascar, Zambia, and Mozambique (Choi et al., 2012, Koekemoer et al., 2006, Michel et al., 2005) (**Figure 2-2**). Similarly, Jones *et al* analyzed 43 complete mitochondrial genomes of *An. funestus* from Zambia, the Democratic Republic of Congo, and Tanzania, identifying 41 unique haplotypes, comprising 567 polymorphisms (Jones et al., 2018). This study also detected two distinct yet partially sympatric, lineages of *An. funestus*, lineage I and II, estimating their divergence to half a million years ago. An analysis of these lineages in the context of plausible introgression within the AFG indicates a genetic exchange between *An. parensis* and *An. funestus* before its rapid geographic range expansion (Small et al., 2020). Recently, a PCR-based diagnostic using hydrolysis probe analysis has been developed to identify these lineages in field-collected samples (Choi et al., 2013) that could be expanded to improve the identification of lineages in *An. funestus*.

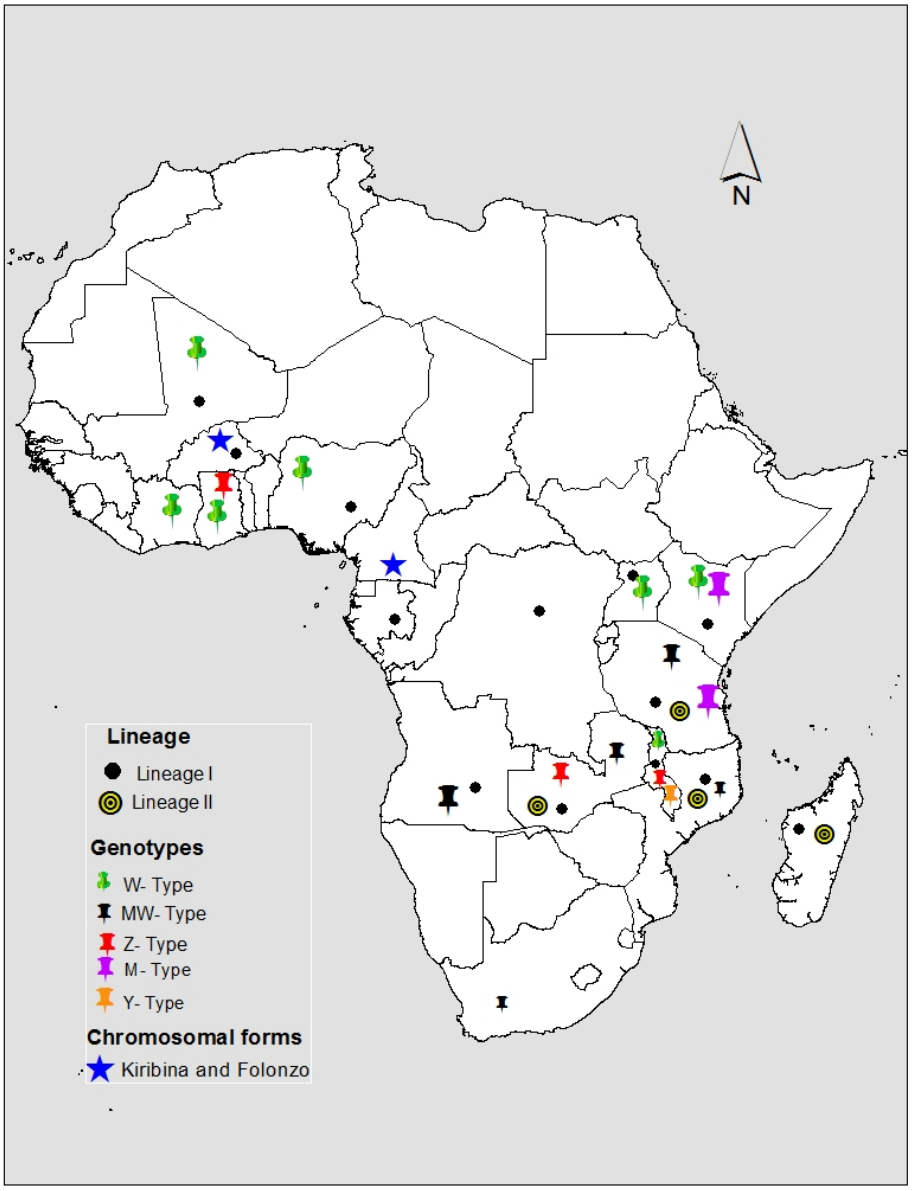


Figure 2-2: The distribution of *An. funestus*. (a) Lineages I and II (Jones et al., 2018, Michel et al., 2005) (b) RFLP genotypes, W - type, MW type, Z-type, M - type, and Y – type (Koekemoer et al., 2006) and (c) Chromosomal forms Kiribina and Folonzo (Costantini et al., 1999). The map was created using QGIS (v 3.18).

2.4.3 Restriction fragment length polymorphisms (RFLP) of ribosomal DNA

Geographical barriers to gene flow such as the Great Rift Valley have been hypothesized to influence how mosquitoes interact in space and time, shaping their population structure and adaptation. An RFLP analysis of a variable domain (D3) of the 28S ribosomal nuclear DNA in *An. funestus* mosquitos sampled from either the eastern or western sides of the Rift Valley found different RFLP profiles;

specifically W, M, and MW-types in the West, East, and Southern African countries respectively (Koekemoer et al., 2006). Notably, samples from Malawi, which is at the southern tip of the Rift Valley, had all the Y, Z, M, W, and MW genotypes (**Figure 2-2**), suggesting that major landscape features could be guiding the directional flow and geographical convergence of genes in mosquito populations and shaping their genetic profiles. Natural barriers to gene flow other than the Rift Valley, such as wildlife reserves and forests, urban landscapes, lakes, and, mountain ranges, should also be investigated to assess their effect on *An. funestus* diversity.

2.4.4 Microsatellite genotyping analysis

Once *An. funestus* microsatellite markers were physically mapped (Wondji et al., 2005, Sharakhov et al., 2004), they became the indicator of choice for studying population diversity, gene flow patterns, migration rates, population size, bottlenecks, and kinship. These markers are robust due to their codominant nature, neutrality, random repeats across genomes, and conformity to Mendelian inheritance (Selkoe and Toonen, 2006).

Continentally, microsatellite analysis shows that *An. funestus* subdivides into eastern, western, and central African genetic populations, broadly consistent with mitochondrial DNA patterns, but offering clearer resolution (Michel et al., 2005). Genetic diversity studies in southern African countries have revealed finer scale variation. Barnes *et al* analyzed samples from Malawi, Zambia, and Mozambique and found strong north-south segregation within mosquitos from Malawi and Zambia, indicating high levels of gene flow (Barnes et al., 2017a). However, within Malawi, they also found high F_{ST} values between southern populations when compared to those in the north, indicating the presence of a gene flow barrier (Barnes et al., 2017a). Analysis of additional *An. funestus* samples from Uganda and Zimbabwe later corroborated the same diversity observed in the southern African region (Kaddumukasa et al., 2020). In Kenya, *An. funestus* collected from the western part of the country were found to be genetically distinct from coastal populations (Ogola et al., 2019); similar to the pattern observed previously using chromosomal inversion analysis (Kamau et al., 2002). These studies implicate the Rift Valley as a possible barrier to gene flow between populations. Genetic

structuring has also been observed within and between island *An. funestus* populations in Comoros and Madagascar (Ayala et al., 2006). Ayala *et al* found samples from the two islands to cluster separately but also observed in-country structure within Madagascar, which might be driven by landscape features. As *An. funestus* undergoes speciation across the continent, the role of these observed diversities should be investigated on how they influence vector adaptation, dispersal, and potential vectorial capacity.

2.4.5 Whole genome sequence analysis

Currently, WGS studies investigating population diversity in *An. funestus* are rare in contrast with the extensive investigation that has been done on the *An. gambiae* complex (Anopheles gambiae Genomes, 2020, Anopheles gambiae Genomes et al., 2017). However, this is likely to change rapidly given the rise of collaborative programs such as the recent inception of the MalariaGEN *Anopheles funestus* genomic surveillance project (https://www.malariagen.net/projects/anopheles-funestus-genomic-surveillance-project) which is sequencing samples of this species from across Africa. A recent success of this program is the improved genome assembly from an individual female *Anopheles funestus* (specimen from Gabon; 251 megabases) complete with the mitochondrial genome (15.4 kilobases) (Ayala et al., 2022a).
Genomic analysis has cross-cutting potential of providing new insights into the genetic diversity of mosquito vectors, improving the surveillance of vectors and insecticide resistance, and providing an open resource for the development of new control tools. For example sequence analysis of *An. funestus* collected across Africa has shown Southern African mosquitoes to cluster separately from other African populations (Weedall et al., 2020) with a similar clustering pattern found using microsatellite analysis suggesting a barrier to gene flow (Barnes et al., 2017b). Several WGS in the *An. gambiae* complex have shown its potential. For example, genome-wide sequence analysis in the *An. gambiae* complex showed that *An. coluzzii* is largely restricted to West Africa whilst West, Central, and Eastern *An. gambiae* are genetically similar (Anopheles gambiae Genomes, 2020). Additionally, WGS in the *An. gambiae* complex has revealed new mechanisms of insecticide resistance and associated alleles such as copy number variants (Lucas et al., 2019).

Thus, WGS analysis on *An. funestus* has the potential of discovering fine-scale population structuring which is vital for elucidating population history and the spread of insecticide-resistance genes across the region. Unlike other approaches such as the use of microsatellites, WGS can allow delineation of more recent genetic changes in a population. However, from a practical perspective, simpler options involving specific amplicons are likely to be more widely used given their quicker turnaround times. WGS will remain an important research tool but is unlikely to be practicalized at scale.

2.5 Insecticide resistance profiling in *Anopheles funestus*

Five classes of insecticides are recommended for the control of adult *Anopheles* vectors: pyrethroids, carbamates, organophosphates, organochlorines, and neonicotinoids. Given the limited options within these classes, and the widespread insecticide resistance (Ranson and Lissenden, 2016b), control programs need integrated measures to monitor phenotypic insecticide susceptibility and underlying resistance mechanisms to select appropriate insecticide products for the management of resistance.

Bioassays to determine phenotypic resistance are conducted following either the WHO or Centres for Disease Control and Prevention (CDC) bottle assays; in which mosquitoes are exposed to standard concentrations of an insecticide and either mortality or knock-down measured after a specific time (WHO, 2022b). The mechanisms of resistance responsible for resistance phenotypes include metabolic detoxification (Wondji et al., 2009), target site mutations (Martinez-Torres et al., 1998), cuticular thickening which reduces insecticide penetration (Wood et al., 2010), or behavioral avoidance to reduce exposure to insecticides (Gatton et al., 2013).

2.5.1 Metabolic resistance

Metabolic resistance occurs when a mosquito produces high levels of detoxifying enzymes that chemically modify and deactivate the insecticides. Three families of metabolizing enzymes are associated with resistance: esterase, monooxygenases, and glutathione-S-transferases (GSTs) (Hemingway and Ranson, 2000a).

Increased expression of monooxygenase enzymes belonging to the cytochrome P450 gene family (*CYP450*) is the most common cause of resistance to pyrethroids in *An. funestus* (Weedall et al., 2020, Djuicy et al., 2020, Mugenzi et al., 2019, Riveron et al., 2014b, Wondji et al., 2009). The expression of these genes varies considerably across Africa reflecting possible barriers to gene flow amongst populations (Weedall et al., 2020, Mugenzi et al., 2019, Barnes et al., 2017a). For instance, the *CYP450* genes *CYP6P9a, CYP6M7,* and *CYP6P9b* are overexpressed (i.e., exhibit elevated transcription compared to susceptible strains) in *An. funestus* resistant populations in southern African populations from Zambia, Malawi, and Mozambique (Mugenzi et al., 2019, Riveron et al., 2014a, Christian et al., 2011). In contrast, the *CYP450* genes *CYP6M4, CYP9K1, CYP6P9b, CYP304b1, CYP6N1, CYP6M1, CYP6Z1,* and *CYP6M7* are overexpressed in resistant populations from Uganda and Tanzania in East Africa (Sandeu et al., 2020, Morgan et al., 2010, Matowo et al., 2022). Recently, *CYP6Z1* has also been shown to confer carbamate and pyrethroid cross-resistance in the laboratory (Ibrahim et al., 2016) though further field evaluation is needed.

In West Africa (Benin and Nigeria), the *CYP450 CYP6P9a, CYP6P9b,* and a glutathione S-transferase gene family (*GSTe2*) are overexpressed in resistant *An. funestus* mosquitoes and confer pyrethroid and dichlorodiphenyltrichloroethane (DDT) cross-resistance (Tchigossou et al., 2020, Atoyebi et al., 2020, Tchigossou et al., 2018). However, overexpression of *CYP6P9a* was not subsequently detected in West African samples in an analysis by Weedall *et al*; indicating this mechanism may be restricted to East and Southern African countries (Weedall et al., 2020). Similarly, overexpression of *CYP6P4a* appears to be restricted to Ghana, and *CYP6P5* overexpression is only found in East and West African samples (Weedall et al., 2020). In East and West African *An. funestus* populations overexpression of *CYP6P9a/b,* and glutathione S-transferase epsilon *(GSTE-L119F)* genes confer resistance to DDT (Tchigossou et al., 2018, Mulamba et al., 2014b). Contrastingly, in southern African countries, *An. funestus* populations remain susceptible to DDT insecticides (Ippolito et al., 2022, Casimiro et al., 2006). This demonstrates how diverse the genes involved in metabolic resistance across the continent are with *CYP6P9a/b* having a continental distribution.

Despite the crucial role of *CYP450* and *GSTs* genes in mediating insecticide resistance in *An. funestus*, simplified and field-applicable DNA-based assays for

tracking metabolic resistance have not been available for this species. An assay for field tracking *CYP6P9a* in *An. funestus* has only recently been developed (Mugenzi et al., 2019). The assay is based on PCR-RFLP where a *Taq* I restriction enzyme cuts a 450bp region of *CYP6P9a*-resistant mosquitoes but not in susceptible ones, allowing the distinction (Weedall et al., 2019). Such assays can easily advance monitoring of resistance alleles in the field without requiring sophisticated equipment, however, they might not be straightforward to design, depending on the genetic basis of the resistance. Considering the ubiquity of these resistance genes, the DNA-based assays should have a multigene panel approach where the most common genes can be amplified in the same assay.

2.5.2 Target site resistance

Target-site resistance is caused by point mutations in insecticide-binding proteins which thereby inhibit the binding and toxic activity of the insecticide. The most widely studied target site mutation is knockdown resistance (*kdr*), which is based on a point mutation changing leucine to phenylalanine or serine at codon 1014 (995 using *An. gambiae* codon numbering) of the voltage-gated sodium channel (VGSC) in *An. gambiae* mosquitoes. The mutation reduces sensitivity to pyrethroids and DDT (Ranson et al., 2000). However, analysis of *VGSC* gene at the *1014* codon has not detected any mutation in *An. funestus* (Irving and Wondji, 2017, Menze et al., 2016). This suggests that *kdr* might not be involved in DDT and pyrethroid cross-resistance in *An. funestus* (Ranson et al., 2000, Martinez-Torres et al., 1998). Other non-synonymous mutations in the *VGSC* gene in *An. funestus* such as I877L, V881L, and A1007S have been detected, and though require further investigation, do not appear to have a substantial impact on insecticide resistance (Menze et al., 2016).

Despite dieldrin not currently being used for vector control, *An. funestus* resistance to this insecticide remains high, especially in Central and West Africa. This is caused by an A296S-*rdl* mutation in γ-aminobutyric acid (GABA)-gated chloride channel in *An. funestus* from West Africa (Burkina Faso), Central Africa (Cameroon), and Southern Africa (Malawi), but not in East Africa (Amvongo-Adjia et al., 2020, Riveron et al., 2015, Wondji et al., 2011) (**Table 2-2**). The mutation is likely to persist in the population even in absence of selection pressure due to

its chromosomal location, that is close to the centromere, which reduces any cross-over event (Wondji et al., 2011, Brooke et al., 2002). Organophosphate resistance in *An. gambiae* and *An. coluzzii*, is driven by a *G280S*/*G119S ace*-1 mutation (Ibrahim et al., 2022, Grau-Bove et al., 2021), with little known in *An. funestus*. There is a need for research to understand the resistance mechanism of the commonly used IRS insecticide (pirimiphos-methyl), even though it remains efficacious against *An. funestus* (Abong'o et al., 2020).

The evolution of resistance in *An. funestus* populations primarily through metabolic resistance mechanism via P450s, makes them liable to cross-resistance with other insecticides. Looking into the future, the use of the WGS approach has the potential of discovering novel resistance mechanisms in *An. funestus* whilst also providing new insights into genes already implicated in resistance. In *An. gambiae* and *Aedes aegypti*, for instance, this technique has led to the discovery of a new resistance mechanism through gene duplication or copy number variation (CNV) in metabolic resistance genes (Lucas et al., 2019, Faucon et al., 2015). Copy number variations can lead to resistance, as the presence of more copies of a detoxifying gene will result in its overexpression (Bass and Field, 2011). As WGS costs are decreasing, its application for *An. funestus* resistance monitoring should be prioritized.

Table 2-2: Selected genes involved in insecticide resistance in *An. funestus* and their geographical distribution across Africa.

Geographical distribution	Insecticide class	Resistance mechanism	Resistance genes	Reference
Southern Africa	Pyrethroid	Metabolic	*CYP6P9a*, *CYP6M7*, & *CYP6P9b*	(Weedall et al., 2019, Riveron et al., 2014a)
Eastern Africa	Pyrethroid	Metabolic	*CYP6M4*, *CYP9K1*, *CYP6P9b*, *CYP304b1*, *CYP6M7*, *CYP6N1*, *CYP6M1*, & *CYP6Z1*	(Matowo et al., 2022, Sandeu et al., 2020, Morgan et al., 2010)
West Africa	Pyrethroid & Organochloride	Metabolic	*CYP6P9a*, *CYP6P9b*, & *GSTe2*	(Tchigossou et al., 2020, Atoyebi et al., 2020, Tchigossou et al., 2018)
Central, South & West Africa	Organochloride	Metabolic & target-site	*CYP6P9a/b*, *A296S RDL*, & *GSTe2*	(Tchigossou et al., 2018, Mulamba et al., 2014b)

2.6 The potential of genetic technologies for the surveillance and control of *An. funestus*

Genetic manipulation of disease vectors involves the deliberate release of individuals containing a desirable genetic trait to spread it through the wild-type population via mating (James, 2005). Such approaches can include either

population suppression through the spread of genes reducing vector reproduction, or modification of vector by introducing genes that confer refractoriness to pathogens (Bier, 2021). Many of these gene drive approaches are based on the use of CRISPR-Cas9-based elements that can copy themselves from one chromosome to another in the germline and thereby increase their representation among the gametes. This type of approach ensures accuracy and super Mendelian inheritance leading to a rapid increase in the frequency of the desired traits in the target population (Hammond et al., 2016, Gantz et al., 2015, Esvelt et al., 2014, Burt, 2003).

The development of CRISPR-based genome editing tools and gene drives in *Anopheles* mosquitoes rapidly advanced over the past six years albeit with a focus on mosquitoes of the *An. gambiae* complex (Hammond et al., 2021, Hammond et al., 2016). However, a more holistic gene drive program for malaria control will need to equally target the increasingly important African malaria vector, *An. funestus*. Fortunately, the transgenic pipelines and technologies that are already established for *An. gambiae* can be adapted to this species.

Only a handful of studies have been published that demonstrate the use of gene editing technologies in *An. funestus* (Quinn et al., 2021, Li et al., 2018). Using CRISPR/Cas9, Li *et al* showed for the first time that heritable germline mutations could be introduced into *An. funestus* genome by microinjection of guide RNAs (gRNAs) and Cas9 protein into eggs (Li et al., 2018). This resulted in a stable colony that can be used for reverse genetics studies. This was achieved through a nonhomologous end-joining (NHEJ) repair process, following Cas9 cleavage of a target site that is determined by the sequence of the gRNA, which leads to small insertions or deletions. Quinn *et al* have also recently demonstrated the successful use of homology-directed repair (HDR), also known as knock-in, for the generation of transgenic *An. funestus* (Quinn et al., 2021). HDR has the advantage of introducing the desired transgenic DNA sequence that is incorporated into the mosquito germline during the repair process. Since the copying mechanism of HDR is like what many of the CRISPR-based gene-drive rely on to increase their copy number, the high rates of HDR observed in *An. funestus* to date augur well for its amenability to gene drives of this type.

Assuming successful development of gene-drive constructs for *An. funestus*, several entomological and regulatory questions will need to be addressed before

large-scale deployment. A major challenge is the mass rearing of modified *An. funestus* mosquitoes under laboratory settings. Currently, there are only two colony lines of the vector successfully established in the laboratory, *An. funestus* from Mozambique (FUMOZ) and *An. funestus* from southern Angola (FANG) (Hunt et al., 2005, Hargreaves et al., 2000). This is mainly due to the bottlenecks of adapting *An. funestus* into a laboratory colony which includes larval survival, mating success in cages, and low adult survival rates (Ngowo et al., 2021). Overcoming these challenges will be key to establishing transgenic colony lines in the laboratory for experimentation and large-scale vector control use.

Additionally, the high levels of genetic diversity within *An. funestus* populations across Africa could impact the application of gene drive control strategies (Weedall et al., 2020, Barnes et al., 2017a, Michel et al., 2005). Sex-linked gene drive approaches are dependent on gene flow which is shaped by natural barriers such as large water bodies, large forests cover, aridity, valleys, and mountains (Anopheles gambiae Genomes et al., 2017). Hence, fine-scale population genetics surveys of populations at target release sites must be undertaken as an integral part of the deployment strategy. Overall, the application of gene drives will need to be tailored depending on the local vector population and environmental and geographical features (Eckhoff et al., 2017). Similarly, it will be vital to resolve regulatory issues around ethics, and environmental impact, and importantly allow the communities living in malaria-endemic areas to have a leading voice in the development and deployment of such tools (WHO, 2021b, James et al., 2018).

2.7 Conclusion

The last decade has seen an upsurge in *An. funestus* group research to understand mechanisms of insecticide resistance, taxonomy, and population biology. A combination of robust morphological identification, allele-specific PCR, and small-scale WGS should be used in tandem with geographical information when profiling mosquito identity. Cytochrome *P450*-mediated metabolic resistance starkly varies across the continent hence the development of field adaptable DNA-based assay to diagnose it should be a priority to help in resistance management and surveillance. Similarly, a detailed analysis of *An. funestus* population genetics should be undertaken on how it influences the spread of resistance genotypes and

as a prerequisite to the deployment of genetic control tools. Attempts at malaria control and elimination need to be holistic bringing together current and emerging vector control approaches, a pool of empowered human personnel, and most importantly involving communities who bear the burden of this disease.

3 Genetic Markers Associated with the Widespread Insecticide Resistance in Malaria Vector *Anopheles funestus* Populations across Tanzania

3.1 Abstract

Anopheles funestus is a leading vector of malaria in most parts of East and Southern Africa, yet its ecology, and responses to vector control remain poorly understood compared to other vectors such as *Anopheles gambiae* and *Anopheles arabiensis*. This study presents the first large-scale survey of the genetic and phenotypic expression of insecticide resistance in *An. funestus* populations in Tanzania. We performed insecticide susceptibility bioassays on *An. funestus* mosquitoes in nine districts with moderate to high malaria prevalence in Tanzania, followed by genotyping for resistance-associated mutations (*CYP6P9a, CYP6P9b, L119F-GSTe2*) and structural variants (SV4.3kb, SV6.5kb). Generalized linear models were used to assess relationships between genetic markers and phenotypic resistance. An interactive R Shiny tool was created to visualize the data and support evidence-based interventions. Pyrethroid resistance was universal but reversible by piperonyl-butoxide (PBO). However, carbamate resistance was observed in only five of the nine districts, and DDT resistance was found only in the Kilombero valley, south-eastern Tanzania. Conversely, there was universal susceptibility to the organophosphate, pirimiphos-methyl in all sites. Genetic markers of resistance had distinct geographical patterns, with *CYP6P9a*-R and *CYP6P9b*-R alleles, and the SV6.5kb structural variant absent or undetectable in the northwest but prevalent in all other sites, while SV4.3kb was prevalent in the northwestern and western regions but absent elsewhere. Emergent *L119F-GSTe2*, associated with deltamethrin resistance, was detected in heterozygous form in districts bordering Mozambique, Malawi, and the Democratic Republic of Congo. The resistance landscape was most complex in western Tanzania, in Tanganyika district, where all five genetic markers were detected. There was a notable south-to-north spread of resistance genes, especially *CYP6P9a*-R, though this appears to be interrupted, possibly by the Rift Valley. This study underscores the need to expand resistance monitoring to include *An. funestus* alongside other vector species; and to screen for both the genetic and phenotypic signatures of resistance. The findings can be visualized online via an interactive user interface and could inform data-driven decision-making for resistance management and vector control.

Since this was the first large-scale survey of resistance in Tanzania's *An. funestus*, we recommend regular updates with greater geographical and temporal coverage.

3.2 Background

Vector control constitutes one of the key pillars of malaria control in Africa (Wilson et al., 2020), where it primarily constitutes the deployment of insecticide-treated nets (ITNs) and indoor residual spraying (IRS). Despite great successes, in particular, since 2000, malaria remains a significant challenge (WHO, 2023f), partly due to biological threats, notably the evolution and spread of insecticide resistance (Hemingway et al., 2016, WHO, 2022a). To accelerate progress, affected countries must adopt proactive strategies to manage the widespread resistance and optimize the allocation of insecticidal interventions.

In the east and southern African region, malaria transmission is mediated primarily by *Anopheles funestus* (Msugupakulya et al., 2023), which, like *Anopheles gambiae*, is highly adapted to human habitations (Kahamba et al., 2022). In Tanzania, studies in the high prevalence settings in the northeast and south-eastern regions have shown that this vector species can mediate as high as 90% of the local malaria infections or more (Matowo et al., 2021, Kaindoa et al., 2017). Unfortunately, compared to *An. gambiae* and *Anopheles arabiensis,* for which there have been several insecticide resistance surveys in Tanzania (Tungu et al., 2023), only a handful of studies have profiled the resistance patterns in *An. funestus* (Pinda et al., 2020, Kaindoa et al., 2017, Matowo et al., 2022, Matowo et al., 2021). This lack of data, especially the phenotypic data, can be attributed to difficulties associated with rearing *An. funestus* and identifying its aquatic habitats (Ngowo et al., 2021, Nambunga et al., 2020), which complicates the collection of age-synchronized larvae required for age-standardized resistance bioassays (WHO, 2022b). The resulting information gap can derail the effective planning, implementation, and monitoring of vector control programs in settings where *An. funestus* dominates.

Comprehensive information on molecular mechanisms driving resistance in *An. funestus* in Tanzania is similarly scarce (Matowo et al., 2022). Unlike phenotypic bioassays, the genetic signals of insecticide resistance can enable early detection and mitigation before the problem is widely established in vector populations

(Vontas and Mavridis, 2019). By establishing the exact mechanisms underlying the observed phenotypes, molecular monitoring also enables the elucidation of causes and risk factors of resistance in specific locations. However, establishing direct correlations between the phenotypes and molecular resistance can be challenging due to the vast number of mechanisms and gene families, as well as the stochastic nature of the genetic interactions leading to resistance (Liu, 2015, Hemingway et al., 2004). In *An. funestus*, the cytochrome P450 (*CYP450*) monooxygenases gene family constitutes the dominant family of pyrethroid resistance genes and has been associated with reduced efficacy of pyrethroid-based ITNs (Weedall et al., 2019). Notably, *CYP6P9a* and *CYP6P9b* are highly expressed in pyrethroid-resistant *An. funestus* populations in east and southern Africa (Mugenzi et al., 2019), leading to operational failures in vector control in the region (Wondji et al., 2012). Another cytochrome P450 gene, the *CYP9K1* is significantly overexpressed in pyrethroid-resistant *An. funestus* populations from Uganda (Sandeu et al., 2020, Mulamba et al., 2014a) while *CYP6P4a* and *CYP6P4b* have been found in Ghana (Mugenzi et al., 2022). While many of these genes are associated with resistance to a narrow range of insecticides, certain mutations can confer resistance to multiple insecticide classes. For instance, the L119F mutation in the Glutathione S-Transferases epsilon 2 (*GSTe2*) facilitates broad detoxification of pyrethroids, organochlorides, and organophosphates in *An. funestus* (Riveron et al., 2014c). Until now, genetic analysis of *L119F-Gste2* in field-collected mosquitoes is geographically restricted to west and central Africa (Tchigossou et al., 2020, Tchouakui et al., 2019, Tchigossou et al., 2018, Riveron et al., 2014c) with its spread resistance role in east and southern Africa remaining unclarified.

Novel IR mechanisms such as copy number variants have also recently been demonstrated to increase the expression of insecticide-detoxifying genes, leading to resistance in *An. funestus* (Mugenzi Leon et al., 2023, Mugenzi et al., 2020). Monitoring the evolution and spread of these resistance genes in the face of sustained pressure from insecticide-based vector control is vital to managing resistance. Fortunately, field-adaptable molecular assays are now available that can track some molecular resistance genes in *An. funestus* (Mugenzi Leon et al., 2023, Mugenzi et al., 2020, Weedall et al., 2019, Tchouakui et al., 2019, Mugenzi et al., 2019) - and can be leveraged to monitor resistance and inform appropriate deployment of the limited vector control tools.

In this study, we comprehensively analysed the insecticide resistance phenotypes and genotypes *An. funestus* mosquitoes in nine regions representing different eco-epidemiological settings in Tanzania. We aimed to understand the potential responsiveness of these mosquitoes to public health insecticides, generate insights for more effective strategies for malaria control, and establish a baseline chart of insecticide resistance in Tanzania's *An. funestus*. We screened for five major genes and structural variants known to confer metabolic resistance and conducted standard susceptibility tests against pyrethroids, carbamates, organophosphates, and organochlorides. This study represents the first large-scale survey of the insecticide resistance patterns in *An. funestus*, which despite being a dominant vector in the country, remains far less investigated compared to other malaria vectors.

3.3 Methods

3.3.1 Study area and mosquito collection

Mosquitoes were collected from November 2021 to December 2022 in selected villages across nine administrative districts of Tanzania (**Figure 3-1**). The locations represent a comprehensive geographical and epidemiological cross-section of the country and were selected for their high prevalence of *An. funestus* (Tanzania-NMCP, 2020). Due to difficulties in finding *An. funestus* immature stages and unsuccessful attempts to get sufficient offspring from wild blood-fed females, we adopted a previously tested approach relying on unfed and non-gravid females of unknown ages for the resistance bioassays (Pinda et al., 2020, Kaindoa et al., 2017). Following consent of household heads, adult mosquitoes were sampled inside houses using a combination of CDC-miniature light traps (CDC-LT) (Sudia and Chamberlain, 1988) and a miniaturized double-net trap (DN-Mini) (Limwagu et al., 2024). The collected mosquitoes were first sorted by taxa using taxonomic keys (Coetzee, 2020, Gillies and Coetzee, 1987, Gillies and De Meillon, 1968), and the *An. funestus* mosquitoes were further sorted based on physiological features (Coetzee, 2020, Gillies and Coetzee, 1987, Gillies and De Meillon, 1968) and abdominal status (unfed). The unfed and non-gravid females were maintained *ad libitum* on 10% sucrose-soaked cotton wool for a day to acclimatize and select weak individuals not suitable for the resistance tests.

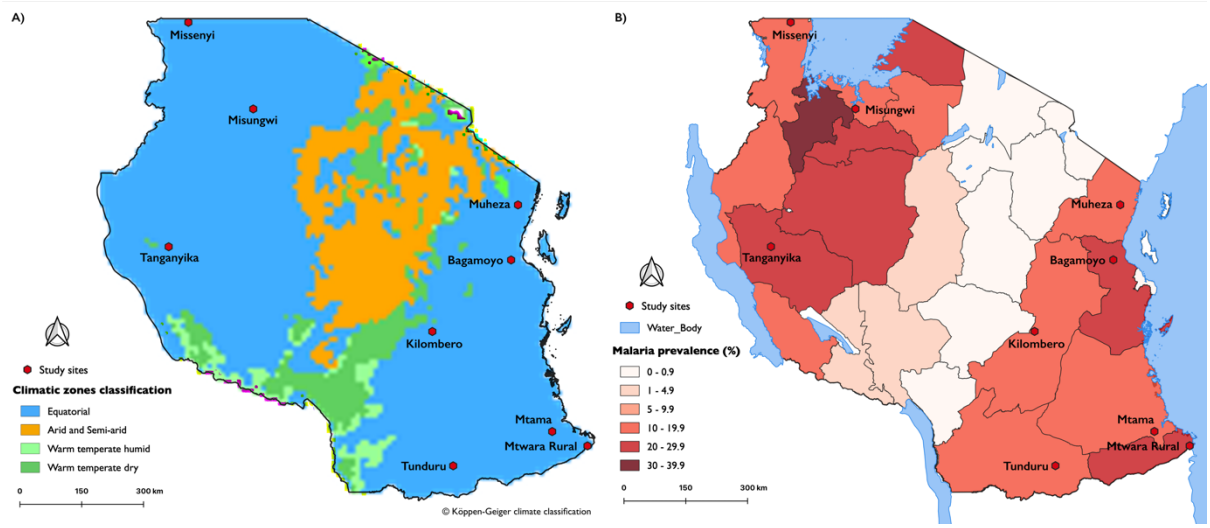


Figure 3-1: Map of Tanzania showing the districts (red dots) selected for insecticide resistance surveillance. A) The sites were selected based on sentinel data from Tanzania's national malaria control program that confirmed the presence of *An. funestus* group mosquitoes (all sites) and B) national malaria prevalence data encompassing moderate (10 – 19.9%) (Missenyi, Misungwi, Kilombero, Tunduru, and Muheza) and high (>20%) (Tanganyika, Mtwara rural, Bagamoyo, and Mtama) malaria burden. Malaria prevalence data was adopted from the Tanzanian Ministry of Health report on the School Malaria and Nutrition Survey (SMNS) (Tanzania-MoH, 2022).

3.3.2 Tests for susceptibility of Anopheles funestus to different insecticides

The acclimatized mosquitoes were sugar-starved for at least two hours before being utilized in the bioassays, following the guidelines for WHO tube tests for mosquito susceptibility (WHO, 2022b). Room temperature and relative humidity were recorded at the time of each test. The candidate insecticides included those commonly used for vector control either on ITNs (permethrin, piperonyl butoxide, and deltamethrin) or for IRS (bendiocarb, pirimiphos-methyl,) (Tanzania-NMCP, 2020). In addition, we included dichloro-diphenyl-trichloroethane (DDT), the persistent organic pollutant, which is no longer used for vector control in Tanzania (Oxborough, 2016).

The susceptibility of adult *An. funestus* mosquitoes were determined by exposing the acclimatized females for 60 mins to WHO-approved insecticide-impregnated papers containing the diagnostic doses of the candidate insecticides: bendiocarb (0.1%), pirimiphos-methyl (0.25%), DDT (4%), permethrin (0.75%), and deltamethrin (0.05%). For each round of the bioassays and each insecticide, we included four test tubes lined with the impregnated papers and two control tubes

lined with untreated control papers containing respective solvent oils. Twenty mosquitoes were exposed in each tube. The tests were thus conducted in four test replicates with two control tubes. After the one-hour exposure and knock-down monitoring, the mosquitoes were maintained in insecticide-free tubes for 24hrs to monitor mortality.

3.3.3 Tests for the intensity of pyrethroid resistance in Anopheles funestus

Intensity assays are generally used to determine the operational significance of the resistance phenotypes once resistance is confirmed using the discriminating insecticide doses. In line with WHO guidelines, the intensity of resistance against the two pyrethroids was determined by exposing the mosquitoes to increasing concentrations of the insecticides. We used either 5x and 10x the doses of permethrin (i.e. 3.75% and 7.5%, respectively) and 5x and 10x the doses of deltamethrin (i.e. 0.25% and 0.5%, respectively). Knockdown was recorded after one hour following insecticide exposure and mortality was recorded after a further 24 hours of monitoring. The tests were also conducted in four test replicates with two control tubes in which mosquitoes were exposed to papers containing silicone oil but no insecticides.

3.3.4 Tests of the synergistic effect of piperonyl butoxide (PBO) on pyrethroid-resistant Anopheles funestus

Piperonyl butoxide (PBO) is a synergist commonly used on ITNs to enhance their insecticidal activity on resistant mosquitoes by inhibiting the function of cytochrome P450 monooxygenases. In this study, the role of PBO in reversing pyrethroid resistance phenotype was determined by first exposing the female *An. funestus* mosquitoes to 4% PBO for 1 hour, followed by a 1-hour exposure to the diagnostic dose of deltamethrin (0.05 %). The tests were conducted in three replicate tubes with two control tubes, in which mosquitoes were exposed to control papers containing only the solvent oil. Knockdown was recorded after one hour following insecticide exposure and mortality was recorded after a further 24 hours of monitoring (WHO, 2022b).

3.3.5 Tests to confirm the genetic identity of the Anopheles funestus mosquitoes.

To enhance the probability of the collected mosquitoes being *An. funestus* s.s., rather than the other species within the *An. funestus* group, which comprises at least 11 known members (Gillies and Coetzee, 1987, Gillies and De Meillon, 1968), the field collections had been strategically conducted in villages with high densities of *An. funestus s.s.* The collections were specifically done indoors, aligning with the species' endophilic behaviour. Nonetheless, to confirm their genetic identity, polymerase chain reaction (PCR) assays were performed on the mosquitoes once the resistance assays had been completed.

A subset of the mosquitoes (~20%) that were either alive or dead after 24 hours following insecticide exposure were randomly selected from the replicates and preserved in 80% ethanol. Genomic DNA was extracted from individual mosquitoes using a DNeasy Blood & Tissue Kit (Qiagen, UK). Species identification followed a PCR-based protocol utilizing species-specific primers targeting the non-coding internally transcribed spacer region (ITS2) between the 5.8S and 28S ribosomal DNA sequence to distinguish between at least seven members of *An. funestus* group (Cohuet et al., 2003, Koekemoer et al., 2002b). Briefly, 0.33µl of each 10µm primer was added to 6.25µl of 2X GoTaq® G2 Green Master Mix (Promega, USA) in a 20µl reaction volume. The cycling conditions were as follows: 5min at 95°C followed by 30 cycles of 94°C denaturation, 45°C annealing, and 72°C extension, and a final extension at 72°C for 5min. The PCR products were separated on a 2% agarose gel stained with SYBR™ Safe DNA Gel Stain (Invitrogen, UK).

In addition to testing the subset of mosquitoes used for the actual resistance bioassays, we also tested a larger sub-sample of mosquitoes originating from a broader field survey that had been completed in these same areas to provide more representative data on the diversity of the *An. funestus* group.

3.3.6 Genotyping of selected metabolic resistance genes and structural variants (SV)

Twenty randomly selected mosquitoes that were either alive (10) or dead (10) 24 hours post-exposure to discriminating insecticide doses of deltamethrin were identified by PCR to species level and genotyped to screen for the insecticide

resistance genes, *L119F-GSTe2*, *CYP6P9a,* and *CYP6P9b*, as well as structural variants, SV4.3kb, and SV6.5kb, known to be associated with the overexpression of the resistance genes (Mugenzi Leon et al., 2023, Mugenzi et al., 2020). Increased expression of these genes, or transposons in the case of SV, has been associated with resistance phenotypes in *An. funestus* (Mugenzi et al., 2020, Weedall et al., 2019, Mugenzi et al., 2019).

The *L119F-GSTe2* mutation was genotyped following an allele-specific PCR protocol using two inner and outer primer pairs resulting in two fragments (850bp and 312bp) for the susceptible mosquitoes and three bands (850bp, 12bp, and 523bp) for the resistant haplotype (Tchouakui et al., 2019). The *CYP6P9a* gene was genotyped following a PCR assay targeting a restriction fragment length polymorphism (RFLP) - where a restriction enzyme *Taq*I-V2 cuts a 450bp fragment into 350 and 100bp in mosquitoes carrying the resistance alleles but not in susceptible where the haplotypes remain uncut (Weedall et al., 2019). A similar assay was used for detecting *CYP6P9b*, but with restriction enzyme Tsp45I which digests a 550bp fragment into 400bp and 150bp in susceptible mosquitoes whereas resistant haplotypes remain uncut (Weedall et al., 2019). Finally, the detection of SV 6.5kb and 4.3kb was performed following methods described by Mugenzi *et al.*,(Mugenzi Leon et al., 2023, Mugenzi et al., 2020). Mosquitoes carrying the SV 6.5kb variant had an amplicon fragment size of 569bp for positive samples, 266bp for negative samples, and both fragment sizes in heterozygotes. Similarly, the presence of the SV 4.3kb variant was indicated by a 780bp amplicon in positive individuals, and a 280bp amplicon in negatives, and both amplicons were observed in heterozygous individuals.

3.3.7 Data analysis

In the initial susceptibility tests, we assessed the observed mortality in both treatment and control tests and used Abbott's mortality adjustments in cases where control mortality exceeded ≥5% (WHO, 2022b). Tests were discarded if control mortality was 20% or more. The data was summarised, and mosquito populations were considered susceptible to an insecticide if mean mortality was ≥ 98% and resistant if mean mortality was ≤90% mortality (WHO, 2022b). In the resistance intensity assays, resistance was recorded as low if 24-hour mortality at

5x was between ≥98%, moderate if mortality was below ≤98% at 5x but above ≥98% at 10x the dose, and high if 10x mortality was below 98%.

Generalised linear mixed models within the *lme4* package (36) were used to analyse the phenotypic resistance levels against different insecticides in R statistical software (version 4.1.1). Since these tests were done in different study sites, it was not possible to maintain a standard microclimatic condition in all cases, and as such, statistical analysis was used to discern the potential influence of these factors on the mortality outcomes. Mortality to each candidate insecticide was determined at the group level (i.e. averages of replicates for a given insecticide) in a binomial model with the response as the number of dead out of the number exposed and explanatory variables insecticide type (including controls), location of the test (district), temperature, relative humidity (RH) recorded at the time of test and interaction between insecticide and district; replicates were fitted as a random effect. The binomial model for the association between phenotype and the IR genotypes had mortality (alive/dead) response variable and explanatory variables as either resistant or susceptible genotypes of the five genes (*CYP6P9a, CYP6P9b, L119F-GSTe2,* SV 6.5kb, and SV 4.3kb). *Drop1* command was used to examine the models and determine the variable to remove in a stepwise format starting with the most complex model. Additionally, the likelihood ratio test (LRT) was used to compare two nested models. The R command *ggemmeans* was used to run the model, and *gglot2* was used to plot the predictions. The variation in frequency of the five resistance genes by district was determined by ANOVA test on two logistic regressions one with genes as the response and district as the predictor, and the other with just the intercept term.

3.4 Results

3.4.1 Confirmation of the molecular identity of the mosquitoes tested

Overall, PCR analysis showed that the proportions of *An. funestus* s.s in the sub-sample of mosquitoes collected in the nine districts (N = 6,724 *An. funestus* group females) were as follows: Bagamoyo 89.8%, Kilombero 98.7%, Missenyi 99.4%, Misungwi 91.3%, Mtama 95.5%, Mtwara Rural 89.7%, Muheza 91%, Tanganyika 80.5%, and Tunduru 97.7%. The residual proportions from these represented other

sibling species within the *An. funestus* group, which included *An. leesoni, An. rivulorum, An. parensis* and *An. funestus*/*An. leesoni* hybrid.
However, the mosquitoes used in the insecticide resistance bioassays were identified by PCR to be 100% *An. funestus s.s.* in all the study locations except in Tanganyika District, Katavi region in western Tanzania, where 15% were *An. parensis*. In Misungwi district, Mwanza region in north-western Tanzania, over 90% of the mosquitoes of the samples were found to be *An. parensis* in the initially selected village (Ngaya), leading to the exclusion of this data set and relocation to an alternative village with a higher occurrence of *An. funestus* (Nyang'omango). These notable instances of unusually high prevalences of *An. parensis* are discussed separately in an upcoming publication by Mapua *et al* (unpublished).

All subsequent genetic screening for insecticide resistance markers for all study sites was performed only *An. funestus s.s* from the resistance tests and did not include any other sibling species in the *An. funestus* group.

3.4.2 Resistance of *An. funestus* to pyrethroids

An. funestus mosquitoes showed resistance to pyrethroid insecticides in all study sites. One-hour knock-down (KD_{1h}) to permethrin was lowest in Morogoro (35%) and highest in Muheza district (84%), while KD_{1h} deltamethrin was lowest in Mtama district 9% and highest in Tanganyika (48%). In tests against permethrin, the lowest 24hour mortality was 29% (CI 19.8 -39.6) in Tunduru district, Ruvuma region in southern Tanzania, while the highest mortality was 76% (CI 67.1 – 85.4) in Missenyi district, in Kagera region, north-western Tanzania. On the other hand, in tests against deltamethrin, the lowest mortality was 7% (3.4 - 15.7) in Mtama district, Lindi region in south-eastern Tanzania, while the highest was 55% (CI 43.9 – 65.5) in Tanganyika district, Katavi region in western Tanzania (**Figure 3-2**). In the analysis of factors influencing the observed mortality, we observed that the percentage mortality observed against the candidate insecticides varied between sites (insecticide*site interaction: χ^2=187.4, p<0.00005) but was not influenced by temperature (χ^2=0.6168, p=0.4345) or relative humidity (χ^2=0.2326, p=0.6296).

3.4.3 Intensity of resistance to pyrethroids

The intensity of pyrethroid resistance was determined as low if 24-hour mortality at the 5x dose was between ≥98%, moderate if the mortality was below 98% at 5x but above ≥98% at 10x the dose, and high if 10x mortality is <98%. KD_{1h} was greater than 80% in all locations for 5x and 10x permethrin, lowest in Misungwi district (34%) and highest in Tanga (91%) for 5x deltamethrin and ranged 35% in Mtwara Rural and 84% in Tanganyika for 10x deltamethrin. In tests using permethrin, high-intensity resistance was observed in the coastal region, in Bagamoyo district (5x mortality: 86% (76.1 – 92.4); 10x Mortality: 95% (87.4 – 98.1)), and Tanganyika district in the western part of the country (5x mortality: 83% CI 72.5 – 89.3); 10x Mortality: 95% (87.4 – 98.1)). The rest of the study sites had only low-intensity resistance to this insecticide. In contrast, the resistance to deltamethrin was of high intensity in five out of the nine study sites districts. These included Bagamoyo, Mtama, and Mtwara rural districts in eastern Tanzania, Tanganyika district in western Tanzania, and Misungwi district in the north of the country, where 24-hr mortality was below 98% even at 10x the deltamethrin dose (**Figure 3-2**).

3.4.4 Synergistic effect of PBO and the reversal of pyrethroid resistance

Pre-exposure of the mosquitoes to PBO (synergist) for one hour, followed by exposure to the candidate insecticide, resulted in the reversal of the initially observed resistance to the discriminating doses of deltamethrin (**Figure 3-2**). One-hour knock-down (KD_{1h}) was greater than 98% in all locations. The resulting 24hour mortality in the PBO pre-exposed mosquitoes was greater than 98% in all sites except in Tanganyika, where it was only 80% (CI 68 – 88.3). Due to limited supplies and the difficulty of obtaining sufficient mosquitoes, these PBO tests were conducted only for deltamethrin.

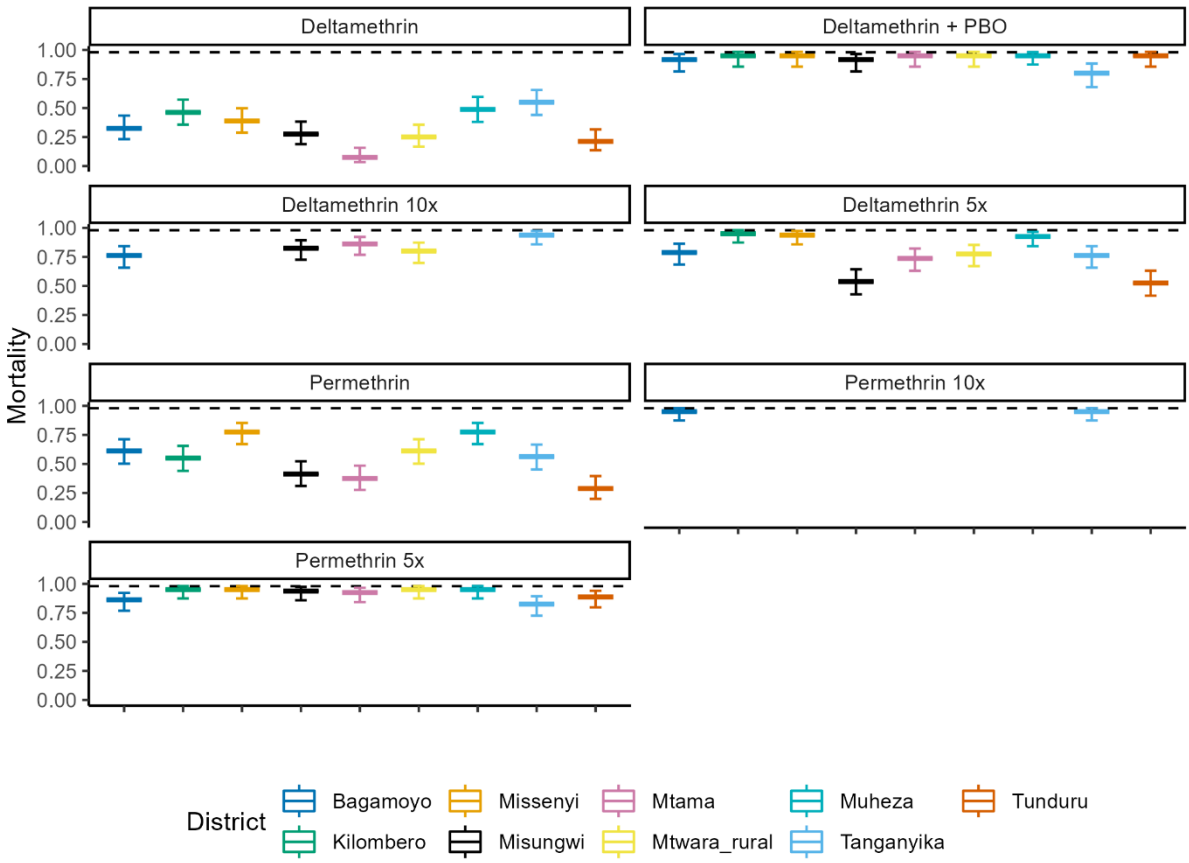


Figure 3-2: Insecticide resistance profile of *An. funestus*. Figure shows resistance to the discriminating insecticide doses, the intensity of resistance as observed in tests at 5x and 10x the diagnostic doses, and the synergistic effect of PBO in reversing the observed resistance. The colours represent the different districts where the bioassays were conducted, the error bars represent the 95% confidence intervals of the predictions, and the dotted line on the y-axis indicates a 98% mortality threshold.

3.4.5 Resistance of An. funestus to bendiocarb, DDT, and pirimiphos-methyl

One-hour knock-down (KD_{1h}) to bendiocarb was greater than 80% in all locations, but DDT KD_{1h} least in Morogoro (55%) with complete knock-down in Mtama, Lindi region. Least KD_{1h} to pirimiphos-methyl was in Missenyi district (74%) but greater than 90% in all other districts. Phenotypic resistance to carbamates (bendiocarb) was observed in five of the nine study sites across the country, namely the southern sites of Mtama, Mtwara Rural, and Tunduru, as well as the western site of Tanganyika and the north-western site of Misenyi, all showing less than 90% mortality. Conversely, high mortality rates, indicative of susceptibility to bendiocarb were observed in the eastern sites of Bagamoyo, Kilombero, and

Muheza, as well as in the northern site of Misungwi (Figure 3-3). Resistance to the organochlorine, DDT was observed exclusively in the Kilombero district, in south-eastern Tanzania (mortality: 68% (57.7 – 77.9). This study also showed the universal susceptibility of *An. funestus* populations in all the study sites to the organophosphate, pirimiphos-methyl, as evidenced by the 24-hour mortalities exceeding 98% (Figure 3-3).

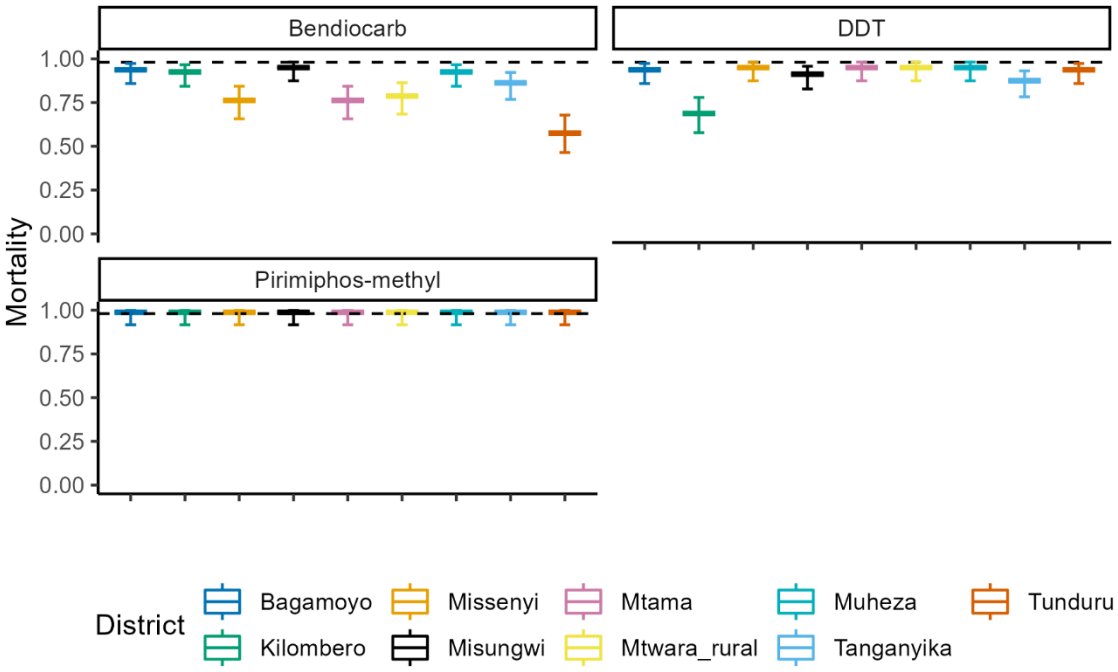


Figure 3-3: Insecticide resistance of *An. funestus* to bendiocarb, DDT, and pirimiphos-methyl. The colours represent the different districts where the bioassays were conducted, the error bars represent the 95% confidence intervals of the predictions, and the dotted line on the y-axis indicates a 98% mortality threshold.

3.4.6 Genetic markers of insecticide resistance in An. funestus in Tanzania

The resistant allele *CYP6P9a*-R was prevalent in the eastern part of the country and was fixed (100% prevalence) in both phenotypically resistant (alive), and susceptible (dead) mosquitoes collected from Mtama, Mtwara rural and Kilombero districts, but was also observed in Tunduru, Bagamoyo, and Muheza at very high frequencies (Figure **3-4**). In addition to the *CYP6P9a*-RR genotype, mosquitoes in Tunduru, Bagamoyo, and Muheza districts also carried the heterozygous resistant genotype *CYP6P9a*-RS. This heterozygous form was also prevalent in the western district of Tanganyika. The *CYP6P9a*-RR genotype was completely absent in the

northwestern sites and was observed only at very low levels in Tanganyika in western Tanzania. Instead, *An. funestus* from Missenyi and Misungwi districts were all homozygous susceptible *CYP6P9a*-SS, with Tanganyika having all three genotypes, RR, RS, and SS (Figure **3-4**). Similarly, *CYP6P9b*-R alleles were fixed in all districts except the north-western districts of Misungwi and Missenyi, where the gene did not amplify. In Tanganyika, both *CYP6P9b*-RR and *CYP6P9b*-RS genotypes were identified alongside a small number of non-amplifications (i.e. na) (Figure **3-4**). Contrastingly, the *L119F-GSTe2*-RR resistance genotype was absent in all study sites. Instead, *An. funestus* in six districts were homozygous susceptible for *L119F-GSTe2*-SS, while those in Tunduru, Mtwara rural, and Tanganyika districts had the heterozygous haplotype of the gene *L119F-GSTe2*-RS in some surviving mosquitoes (Figure **3-4**).

In tests for the structural variants, it was observed that the homozygous resistant genotypes of SV 4.3kb (RR) were near fixation and restricted to the northwestern districts of Missenyi and Misungwi and were also found at low frequencies in the western district of Tanganyika (Figure **3-4**). Conversely, the homozygous resistant genotypes of SV 6.5kb (RR) were widespread and fixed across most districts except the north-western districts of Missenyi and Misungwi, where the SV did not amplify in the PCR (i.e. na) (Figure **3-4**).

Further analysis revealed statistically significant geographic variations in the frequencies of *CYP6P9a* (χ^2=172.62, p<0.0005), *CYP6P9b* (χ^2=16.04, p=0.0135), and SV 4.3kb (χ^2=169.35, p<0.0005). In contrast, no significant differences were observed for *L119F-GSTe2* (χ^2=0.2324, p=0.9116) or SV 6.5kb (χ^2=9.39, p=0.1518) by district.

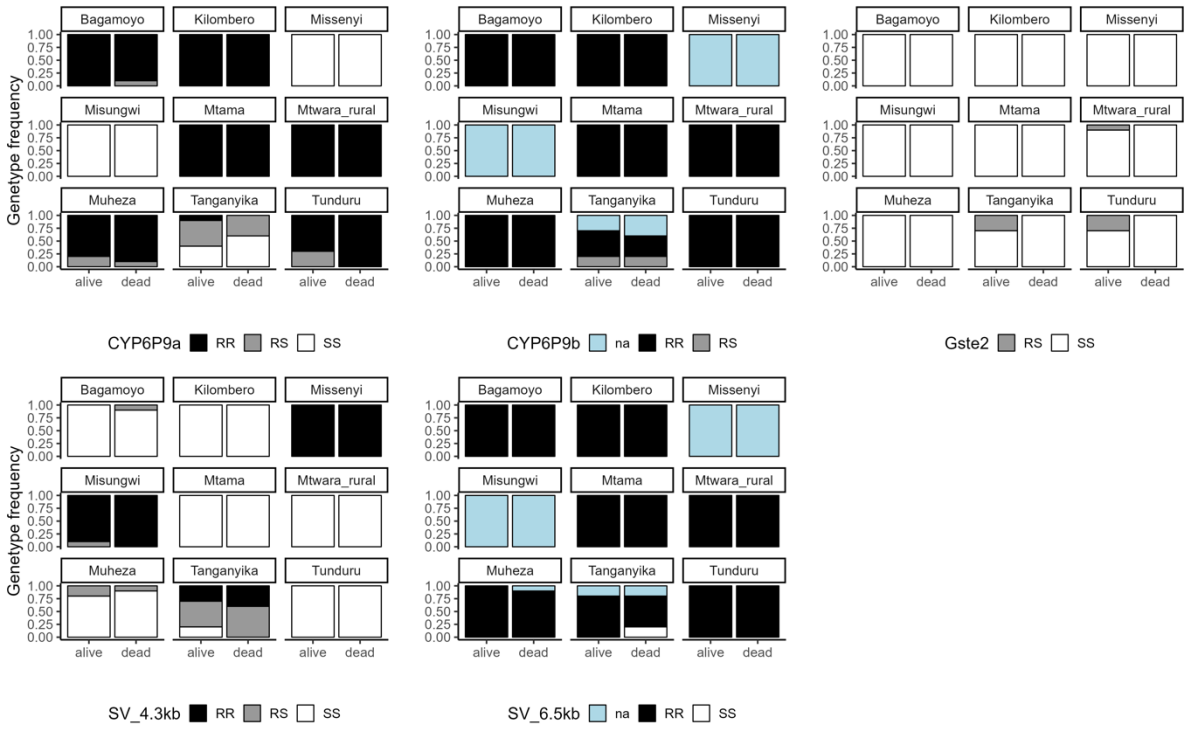


Figure 3-4: The distribution of metabolic resistance genotypes, *CYP6P9a*, *CYP6P9b*, and L119F-*GSTe2*, SV 4.3kb and SV 6.5kb in phenotypically resistant (Alive), and susceptible (Dead) *An. funestus* across nine districts in Tanzania.

3.4.7 Associations between genotypic and phenotypic expression of insecticide resistance

Using equal samples of live and dead *An. funestus* following exposure to the candidate insecticides, and the subsequent genotyping as described above, we used generalised linear mixed models to test for associations between phenotypic expression of resistance (i.e. percentage surviving exposure) and the presence of specific genotypes (i.e. *CYP6P9a*, *CYP6P9b*, *L119F-GSTe2*, SV 4.3kb and SV 6.5kb). This analysis was done only for deltamethrin. The final model had only the *L119F-GSTe2* gene; indicating only variants of this gene were predictive of surviving lethal deltamethrin insecticide doses (χ^2=9.0482, OR=2.5875, p=0.0026) (Figure **3-5**). However, there was no significant association between either *CYP6P9a* (χ^2=1.465, p=0.4808), *CYP6P9b* (χ^2=1.206, p=0.5471), SV 4.3kb (χ^2=1.065, p=0.587), or SV 6.5kb (χ^2=1.795, p=0.4076) with survival to lethal deltamethrin doses (Figure **3-5**).

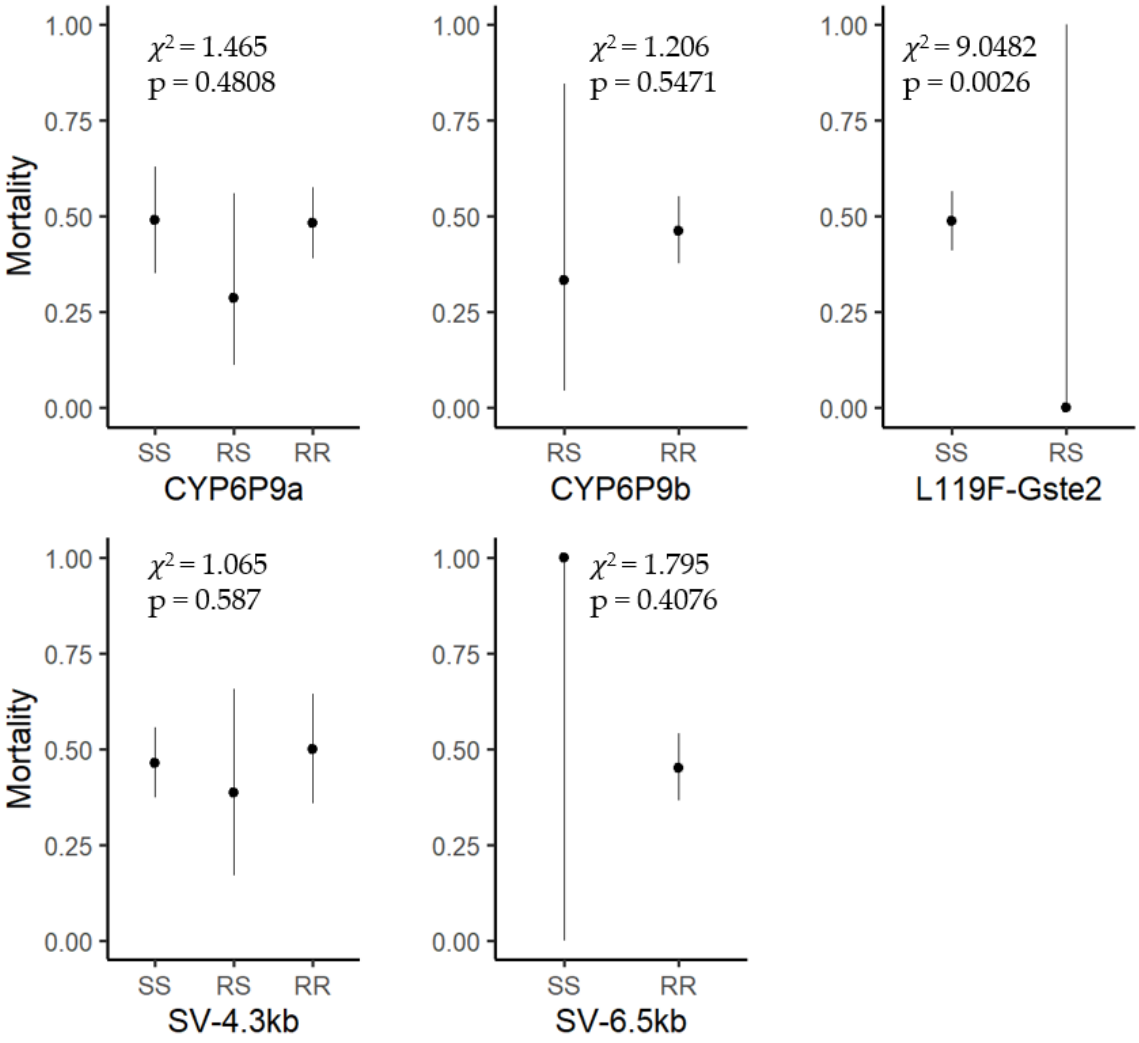


Figure 3-5: The association between metabolic resistance genes *CYP6P9a, CYP6P9b, L119F-GSTe2* and structural variants *SV 6.5kb* and *SV 4.3kb* with deltamethrin resistance phenotype. Y axis indicates the predicted mortalities from each single genotype, with the point indicating the mean and line 95% confidence interval. The genotypes on the X-axis indicate SS = homozygous susceptible, RS = heterozygous resistant, and RR = homozygous resistant.

3.4.8 Interactive map for visualisation of insecticide resistance

Since this was the first large scale survey of insecticide resistance in Tanzania's *An. funestus*, the findings could provide a critical foundation for future updates, preferably with a more granular coverage both geographically and temporally. More importantly, it could contribute to data-driven decision-making for resistance management when deploying vector control interventions. Therefore, to enhance practical application of the findings, we developed an interactive tool using R Shiny (37), enabling users to visualize the predicted insecticide resistance in *An. funestus* across Tanzania – and could, in future, be extended to other vector

species. This user-friendly tool allows the selection of various tested insecticides, displaying their predicted mortality rates on an interactive map. It highlights regional resistance patterns, aiding in the interpretation and application of our research data. The tool is accessible online at https://boydorr.gla.ac.uk/lucanelli/odero_ins_res_map/, and will be a valuable resource for researchers and public health practitioners working on malaria control.

3.5 Discussion

Anopheles funestus is a major malaria vector in Tanzania and is particularly dominant in the north-western region and southeastern regions (Mwalimu et al., 2024, Matowo et al., 2021, Kaindoa et al., 2017). Yet, its resistance to key public health insecticides has been, at best, patchy in comparison to the other malaria vectors such as *An. arabiensis* and *An. gambiae* for which the resistance data is more commonly collected. This study provides the first large-scale contemporaneous dataset on both the genotypic and phenotypic expression of resistance in *An. funestus* to a range of insecticides, including two pyrethroids, deltamethrin and permethrin, an organochlorine, DDT, a carbamate, bendiocarb, and an organophosphate, pirimiphos-methyl.

The findings show widespread multiple resistance in wild *An. funestus* mosquito populations in mainland Tanzania and provides a basis for decision-making for national malaria control programs on the management of insecticide resistance and selection of appropriate interventions. The findings show pyrethroid resistance to be particularly common in *An. funestus* with high resistance intensities to deltamethrin in most locations. Similar pyrethroid resistance profiles in this vector have been shown in earlier but separate studies in the south and north of Tanzania (Matowo et al., 2021, Kaindoa et al., 2017). Continentally, phenotypic expression of pyrethroid resistance in *An. funestus* has been reported in other countries including Uganda (Mulamba et al., 2014b), Mozambique (Cuamba et al., 2010), Malawi (Menze et al., 2022, Kumala et al., 2022), Ghana (Mugenzi et al., 2022, Riveron et al., 2016), and Cameroon (Antonio-Nkondjio et al., 2017), with most countries still lacking bioassay data on this vector species. The synergistic effect of PBO, leading to the restoration of susceptibility in pyrethroid-resistant *An. funestus* mosquitoes were evident in all tested

populations, pointing to the predominant involvement of metabolic-mediated resistance mechanisms. Malaria vectors of the *An. gambiae* complex has also shown similar restoration of pyrethroid-susceptibility with PBO (Tungu et al., 2023). In epidemiological studies evaluating ITNs treated with both PBO and pyrethroids in Tanzania (Protopopoff et al., 2018) and Uganda (Staedke et al., 2020), increased community protection against malaria was observed, compared to protection accrued with pyrethroid-only nets. Our results therefore add to the body of evidence on the efficacy of PBO-ITNs against pyrethroid-resistant malaria vector populations (Maiteki-Sebuguzi et al., 2023, Gleave et al., 2021).

The study also shows *An. funestus* across Tanzania to be susceptible to the IRS insecticide pirimiphos-methyl unlike in *An. gambiae*, where resistance has been confirmed in parts of the country (Kisinza et al., 2017). However, the observed resistance to the carbamate (bendiocarb) in parts of the country is concerning and should be factored in by malaria control programs if IRS campaigns are planned in the future. Though Tanzania has recently discontinued the use of IRS (Mwalimu et al.), this remains an important tool and could be especially useful in high transmission settings or emergencies to curb outbreaks. Phenotypic resistance to DDT was isolated to the Kilombero valley in south-eastern Tanzania and has been linked to the recent discovery of knockdown resistance (*kdr*) in *An. funestus* in the same location (Odero et al., 2024a). The *kdr* evolution in Kilombero is thought to be due to agricultural DDT contamination and a legacy of past extensive DDT stockpiles (Odero et al., 2024a). Without proactive management of resistance, the observed resistance situation in the country is likely to worsen, as similar insecticide classes are heavily used for agricultural pest control (Urio et al., 2022).

As vector control evolves in sub-Saharan Africa and as new or improved insecticidal interventions are introduced, it is crucial to monitor the genetic basis of insecticide resistance and track its evolution within and beyond Tanzania. The genotype frequencies of *CYP6P9a/b* and SV 6.5kb IR genes have previously been estimated as low-moderate in Tanzania but fixed in other southern African countries (Mugenzi et al., 2020, Weedall et al., 2019, Mugenzi et al., 2019). Here, we found the resistant alleles of these three genes to be either fixed or near fixation across most localities in the eastern region of Tanzania, indicating how these mutants have recently selectively swept through *An. funestus* populations

in Tanzania. The high frequencies of homozygous susceptible genotypes of *CYP6P9a*-SS in the northern Tanzania districts and the evidence of mixed genotypes in west of Tanzania (near Lake Tanganyika) could suggest a gene flow barrier in the country (possibly including landscape features such as the Great Rift Valley); or an ongoing south-north directional selection of the alternative homozygous resistant genotype *CYP6P9a*-RR arising from the southern Africa region (Barnes et al., 2017a). This potential gene flow restriction could further explain the contrasting distribution of structural variants, with SV 4.3kb fixed and restricted in the northern Tanzania districts (Missenyi and Misungwi) and SV 6.5kb in the rest of the country. The cause of high non-amplifications of SV 6.5kb and *CYP6P9b* in northern Tanzania is unclarified and warrants investigation. Nonetheless, it has been postulated to be due to the SV 4.3kb (Mugenzi Leon et al., 2023). Both structural variants have been demonstrated to exacerbate resistance in *An. funestus* by increasing the overexpression of *CYP6P9a* and *CYP6P9b* (Mugenzi Leon et al., 2023, Mugenzi et al., 2020). Recent analysis had similarly linked such copy number variants with increased expression of the genes encoding metabolic resistance in *An. gambiae* and *An. coluzzii* (Lucas et al., 2023, Lucas et al., 2019). The lack of statistical association of *CYP6P9a/b,* SV 4.3kb and SV 6.5kb genes with pyrethroid resistance in this study is likely due to their already strong selection and fixation in the *An. funestus* populations in Tanzania. This could also be a stronger effect of alternate metabolic genes not analysed here but previously shown to be associated with *An. funestus* pyrethroid resistance in East Africa such as *CYP6M4, CYP9K1, CYP6M1,* and *CYP6Z1* (Matowo et al., 2022, Sandeu et al., 2020).Additionally, this observation could also have resulted from matching the number of samples genotyped which did not consider the natural frequencies of these genes in the populations hence lacking sufficient statistical power to detect a true effect.

L119F-Gste2, which confers cross-resistance to DDT and pyrethroids in *An. funestus* have previously been identified in West and Central Africa (Tchigossou et al., 2020, Tchigossou et al., 2018, Riveron et al., 2014c) and Uganda (Mulamba et al., 2014b) as the only previous detection in eastern Africa. However, findings of this study now establish that the mutation has now spread to eastern Africa, with low frequencies of the *L119F-Gste2* resistance alleles detected in the Tanzanian districts bordering the Democratic Republic of the Congo (Tanganyika),

Mozambique (Mtwara rural), and Malawi (Tunduru). The strong association observed between this genotype and deltamethrin resistance phenotypes is concerning and warrants close monitoring of its spread and evolution. Though it is not possible to conclusively describe the evolutionary path of this resistance allele in the region, its strong association with pyrethroid resistance could allude to a recent selection.

Overall, this study has highlighted the importance of broadening the resistance monitoring efforts to include all principal malaria vectors, including not just the members of the *An. gambiae* complex, but also *An. funestus*. This is particularly important in areas where this vector species dominates malaria transmission. One complementary outcome of this study was the extension to develop a user-friendly online platform for stakeholders, particularly scientists and public health officials to access the data. This platform lays the groundwork for informed decision-making regarding both the management of insecticide resistance and the actual deployment of effective interventions. The study also has the advantage of providing contemporaneous data from multiple sites with both genetic and phenotypic observations. However, it will be essential to regularly update this information, preferably with greater granularity in terms of both the geographical coverage at regional and sub-regional levels, and in terms of the temporal frequency of data collection.

One limitation of this study was the utilization of adult wild female mosquitoes of unknown ages in the bioassays. It is standard practice to utilize age-synchronized, 2-3-day-old mosquitoes in resistance bioassays, to avoid aging-related weaknesses (Pinda et al., 2022) and environmental stresses. However, despite efforts to characterize the breeding habitats of *An. funestus*(Nambunga et al., 2020), this species is notoriously difficult to locate in most ecological settings making it challenging to apply the standard approach. This also explains why resistance data on *An. funestus* has been largely missing in previous Tanzania's sentinel surveys (Tungu et al., 2023). In this study, given the difficulties of finding the larvae, and the logistical challenges due to the geographical expanse covered, a decision was made to use adult samples. Therefore, as insecticide susceptibility increases with age in mosquitoes (Collins et al., 2019) we cannot completely rule out the potential confounding effect of aging in our mortality data, which could have

underestimated the resistant levels of our samples. However, this issue was mitigated by several steps including a) using only unfed-non-gravid adults, b) holding the mosquitoes for at least 24 hrs to eliminate any mortality associated with mosquito handling, c) normalizing excess mortalities using a set of controls, where natural mortality due to senescence was considered, and d) screening for both the genotypic and phenotypic expression of resistance in the mosquitoes.

Another significant constraint of this study was the potentially low statistical power to discern the associations between specific gene variants and phenotypic resistance. Given the sample sizes used, there was a predisposition toward detecting only variants with large effect sizes, while more subtle or moderate effects likely remained unidentified. Lastly, the study design and sample sizes did not permit an exploration of the cumulative or epistatic effects exerted by multiple gene mutations within the same individual mosquito. It is conceivable that a combination of mutations across the five genes investigated, rather than the impact of each mutation in isolation, could more accurately account for the variations observed in the phenotypic resistance. This complexity underscores the need for future studies to consider larger sample sizes and more sophisticated statistical and genetic analyses that can capture these nuances.

One area of improvement is that future tests should incorporate some of the newly approved insecticides for vector control, including the pyrrole, chlorfenapyr, which is used in the dual-active Interceptor G2, nets, and neonicotinoids, such as clothianidin, which is also approved by WHO for IRS (WHO, 2023b). Such expansion will provide essential data for the national malaria control program to guide their selection of interventions in the context of evolving resistance patterns.

3.6 Conclusion

This comprehensive study presents a cross-country landscape of insecticide resistance in *An. funestus*, a principal malaria vector in Tanzania. It marks an important step towards the understanding both the distribution and evolution of insecticide resistance mechanisms in Tanzania and will contribute significantly to evidence-based decision making for improved vector control in the country. The study revealed widespread resistance to pyrethroids, albeit with notable

restoration upon pre-exposure to the synergist, piperonyl butoxide (PBO), underscoring the potential of PBO-impregnated nets in combating pyrethroid resistant populations of *An. funestus*. Additionally, the presence of resistance to carbamates and in some sites, and the emergent resistance to DDT in south-eastern part of the country highlight the complexity of resistance in the vector and the significant threat to the existing arsenal of vector control insecticides. Fortunately, *An. funestus* populations across all study sites appear to remain susceptible to the organophosphate, pyrimiphos-methyl, suggesting that this chemical, which is already widely used in agriculture, may be one of the few remaining options suitable for vector control, should the country reinstate the use of indoor residual spraying. The detection of genetic markers, including *CYP6P9a/b*, associated structural variants, and the mutation *L119F-GSTe2* across different districts provide an explanation for genetic foundation underlying the observed phenotypic resistance patterns. Additionally, these genetic markers reveal some underlying gene flow patterns in the *An. funestus* populations and are suggestive of a barrier (potentially including the Great Rift Valley) as well as an ongoing south-north directional sweep of some genotypes, notably the *CYP6P9a*-RR. The findings of this study have been made readily accessible through an interactive online interface, to make it directly applicable for insecticide resistance management and to emphasize the need for continuous surveillance and updates on this dataset. Given the significance of these insights for the development and deployment of effective vector control tools, further research is required on all major vector species, with a focus on increased granularity in the spatial and temporal analysis, to enable adaptive resistance management and improved malaria control in Tanzania and the region.

4 Discovery of knock-down resistance in the major African malaria vector *Anopheles funestus*.

4.1 Abstract

A major insecticide resistance mechanism in insect pests is knock-down resistance (*kdr*) caused by mutations in the voltage-gated sodium channel (*Vgsc*) gene. Despite being common in most malaria *Anopheles* vector species, *kdr* mutations have never been observed in *Anopheles funestus*, the principal malaria vector in Eastern and Southern Africa, with resistance mainly being conferred by detoxification enzymes. In a parallel study, we monitored 10 populations of *An. funestus* in Tanzania for insecticide resistance unexpectedly found resistance to a banned insecticide, DDT, in the Morogoro region. Through whole-genome sequencing of 333 *An. funestus* samples from these populations, we found eight novel amino acid substitutions in the *Vgsc* gene, including the *kdr* variant, L976F (L995F in *An. gambiae*), in tight linkage disequilibrium with another (P1842S). The mutants were found only at high frequency in one region and were accompanied by weak signatures of a selective sweep, with a significant decline between 2017 and 2023. Notably, *kdr* L976F was strongly associated with survivorship to exposure to DDT insecticide, while no clear association was noted with a pyrethroid insecticide (deltamethrin). The WHO prequalifies no DDT products for vector control, and the chemical is banned in Tanzania. Widespread DDT contamination and a legacy of extensive countrywide stockpiles may have been selected for this mutation. Continued monitoring is necessary to understand the origin of *kdr* in *An. funestus*, and the threat posed to insecticide-based vector control in Africa.

4.2 Introduction

Chemical insecticides are central to the control of agricultural pests and disease vectors, such as mosquitoes. The control of *Anopheles* mosquitoes through the distribution of over 2.9 billion insecticide-treated bed nets (ITNs) has helped avert an estimated 633 million cases of malaria (Bhatt et al., 2015), a disease that still kills 600,000 yearly (WHO, 2023f). However, the widespread use of insecticides

for agricultural pest and disease vector control also has detrimental consequences, including direct lethal and sub-lethal effects on human and animal health and destabilising effects on ecosystem structure and function (Nicholson et al., 2023, Wurster et al., 1965). For example, insecticide exposure is a key stressor affecting the population decline of pollinators, essential for ecosystem health and food production (Nicholson et al., 2023, Douglas et al., 2020).

A key obstacle to sustainable malaria control is the evolutionary arms race between mosquitoes and insecticide-based mosquito control. Strong selection pressures generated by insecticide-based agricultural pest and disease vector control activities have resulted in the independent evolution of a diverse range of mechanisms that confer insecticide resistance (IR) phenotypes in numerous insect species (Hemingway and Ranson, 2000b). One of the earliest described IR mechanisms was the emergence of *kdr*, mediated by mutations in the target site of pyrethroid and organochlorine insecticides, located in the voltage-gated sodium channel gene (*Vgsc*), which play key roles in the transmission of action potentials along neurons, and are an essential component of the nervous system (Davies et al., 2007). These *kdr*-driven resistance phenotypes appeared rapidly after the introduction of the organochlorine dichloro-diphenyl-trichloroethane (DDT) spraying for insect control in the mid-20th century (Busvine, 1951) and eventually evolved to confer resistance to pyrethroids (Grigoraki et al., 2021, Reimer et al., 2008), the key ingredient in ITNs - the first line of defence against malaria. In an era of stalling gains in malaria control (WHO, 2023f), and concerted efforts both to develop a new generation of ITN and IRS products (Mosha et al., 2024, Accrombessi et al., 2023b) and proactively manage the deployment of existing insecticides to maximise efficacy, intensified surveillance, including genomic surveillance (Lucas et al., 2023, Donnelly et al., 2016), of malaria vector populations is critical for providing real-time warning of insecticide resistance emergence.

Resistance to all major insecticide classes is common in *An. funestus* and is primarily mediated through the increased activity of enzymes that bind to and metabolise insecticides (metabolic resistance) (Weedall et al., 2019, Coetzee and Koekemoer, 2013). This contrasts with another major vector *An. gambiae* where resistance is mostly conferred by a combination of metabolic and target-site

resistance (Hemingway and Ranson, 2000b). In a previous study, we reported insecticide resistance phenotypes across Tanzania and found that, in the Morogoro region, resistance to DDT was present(Odero et al., 2024b).

In this study, we report the findings of phenotypic and genomic surveillance done in Tanzania to understand the evolution and spread of insecticide resistance in *Anopheles funestus* - the dominant malaria vector in Eastern and Southern Africa (*16*). We report the first discovery of *kdr* mutations in *An. funestus*. We discover that, in Tanzanian *An. funestus*, *kdr* confers resistance to DDT, but not deltamethrin, despite a complete ban on DDT use for agriculture and vector control in Tanzania since 2008 by the Stockholm Convention (UNEP, 2005b). We suggest environmental contamination from extensive DDT stockpiles (WorldBank, 2012), or unofficial agricultural use, as possible causes. The emergence of *kdr*, which threatens the control of major crop pests and vectors of disease, such as *An. gambiae* and *Aedes aegypti (Soderlund and Knipple, 2003)*, highlights the potential of chemical insecticide contamination or unofficial use to exert unexpected and potentially harmful impacts on public health.

4.3 Materials and methods

4.3.1 Mosquito collection

Anopheles funestus samples analyzed in this study were collected from ten administrative regions in Tanzania: Dodoma, Kagera, Katavi, Lindi, Morogoro, Mtwara, Mwanza, Pwani, Ruvuma, and Tanga (Figure **4-1A**). The collections were done as part of a countrywide *Anopheles funestus* surveillance project in Tanzania and were subsequently incorporated into the MalariaGEN *Anopheles funestus* genomic surveillance project database (https://www.malariagen.net/projects/anopheles-funestus-genomic-surveillance-project). Mosquitoes were collected in households between 2017 and 2023 using CDC light traps and mechanical aspirators. They were sorted by sex and taxa and *An. funestus* group mosquitoes were preserved individually in 96-well plates containing 80% ethanol.

4.3.2 Whole genome sequencing and analysis

Genomic DNA was extracted from individual mosquitoes using DNeasy Blood and Tissue Kits (Qiagen, Germany). A single band confirmed the DNA purity and integrity on 1% agarose gel and a minimum DNA concentration of 20 ng/µl on a Qubit® 2.0 fluorometer. Samples that passed quality control, were individually whole-genome-sequenced commercially at 30X. The sequencing data have been deposited in the European Nucleotide Archive (https://www.ebi.ac.uk/ena/browser/home) under study number PRJEB2141.

Reads were aligned to the *An. funestus* reference genome AfunGA1 (Ayala et al., 2022b) with Burrows-Wheeler Aligner (BWA) version v0.7.15. Indel realignment was performed using Genome Analysis Toolkit (GATK) version 3.7-0 RealignerTargetCreator and IndelRealigner. Single nucleotide polymorphisms were called using GATK version 3.7-0 UnifiedGenotyper. Genotypes were called for each sample independently, in genotyping mode, given all possible alleles at all genomic sites where the reference base was not "N". The aligned sequences in BAM format were stored in the European Nucleotide Archive (study number PRJEB2141).

The identification of high-quality SNPs and haplotypes was conducted using BWA version 0.7.15 and GATK version 3.7-0. Quality control involved removing samples with low mean coverage, removing cross-contaminated samples, running PCA to identify and remove population outliers, and confirming the sex of all samples based on the modal coverage ratio between the X chromosome and the autosomal chromosome arm 3R. Full quality control methods are available on the MalariaGEN vector data user guide (https://malariagen.github.io/vector-data/ag3/methods.html).

We used decision-tree filters that identify genomic sites where SNP calling and genotyping are likely to be less reliable. More information on site filters can be found in the MalariaGEN vector data user guide. Genotypes at biallelic SNPs that passed the decision-tree site filtering process were phased into haplotypes using a combination of read-backed and statistical phasing. Read-backed phasing was performed for each sample using WhatsHap version 1.0

(https://whatshap.readthedocs.io/). Statistical phasing was then performed using SHAPEIT4 version 4.2.1 (https://odelaneau.github.io/shapeit4/).
Complete specifications of the haplotype phasing pipeline are available from the malariagen/pipelines GitHub repository (https://github.com/malariagen/pipelines/tree/master/pipelines/phasing-vector).

4.3.3 Identification of SNPs on *Vgsc*

To identify the *An. funestus Vgsc* gene and the variant that confers target-site resistance we performed alignments between the *An. gambiae* VGSC transcript AGAP004707-RD in AgamP4.12 geneset from the Ag1000 phase 3 data resource (https://www.malariagen.net/data/ag1000g-phase3-snp) and AFUN2_008728 from the *An. funestus* AfunF1.3 dataset. We extracted single nucleotide polymorphism (SNPs) altering the amino acid of VGSC protein from the *An. funestus* dataset and computed the allele frequency on the mosquito cohorts defined by the region and year of collection. Under selection pressure various alleles are expected to increase in frequency; we therefore filtered out variant alleles with a frequency lower than 5% resulting in a list of 8 variant alleles. Multiple sequence alignments of *An. funestus Vgsc* against *An. gambiae* and *M. domestica* were performed using MEGA v11.013 (Kumar et al., 2018).

4.3.4 Population genetic analyses

We searched for signatures of selective sweeps on the *Vgsc* gene using the *G123 and H12* selection statistics (Harris et al., 2018, Garud et al., 2015). H12 selection scans were performed on *An. funestus* genotypes by collection region where sample *n*>20 **(**see Figure **4-1A**) using the *h12_gwss* function in the malariagen_data python API (https://malariagen.github.io/malariagen-data-python/latest/Af1.html). Linkage disequilibrium (Rogers and Huff's R-squared) (Rogers and Huff, 2009) between the 8 *Vgsc* alleles was calculated using the *rogers_huff_r_between* in scikit-allel (https://zenodo.org/record/4759368). Haplotype clustering was performed by performing hierarchical clustering on a Hamming distance matrix, inferred from phased *An. funestus* haplotypes, using the Scipy library (https://scipy.org/citing-scipy/). Clustering dendrogram, and bar plot of amino acid substitutions, were plotted using the seaborn library.

4.3.5 Association of L976F and P1842S alleles with insecticide resistance

Due to difficulties in finding *An. funestus* immature stages and unsuccessful attempts to get sufficient offspring from wild blood-fed females, we adopted a previously tested approach relying on unfed and non-gravid females of unknown ages for the resistance bioassays (Pinda et al., 2022, Kaindoa et al., 2017). To test for associations between the identified mutations with IR, we exposed wild non-blood-fed *An. funestus* mosquitoes of unknown ages to standard doses of deltamethrin and DDT insecticides following the WHO tube assays. The bioassays were conducted as part of a countrywide insecticide resistance surveillance in Tanzania (Odero et al., 2024b). For each insecticide, we randomly separated phenotypically resistant mosquitoes (i.e., alive 24 hours post-exposure) and susceptible (i.e., dead 24 hours post-exposure). For DDT we had 10 alive and 10 dead and for deltamethrin 29 alive and 27 dead. DNA was extracted from individual mosquitoes using DNeasy Blood and Tissue kit (Qiagen, Germany). The mosquitoes were identified at the species level using species-specific primers that can distinguish *An. funestus* from the other members of the group (Koekemoer et al., 2002b). To establish if the two *kdr* variants are associated with insecticide resistance, we designed PCR primers from *An. funestus Vgsc* (Gene ID: LOC125769886) to amplify domain IIS6 (L976F); kdrL976F_FWD 5'-TGTGCGGTGAATGGATCGAA-3' & kdrL976F_REV 5'-CGCTTCAGCGATCTTGTTGG-3', and C-terminal (P1842S) kdrP1842S-FWD 5'-CTACCCGGGAAATTGTGGCT-3' & kdrP1842S_REV 5'-TGCCACCATCGTTTCCGTTA-3'. Each 20µl reaction volume contained 10µl GoTaq® Green Master Mix (Promega, USA), 1µl (0.5µM final concentration) of forward/reverse primer, 1µl of DNA, and 7µl nuclease-free water. The thermocycler conditions were 94℃ 5min, 30 cycles of 94℃ 1min, 58℃ 30sec, and 72℃ 30sec, and a final extension of 72℃ 10min. The DNA fragments were separated on a 1% agarose gel, cut, purified using PureLink™ Quick Gel Extraction Kit (Invitrogen), and commercially Sanger sequenced. Collectively, we sequenced 76 individual mosquitoes: 56 from deltamethrin and the rest from the DDT bioassays. The frequencies of the wild type and mutant alleles were determined and correlated with phenotypes using generalised linear models in R-software v4.1.1.

4.3.6 Data analysis

To determine the phenotypic resistance to DDT and deltamethrin, we calculated the percentage mortality from the bioassays following WHO guidelines (WHO, 2022b). Details of the modelling approach accounting for potential mortality variabilities due to extrinsic factors and the resistance profiles across Tanzania are outlined in Odero *et.al* (Odero et al., 2024b). To explore the genetic association between L976F and P1842S alleles with DDT and deltamethrin resistance phenotype, we used Haploview version 4.1 statistical software (Barrett et al., 2005). Linkage disequilibrium was established by D' and R2 parameters. Genetic association between alleles or haplotypes and the resistance phenotypes were conducted on alleles with allele frequency > 0.05 and in Hardy-Weinberg equilibrium. A Chi-square test was performed, and P-values were calculated for the allelic and haplotype frequencies in the alive and dead mosquitoes. The odds ratio for statistically associated and marginally significant alleles was calculated by comparing the related alleles with the rest.

4.4 Results

As part of an insecticide resistance surveillance study in 10 sites across Tanzania (Odero et al., 2024b) (Figure **4-1A**), we investigated phenotypic resistance (as measured by mosquito survival 24 hours following insecticide exposure) in *An. funestus* to the discriminating doses of DDT, deltamethrin (type II pyrethroid), or deltamethrin together with the piperonyl butoxide (PBO) synergist, which is increasingly used on ITNs (*23*) to restore susceptibility in pyrethroid-resistant populations in Tanzania. The mosquitoes were phenotypically resistant to deltamethrin in all Tanzanian regions, but PBO ubiquitously restored susceptibility (Odero et al., 2024b). Unexpectedly, resistance to DDT was recorded in the Morogoro region (68%, CI 57.8 - 77.9), but possible resistance in other locations (Odero et al., 2024b) (Figure **4-1B**).

To understand the genetic bases of resistance, we analysed whole-genome-sequencing (WGS) data from 333 mosquitoes sampled from 10 sites across Tanzania (Figure **4-1A**). We performed genome-wide selection scans (GWSS) with the H12 and G123 statistics (Supplementary Figure **7-1** and **Supplementary Figure 7-2**) to test for evidence of selective sweeps in the *An. funestus* genome associated with known or novel IR loci (Figure **4-1C**; grouping samples by

administrative region, including those collected at different time points (see **Supp. Table 1** for per-group sample numbers). We detected a signal of elevated H12, indicating a possible selective sweep in the region containing the *Vgsc* gene (Chromosome 3, positions 44105643-44105644) in samples from the Morogoro region in the southeastern part of the country (Figure **4-1C**). Notably, *Vgsc* encodes for the voltage-gated sodium channel, where DDT binds in mosquitoes, and where mutations are strongly linked to resistance in *An. gambiae* (*11*). In Kagera, Katavi, and Mwanza regions, there was no visible sign of a selective sweep at or near the *Vgsc* region. In Dodoma, Lindi, Ruvuma, and Tanga, there were peaks of elevated H12 near *Vgsc*, but these appeared within the context of relatively high H12 across the chromosome **(Figure 4-1C, Supplementary Figure 7-1** and **Supplementary Figure 7-2).**

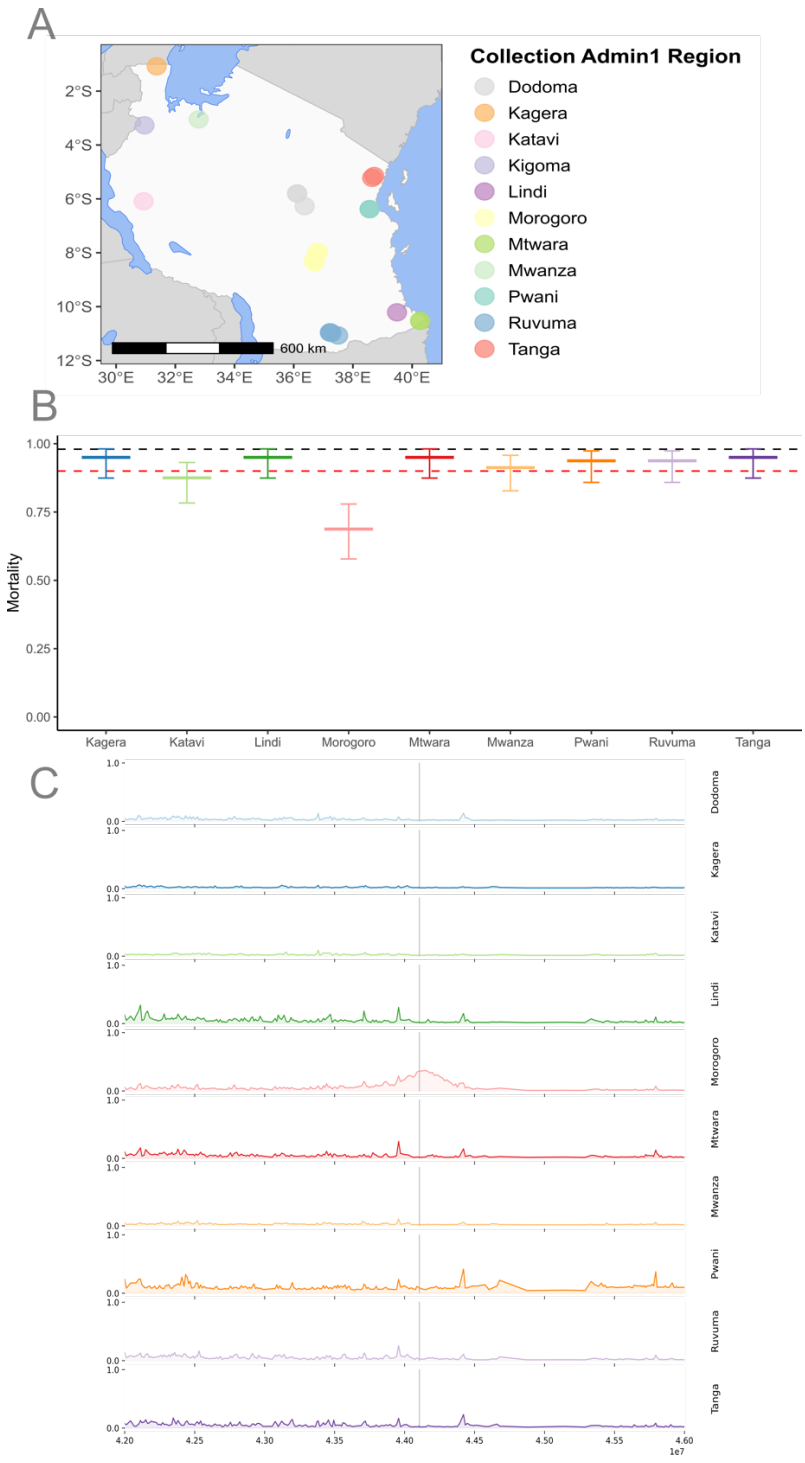


Figure 4-1: (A) Map of *An. funestus* collection locations. Points indicate sample collection locations. The point colour indicates the administrative region from which samples were collected. (B): Phenotypic insecticide resistance profile of *An. funestus* to DDT using bioassay data adopted from our recent surveillance (Odero et al., 2024b). The colours and the X-axis represent the various regions where the bioassays were conducted, and the error bars are 95% confidence interval. The black and red dotted lines on the Y-axis represent the 98 and 90% mortality thresholds. (C) H12 selection scans of *An. funestus* chromosome 3RL, coloured and windowed by sample collection region (where n>20 – see Supp Table 2). X-axis indicates the position (in base-pairs (bp)), Y-axis indicates the selection statistic H12. The Grey line indicates the location of the *Vgsc* gene. Note Mwanza region is absent from panel C as there were too few samples (n<20) to perform a selection scan.

We searched our data for mutations in the *Vgsc* gene and found 8 amino acid substitutions occurring at frequencies greater than 5% (Figure **4-2A**). Of these, two alleles, L976F and P1842S occurred at the highest frequency (Figure **4-2A**). The frequencies of P1824S and L976F were highest in samples collected from Morogoro in 2017 (0.75 and 0.90 respectively) **(**Figure **4-2A)** and declined yearly, reaching their lowest frequency in samples collected in 2023 (0.48 and 0.56 respectively; χ^2 = 12.15, p=0.0005; Figure **4-2B).** These mutations occurred at very low frequencies or were absent in all other locations (Figure **4-2A).**

To understand their function, we aligned the *An. funestus Vgsc* sequence (Gene ID: AFUN2_008728.R15290) with that of *Musca domestica* (Gene ID: X96668) and *An. gambiae* (AGAP004707-RD AgamP4.12 gene set) (Clarkson et al., 2021). We found that the amino acid change at *An. funestus* L976F corresponded to L1014F in *M. domestica* and L995F in *An. gambiae* in domain II subunit 6 (IIS6) of the *Vgsc* gene (Table **4-1**), which in *An. gambiae* species complex drastically increases IR to DDT and pyrethroids (Mitchell et al., 2014, Reimer et al., 2008). The second variant P1842S corresponded to P1874S in *An. gambiae* and P1879 in *M. domestica* and were all in the C-terminal domain (Table **4-1**).

Table 4-1: Comparative non-synonymous nucleotide variation in the voltage-gated sodium channel gene. The position is relative to the *Anopheles funestus* strain FUMOZ reference, chromosome arm 3RL. Codon numbering according to *Anopheles funestus Vgsc* transcript AFUN2_008728.R15290, *Anopheles gambiae* transcript AGAP004707-RD in gene set AgamP4.12, and *Musca domestica* EMBL accession X96668 Williamson *et al.* (Williamson et al., 1996).

Position	***An. funestus***	***An. gambiae***	***M. domestica***	**Domain**
3RL:44,115,564 T>C	I2030V	I2061	P2063	COOH
3RL:44,115,768 C>A	G1962C	A1994	P1997	COOH
3RL:44,116,128 G>A	P1842S	P1874S	P1879	COOH
3RL:44,116,334 T>C	N1773S	N1805	N1810	
3RL:44,116,923 A>T	F1638Y	F1670	V1675	
3RL:44,117,167 A>T	W1557R	W1589	W1594	
3RL:44,122,391 C>A	G1144C	G1173	G1180	
3RL:44,125,475 T>A	L976F	L995F	L1014	IIS6

To explore the possible association between L976F and P1842S alleles with DDT and deltamethrin resistance, we genotyped surviving (resistant) and dead

(susceptible) mosquitoes from IR bioassays for both L976Fand P1842S loci). Neither locus was associated with deltamethrin resistance: L976F (χ^2 = 0.04, p = 0.84) and P1842S (χ^2 = 0.59, p = 0.44). (Figure **4-2C**). We found a strong association with DDT resistance in mosquitoes carrying L976F (χ^2 = 9.23, odds ratio = 11.0, p = 0.0024) and a marginally non-significant positive association for P1842S (χ^2 = 3.75, p = 0.0528) (Figure **4-2C**).

To elucidate *Vgsc* haplotype structure in *An. funestus,* we computed pairwise linkage disequilibrium (LD) using the Rogers and Huff method (briefly, derived from r, the correlation across unphased genotypic values) (Rogers and Huff, 2009), between nonsynonymous variants occurring at a frequency of > 5% in Tanzanian *An. funestus* **(**Figure **4-2D**). We found that P1824S occurred in tight LD with L976F (*D'*=0.95) **(**Figure **4-2D).** Of other non-synonymous polymorphisms, F1638Y and W1557R exhibited only weak LD with L976F (Figure **4-2D)**.

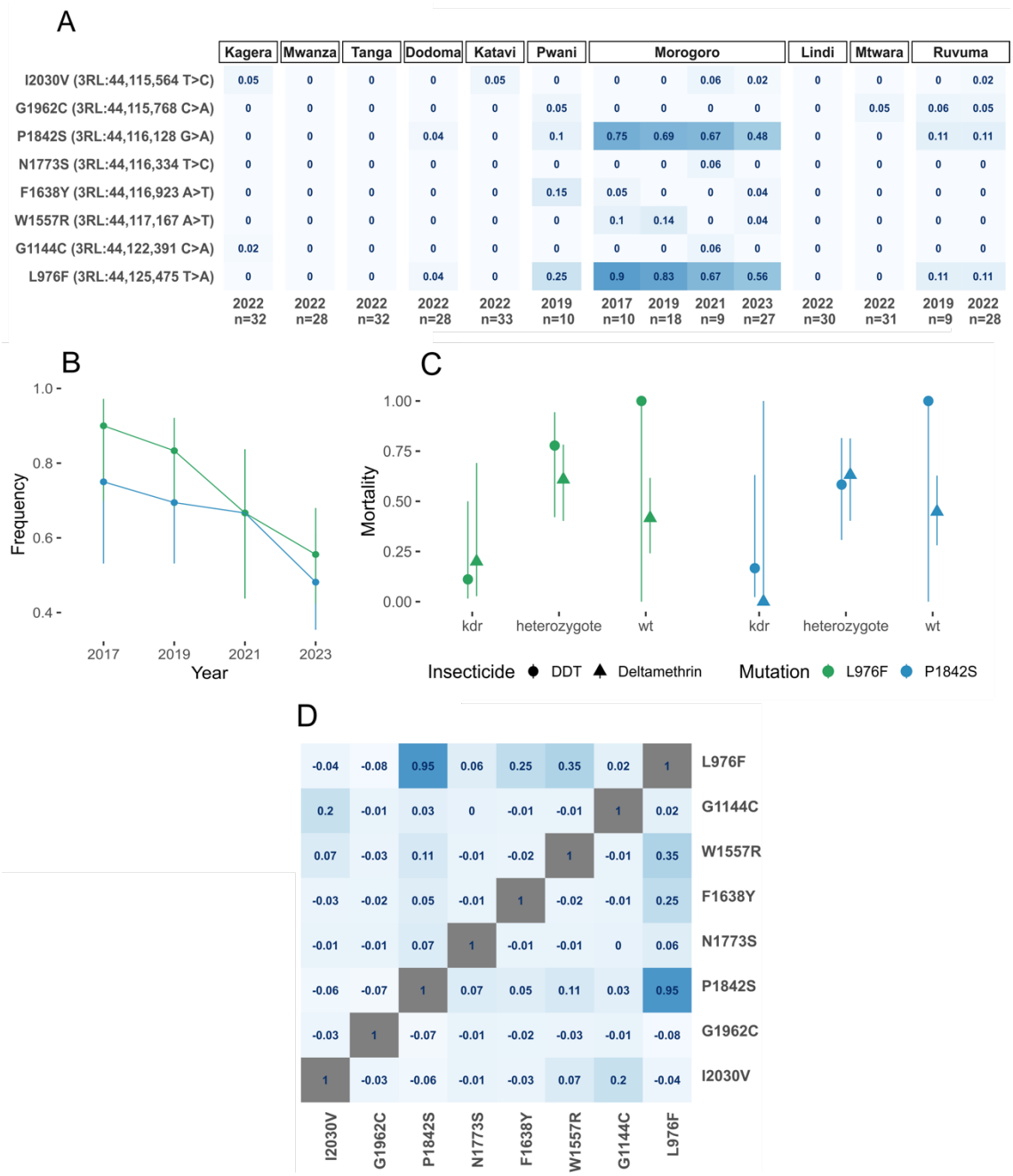


Figure 4-2: (A) Heatmap of *Vgsc* allele frequencies. Y-axis labels indicate mutation effect, chromosome position, and nucleotide change. X-axis labels indicate the collection date and heatmap intensity indicates frequency where darker = higher, with frequency labelled in each heatmap facet. The heatmap is panelled by the sample collection region. (B) L976F and 1842S frequencies, in the Morogoro region, over time. The y-axis indicates allele frequency, X-axis indicates the date. Line and point colour refer to mutation, specified in the legend. Bars indicate 95% confidence intervals. (C) Denotes the association of L976F and P1842S with resistance to Deltamethrin and DDT. Colour and panelling are by mutation, the x-axis indicates genotype, the y-axis indicates mortality, the point shape indicates the mean for each insecticide and the line indicates the 95% CI based on generalised mixed model prediction. (D) Heatmap of linkage disequilibrium (LD) (Rogers and Huff R) between nonsynonymous variants in the *Vgsc* gene at frequency > 5%. LD is indicated by fill colour. SNP effects and positions are labelled on the X and Y axes.

We constructed a haplotype clustering dendrogram from haplotypes in all 333 individuals, from the *Vgsc* gene **(**Figure **4-3).** The clustering dendrogram disclosed three major clades and three main combinations of the four most prevalent *Vgsc* alleles **(**Figure **4-3).** The most striking signal was a subclade of identical, or near-identical haplotypes containing both L976F and P1842S (Figure **4-3),** indicating a selective sweep on a combined L976F/P1842S haplotype. This combined haplotype was present at higher frequencies in the Morogoro region relative to the neighbouring regions of Pwani, Ruvuma, and Dodoma (Figure **4-3**). Most amino acid substitutions were present in a single clade in samples from Pwani, Dodoma, Ruvuma, and especially Morogoro (Figure **4-3**).

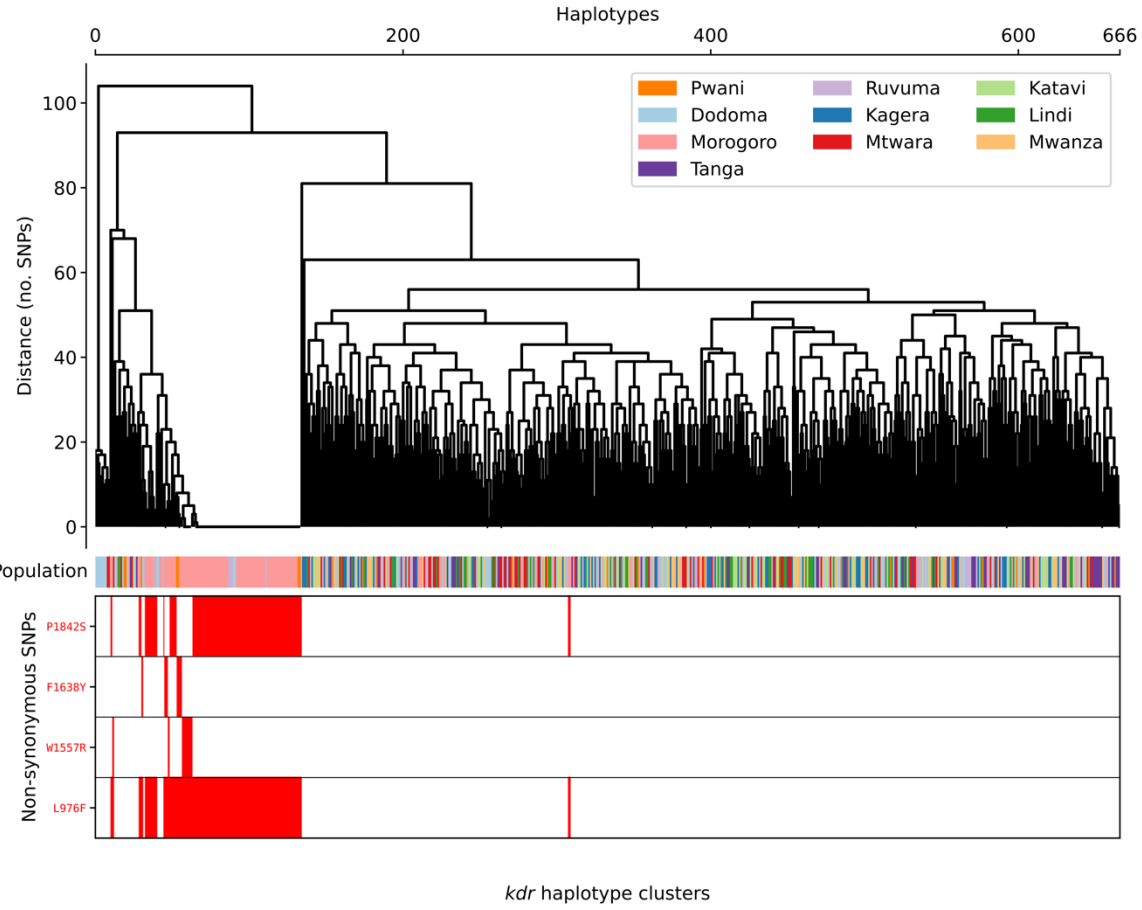


Figure 4-3: Clustering of haplotypes at the *Vgsc* gene (LOC125769886, 3RL:44105643-44156624). The dendrogram branch length corresponds to no. SNPs difference (y-axis). Tips correspond to individual haplotypes (x-axis). The coloured Population bar denotes the administrative region of origin (as described by the legend). Red blocks at the bottom indicate the presence of the labelled non-synonymous SNPs in the *Vgsc* gene.

4.5 Discussion

In a genomic surveillance study in Tanzanian *An. funestus*, we discovered eight novel *Vgsc* mutations. L976F, confers *knockdown resistance* (*kdr*), occurring in tight linkage disequilibrium with, P1842S, and at high frequencies (up to 90%) in the Morogoro region over 4 years, with limited spread to neighbouring regions. The mutation L976F showed an association with resistance to DDT, but not to pyrethroid insecticides. The role of *kdr* in pyrethroid resistance phenotypes in other *Aedes, Culex* and *Anopheles* vectors, makes the discovery of *kdr* in *An. funestus* a significant and unwelcome development that has the potential to pose a new threat to vector control in the region. Reassuringly, a lack of association between *kdr* and deltamethrin resistance indicates that the emergence of *kdr* is not linked to, nor is presently likely to threaten, the mass rollout of PBO-pyrethroid bed nets currently underway in Tanzania as a response to IR (Tanzania-NMCP, 2020). The emergence of *kdr* resistance to DDT suggests that future use of DDT for IRS may become even less favoured. However, this does not preclude a role for *kdr* in the *An. funestus* IR armamentarium in the future and an urgent follow-up study is required to monitor the evolution of vector DDT resistance and determine whether *kdr confer* resistance phenotypes to other widely used pyrethroids, such as permethrin, and alpha-cypermethrin, as well as other insecticide families, especially PBO and pyrrole formulations currently being rolled out in new ITN products across the African continent (WHO, 2023c). The lack of association of *kdr* with pyrethroid resistance might be due to the strong metabolic resistance shown to pyrethroids in *An. funestus*, reducing the benefit of *kdr* (Weedall et al., 2019). The association of *kdr* with resistance to DDT but not pyrethroids, combined with selection signals and recently declining *kdr* allele frequencies where we have time series, suggests recent to past, rather than contemporary selection, perhaps due to factors other than the current use of public health pesticides.

This discovery raises intriguing questions about the conditions that have enabled the emergence of *kdr* in *An. funestus*. Our data suggests that *Vgsc* mutation in *An. funestus* do not confer target-site resistance to pyrethroids, indicating a possible explanation as to why, despite extreme selection pressures imposed by pyrethroid control have facilitated widespread propagation of resistant *Vgsc* haplotypes

across the African continent in *An. gambiae (Clarkson et al., 2021)*, the emergence of *kdr* in Tanzanian *An. funestus* remains relatively localised. Mechanistic studies, including expression studies of mutant *Vgsc* proteins in *Xenopus* oocytes (Miller and Zhou, 2000), will enable comparisons between taxa that will elucidate this further.

If the ubiquitous use of pyrethroids in vector control did not select for the emergence of *kdr*, whence *kdr?* Even more curiously, the apparent decline of *kdr* allele frequencies between 2017 and 2023 suggests that the selection pressure causing the emergence of *kdr* has eased (although non-uniform sample sizes per time-point make confident assertion of this difficult). DDT is a largely obsolete, banned, pesticide that is no longer widely used for vector control in Tanzania, or in Africa as a whole, due to its bio-accumulative and toxic properties - with the most well-known effects being egg-shell thinning properties among birds (Grier, 1982), and associations with human cancers (Cohn et al., 2019). There is no record of DDT use in the last decade for agriculture or vector control in the Morogoro region, or Tanzania as a whole, where the production, importation, and usage of DDT have been banned since 2009 (UNEP, 2005a), except for limited use in malaria vector control. In 2008, Tanzania rolled out an ambitious malaria vector control strategy relying on large-scale use of DDT for indoor residual spraying (IRS), implemented in 60 districts across the country (Figure **4-4A**), and discontinued in 2010 (Oxborough, 2016). Morogoro, where we detected *kdr,* was not part of this expanded campaign. Before the ban, Tanzania imported large stockpiles of DDT mostly for agricultural pest control (Figure **4-4C**). Following the ban, there have been anecdotal reports of continued illegal use of DDT amongst farmers to date (Lahr et al., 2016). The Africa Stockpiles Programme (ASP) was launched in 2005 to eliminate stockpiles of obsolete pesticides, including DDT. At this time, it was estimated that Tanzania still possessed approx. 1,500 tonnes of obsolete pesticides (FAO, 2005), including a DDT stockpile of 30 tons (as of 2012) (WorldBank, 2012), approximately 50 km away from where DDT-resistant *An. funestus* were detected in this study, and 156 tons were in Morogoro town (WorldBank, 2012) (Figure **4-4C**). The ASP and the Tanzanian Government eliminated 100% of inventoried publicly held DDT stockpiles and conducted extensive environmental remediation by programme closed in 2013 (WorldBank, 2016). However, extensive DDT contamination remains (Elibariki and Maguta,

2017), and DDT remains in widespread use by private individuals (Lahr et al., 2016). The coincident proximal location of high levels of *kdr* in *An. funestus* with large past DDT stockpiles as well as the presence of widespread DDT contamination and private usage, leads us to hypothesise that the two most likely scenarios of *kdr* emergence in *An. funestus* are contamination of local larval breeding sites from agricultural or stockpiled DDT (Figure **4-4B, C**), and further investigation is needed to establish why *kdr* did not emerge and sweep through the population during periods of widespread DDT usage in the 20th century The removal of DDT stockpiles by the ASP, and ongoing environmental remediation, may have contributed to reduced selection pressure on *kdr*, evident from declining frequency in Morogoro (although small and uneven sample sizes make confident assertion of this trend difficult). Continued monitoring of allele frequencies and future studies of *kdr* frequencies targeted towards sites of known DDT contamination will establish whether this hypothesis is correct.

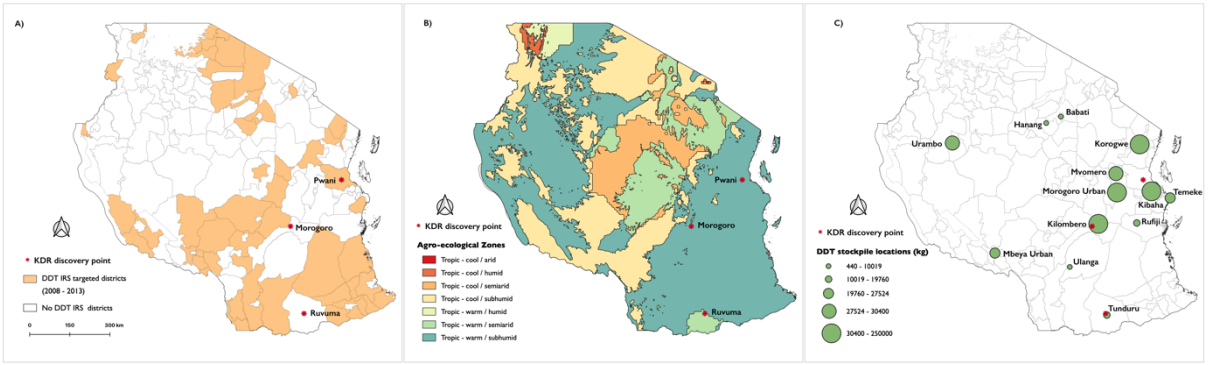


Figure 4-4: (A) Agro-ecological zones in Tanzania with colours on the map denoting the different categories indicated in the figure key. (B) Tanzanian National Malaria Control Programme (NMCP) indoor residual spraying strategy 2008 – 2012. The colours indicate districts where DDT spraying was planned. (C) DDT stockpile locations with the size of the circle indicating the stockpile quantities.

In *Silent Spring* (1962), Rachel Carson brought for the first time into the public eye the unpredictable and often remote impacts of anti-insect chemical agents on human health and nature "On one hand delicate and destructible, on the other miraculously tough and resilient, and capable of striking back in unexpected ways" (Carson, 1962). Further study of *kdr* in *An. funestus* will enable the identification of the origin of this mutation and make clear the full implications of its presence in the population for vector control. Whether the emergence of *kdr* in *An. funestus* is caused by vector control, unlicensed DDT usage in agriculture, or exposure to stockpiled DDT, our findings underscore the legacy of *Silent Spring* by reinforcing

the potential for pesticides and organic pollutants to exert inadvertent influences on animal biology that may have profound and unfortunate consequences for public health.

5 Genomic Analysis Reveals Distinct Genetic Populations of The Malaria Vector *Anopheles funestus* in Tanzania

5.1 Abstract

Anopheles funestus is the leading malaria vector in Tanzania and in most parts of East and Southern Africa. Yet, the genetic diversity and structure of its populations are less understood, compared to other major vectors such as *Anopheles gambiae*. Elucidating the population genetic diversity in malaria vectors is important for understanding the distribution of insecticide resistance genes, how vector populations are structured in space for targeted interventions and devising sustainable insecticide-based vector control approaches. We sequenced the whole genomes of 334 individual *An. funestus* mosquitoes from 11 regions across Tanzania and demonstrated the existence of two genetically differentiated populations; the inland, high-altitude populations (found in Katavi, Kagera, Kigoma, and Mwanza) and coastal, low-altitude populations (found in Pwani, Morogoro, Tanga, Ruvuma, Mtwara, Dodoma, and Lindi), potentially separated by an area of unsuitable climate, geographically coincident with the eastern arm of the Great Rift Valley. The two populations showed demographic differences and divergence, likely due to differentiation at insecticide resistance genes reflecting diverse historical and contemporary dynamics. In addition, we revealed genetic variation in insecticide resistance markers. Specifically, i) positive selection of haplotypes in the *Cyp6* gene cluster, ii) marked geographic variations in allele frequencies of the G454A-*Cyp9k1,* iii) multiple mutations in the *Cyp6p* gene. Altogether, these results suggest the likelihood of gene flow barriers between the populations. Such population disconnectedness should be considered for the implementation of resistance management and the rollout of novel genetic-based vector control approaches. Future research should examine the epidemiological relevance of this discontinuity in gene flow and whether these populations have different malaria transmission abilities.

5.2 Introduction

Anopheles funestus stands out as a dominant malaria vector in the East and Southern Africa region, where it is responsible for most malaria transmission in many localities (Msugupakulya et al., 2023). Despite its significant role, the species has been less studied than other major vectors like *Anopheles gambiae*, primarily due to challenges in laboratory colonisation, unresolved taxonomic complexities and challenges in field observations of the species in its natural habitats (Odero et al., 2023, Dia et al., 2013). The control of *An. funestus* is further complicated by widespread insecticide resistance (Coetzee and Koekemoer, 2013). Indeed, sustained use of insecticides in vector control, notably for insecticide-treated bed nets and spraying indoor surfaces with insecticides, and in agriculture has led to the evolution of insecticide-resistant phenotypes and reduced efficacy of these first-line vector control tools (Ranson and Lissenden, 2016a, Hemingway et al., 2016, Edi et al., 2012). Consequently, the fight against malaria remains at a crossroads with over 600,000 annual deaths, mostly in African children under 5 years (WHO, 2023f).

Understanding the genetic diversity in malaria vectors is crucial for elucidating critical aspects of their biology and a basis for developing targeted and effective control strategies. For example, population genetic analysis has led to the discovery of new cryptic species within the *An. gambiae* complex, *Anopheles* TENGRELA (AT), in Burkina Faso (Tennessen et al., 2021). This understanding can also aid in predicting the spread of advantageous variants (Clarkson et al., 2018) in addition to the discovery of novel insecticide resistance mechanisms such as copy number variation (CNV), associated with pyrethroid resistance in *An. gambiae* (Lucas et al., 2019, Weetman et al., 2018). Genetic variations in mosquitoes are crucial in estimating the viability of transgenic approaches for mosquito control such as gene drives (Wang et al., 2021). Recent advances in molecular techniques and the availability of reference genomes (Ayala et al., 2022b) have spurred research interest towards understanding population diversity in otherwise less studied vectors, *An. funestus*.

Anopheles funestus sensu stricto, hereafter *Anopheles funestus,* mediates 9 out of 10 malaria transmissions in parts of Tanzania (Mapua et al., 2022, Matowo et

al., 2021) and is the leading vector even in locations where another primary vector, *An. arabiensis* outnumbers the vector population (Mwalimu et al., 2024, Matowo et al., 2023). The superiority of *An. funestus* in contribution to malaria infection is replicated across the eastern and southern African countries (Msugupakulya et al., 2023). *Anopheles funestus* has a wide ecological range spanning tropical and sub-tropical Africa (Odero et al., 2023). The vector's genome contains enormous diversity and inversions, and previous studies show structuring into eastern, western, and central African genetic populations (Michel et al., 2005), like *An. gambiae* and *An. coluzzii* (Anopheles gambiae Genomes, 2020, Anopheles gambiae Genomes et al., 2017). Though studies investigating population diversity in *An. funestus* are limited, past evidence points to local vector adaptations between *An. gambiae* populations are potentially driven by geographical distance (isolation by distance) (Lehmann et al., 2003), ecological variations and other genetic discontinuities (Pinto et al., 2013). For instance, the Great Rift Valley has been hypothesised as a major gene-flow barrier in East and Southern Africa resulting in the evolution of genetically distinct *An. funestus* populations on either side of the valley (Michel et al., 2005, Kamau et al., 2002). Continental mitochondrial analysis of *An. funestus* has discovered two distinct lineages; a ubiquitous lineage I and an eastern and southern Africa-restricted lineage II (Jones et al., 2018). Such lineages and diversity, which occur due to restricted gene flow and adaptation, potentially impact the spread of beneficial genetic variation across populations.

The major metabolic resistance genes, *Cyp6p9a* and *Cyp6p9b,* are widespread in pyrethroid-resistant *An. funestus* mosquitoes from East and Southern Africa but absent in West and Central Africa (Weedall et al., 2019, Mugenzi et al., 2019). Conversely, the DDT resistance associated with glutathione S-transferase epsilon (*L119F-GSTe2*) mutation is largely restricted to West and Central Africa (Tchigossou et al., 2020) with recent evidence of spread to eastern Africa (Odero et al., 2024b). These patterns depict possibly restricted interaction and limited movement of these genes across the vector range. Our recent analysis of similar resistance markers in Tanzania revealed an ongoing south-north directional spread of the *Cyp6p9a* and *Cyp6p9b* genotypes, suggesting a landscape feature-driven gene-flow barrier or breakdown of past genetic barriers (Odero et al., 2024b). This analysis raised the following questions pertinent to malaria control in Tanzania

and beyond: i) are there specific genetic differences between the inland and coastal *An. funestus* mosquito populations in Tanzania? ii) what are the main drivers of genetic differences? iii) how do genetic variations, relate to patterns of phenotypic insecticide resistance and population size history? and iv) what are the implications of the genetic diversity and population structuring for current and future malaria control strategies?

To answer these questions, we analysed the whole genome sequences of 334 *An. funestus* mosquitoes sampled from 11 administrative regions with varying ecologies and malaria burden across mainland Tanzania. We investigated the genetic differentiation between the populations using principal components analysis (PCA) and *Admixture* ancestry, based on genome-wide single nucleotide polymorphisms (SNPs). We tested for evidence of selective sweeps in the *An. funestus* genome associated with known or novel insecticide resistance loci by performing genome-wide selection scans (GWSS) with the H12 statistic. Additionally, we constructed haplotype clustering dendrograms from haplotypes in all 334 individuals at the *Cyp6* gene cluster and *Cyp9k1* gene and investigated amino acid variation at each gene. We hypothesised that the population clustering could be driven by potential genetic discontinuities such as topography, ecology (inland vs coastal populations), or the Great Rift Valley.

5.3 Methods

5.3.1 Mosquito collection

Anopheles funestus samples analysed in this study were collected from 11 administrative regions in Tanzania: Kigoma, Dodoma, Kagera, Katavi, Lindi, Morogoro, Mtwara, Mwanza, Pwani, Ruvuma, and Tanga (Figure **5-1A**). The locations represent a comprehensive ecological, geographical and epidemiological cross-section of Tanzania. The collections were done as part of a countrywide *An. funestus* surveillance project in Tanzania and were subsequently incorporated into the MalariaGEN *Anopheles funestus* genomic surveillance project database (https://www.malariagen.net/projects/anopheles-funestus-genomic-surveillance-project). Most mosquitoes were collected in households between 2021 and 2023 using CDC light traps and mechanical aspirators. They were sorted

by sex and taxa and *An. funestus* group mosquitoes were preserved individually in 96-well plates containing 80% ethanol.

5.3.2 Whole genome sequencing

Genomic DNA was extracted from individual mosquitoes using DNeasy Blood and Tissue Kits (Qiagen, Germany). A single band confirmed the DNA purity and integrity on 1% agarose gel and a minimum DNA concentration of 20 ng/µl on a Qubit® 2.0 fluorometer. Samples that passed quality control, were individually commercially whole-genome-sequenced at 30X.

5.3.3 Sequence analysis and SNP calling

The sequences were processed following pipelines developed by the Sanger Institute. Detailed specifications of the sequence analysis, SNP calling, and haplotype phasing pipelines are available from the MalariaGEN GitHub repository (https://github.com/malariagen/pipelines/).

Briefly, reads were aligned to the *An. funestus* reference genome idAnoFuneDA-416_04 (Ayala et al., 2022b) with Burrows-Wheeler Aligner (BWA) version v0.7.15. Indel realignment was performed using Genome Analysis Toolkit (GATK) version 3.7-0 RealignerTargetCreator and IndelRealigner. Single nucleotide polymorphisms were called using GATK version 3.7-0 UnifiedGenotyper. Genotypes were called for each sample independently, in genotyping mode, given all possible alleles at all genomic sites where the reference base was not “N”. The aligned sequences in BAM format were stored in the European Nucleotide Archive (ENA) under study number PRJEB2141.

The identification of high-quality SNPs and haplotypes was conducted using BWA version 0.7.15 and GATK version 3.7-0. Quality control involved the removal of samples with low mean coverage, removing cross-contaminated samples, running PCA to identify and remove population outliers, and sex confirmation by calling the sex of all samples based on the modal coverage ratio between the X chromosome and the autosomal chromosome arm 3R. Full quality control methods are available on the MalariaGEN vector data user guide (https://malariagen.github.io/vector-data/ag3/methods.html). We used decision-tree filters that identify genomic sites where SNP calling and genotyping are likely to be less reliable. Genotypes at bi-allelic SNPs that passed the decision-

tree site filtering process were phased into haplotypes using a combination of read-backed and statistical phasing. Read-backed phasing was performed for each sample using WhatsHap version 1.0 [https://whatshap.readthedocs.io/]. Statistical phasing was then performed using SHAPEIT4 version 4.2.1 [https://odelaneau.github.io/shapeit4/].

5.3.4 Population structure

To estimate geographic genetic population structuring in *An. funestus* across Tanzania, we used principal component analysis (PCA) and a model-based *Admixture* ancestry analysis. The understand the initial genetic variation in the samples, PCA analysis was conducted on Google Colab following procedures outlined in the MalariaGEN Python package https://malariagen.github.io/malariagen-data-python/latest/Af1.html.

The PCA was run on an inversion-free region of chromosome 2 (2RL: 57,604,655-90,000,000) obtained from the full data set via random down-sampling to 100,000 SNPs and plotted using the seaborn python package. *Admixture* analysis was performed to classify the individual mosquitoes of unknown ancestry into discrete populations. The analysis was performed using the software, *Admixture* v1.3.0 (https://dalexander.github.io/admixture/publications.html) on 100,000 SNPs randomly down-sampled from chromosome 2RL with *K* (a priori clusters) values ranging from 1-10. The best value of *K* with the lowest cross-validation error (CVE) of 0.10027 was *K* = 3 followed closely by *K* = 4 (CVE 0.10352) and *K* = 2 (CVE 0.10373) **(Supplementary Figure 7-4)**. The R package Starmie (https://github.com/sa-lee/starmie) was used to summarize the results and the output visualised using ggplot2 in R.

5.3.5 Genetic diversity

We estimated the genetic diversity across the populations by computing Nucleotide diversity (π) and Tajima's D statistics. The analyses were conducted on Google Colab following procedures outlined in the MalariaGEN Python package https://malariagen.github.io/malariagen-data-python/latest/Af1.html.

We determined the signatures of recent inbreeding among individuals in the populations by estimating genome-wide runs of homozygosity (ROH). The ROH were inferred using a hidden Markov model implemented in *scikit-allel*. For more

information on the model, see the supplementary material of The *Anopheles gambiae* 1000 Genomes Consortium, 2017 (Anopheles gambiae Genomes et al., 2017). Due to the difficulty of inferring short ROH, ROH < 100,000bp in length was discarded as per this publication.

5.3.6 Measures of population differentiation

We used genome-wide *Fst* measures to determine the relative extent to which allele frequencies differ between groups of individuals in a population and the degree of differentiation between the sampled populations. The *Fst* was conducted for each chromosome using the *fst_gwss* function in the malariagen_data python API (https://malariagen.github.io/malariagen-data-python/latest/Af1.html) with a genomic window size of 5000 and a minimum cohort size of 10. We also searched for genome-wide signatures of selective sweeps using Garud's H12 selection statistic (Garud et al., 2015). H12 selection scans were performed on *An. funestus* genotypes by collection region where sample *n*>10 and a genomic window size of 5000 using the *h12_gwss* function in the malariagen_data python API.

5.3.7 Haplotype clustering and amino acid variations

To investigate the haplotype structure in *An. funestus* around known insecticide resistance genes which can give evidence for recent positive selection, we performed haplotype clustering hierarchical clustering on all 334 individual mosquitoes at transcript LOC125764713_t1, 2RL:8,685,464-8,690,407, and LOC125764232_t1, X:8339269-8341975, using a Hamming distance matrix, inferred from phased *An. funestus* haplotypes, using *plot_haplotype_clustering* function in the malariagen_data python API.

To identify potential nucleotide polymorphisms *Cyp6p* and *Cyp9k1* genes, we extracted single nucleotide polymorphism (SNPs) altering the amino acid of transcripts LOC125764713_t1 and LOC125764232_t1 from the *An. funestus* dataset and computed the allele frequency on the mosquito cohorts defined by the region and year of collection. Under selection pressure various alleles are expected to increase in frequency; we therefore filtered out variant alleles with a frequency

lower than 5% (Anopheles gambiae Genomes et al., 2017). The resulting frequencies of non-synonymous SNPs were visualised in R software.

5.3.8 Data availability

The sequencing data generated in this study have been deposited in the European Nucleotide Archive (https://www.ebi.ac.uk/ena/browser/home) under study number PRJEB2141.

5.4 Results

5.4.1 Population sampling and sequencing

We obtained whole-genome sequences from 334 individual *An. funestus* mosquitoes from 11 locations spanning the eco-geography of Tanzania (Figure **5-1A**). Mosquitoes were individually whole-genome-sequenced on an Illumina NovaSeq 6000s. The reads were mapped to the *An. funestus* reference sequence idAnoFuneDA-416_04 (Ayala et al., 2022b).

5.4.2 Population Structure

We estimated geographic genetic population structure using principal component analysis (PCA) and a model-based *Admixture* ancestry analysis (Alexander et al., 2009). To avoid the likely confounding effects caused by chromosomal inversion polymorphisms and recombination on the *An. funestus* genome, our PCA analysis relied on an inversion-free section of chromosome 2RL (57,604,655 - 90,000,000 bp) and chromosome X (Small et al., 2023).

The PCA on chromosome 2RL revealed two genetic clusters (Figure **5-1B**). The first cluster contained inland *An. funestus* populations from inland higher altitude regions of Katavi, Kigoma, Kagera, and Mwanza. The second cluster contained coastal populations from lower altitude areas of Pwani, Morogoro, Tanga, Ruvuma, Mtwara, Dodoma, and Lindi (Figure **5-1B**). The PCA based on chromosome X similarly showed the inland and coastal genetic clusters, but the samples from inland Tanzania were dispersed along PC1 and PC2 (Supplementary Figure **7-3**). To further investigate population structure within each major PCA grouping

identified in Figure **5-1B**, we constructed separate PCA plots for samples from inland and coastal Tanzania respectively. The samples from inland Tanzania remained in a single cluster but with some dispersed samples from the Kagera region (Figure **5-1C**). However, the second cluster containing coastal samples was separated into three genetic clusters with a pattern of geographic separation (Figure **5-1D**]. The first cluster contained samples from Tanga, cluster two had Pwani, Dodoma, and Morogoro, and cluster three Ruvuma, Mtwara, and Lindi (Figure **5-1D**).

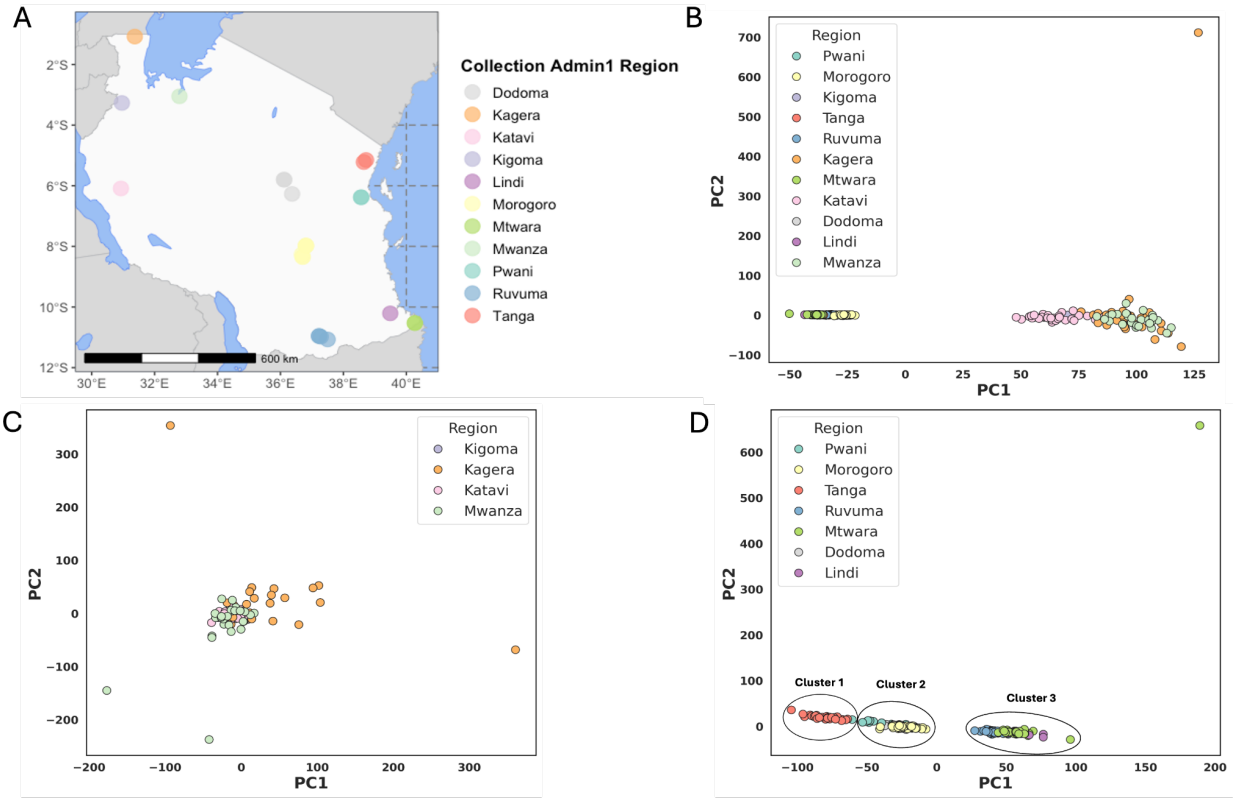


Figure 5-1: The population structure of *An. funestus* in Tanzania. (A) Map of *An. funestus* collection locations. Points indicate sample collection locations. The point colour indicates the administrative region from which samples were collected. (B) PCA plot of the first two principal components from the 2RL chromosome. The colours denote the sampling location (regions). (C) PCA plot of the first two principal components from the 2RL chromosome for inland samples from the north and west; Kigoma, Kagera, and Mwanza. (D) PCA plot of the first two principal components from the 2RL chromosome for coastal samples from the south and east; Pwani, Morogoro, Tanga, Ruvuma, Mtwara, Dodoma, Katavi, and Lindi.

To understand the ancestral origin of the mosquitoes, *Admixture* analysis was performed to classify the individual mosquitoes into discrete populations. The best *Admixture* value of *K* with the lowest cross-validation error (CVE) of 0.10027 was

K = 3 followed closely by *K* = 4 (CVE 0.10352) and *K* = 2 (CVE 0.10373) **(Supplementary Figure 7-4).** The *Admixture* output (*K* = 3) disclosed two major groups of samples, with samples from coastal Tanzania derived from two putative ancestral populations, and samples from inland Tanzania derived from a third (**Figure 5-2**).

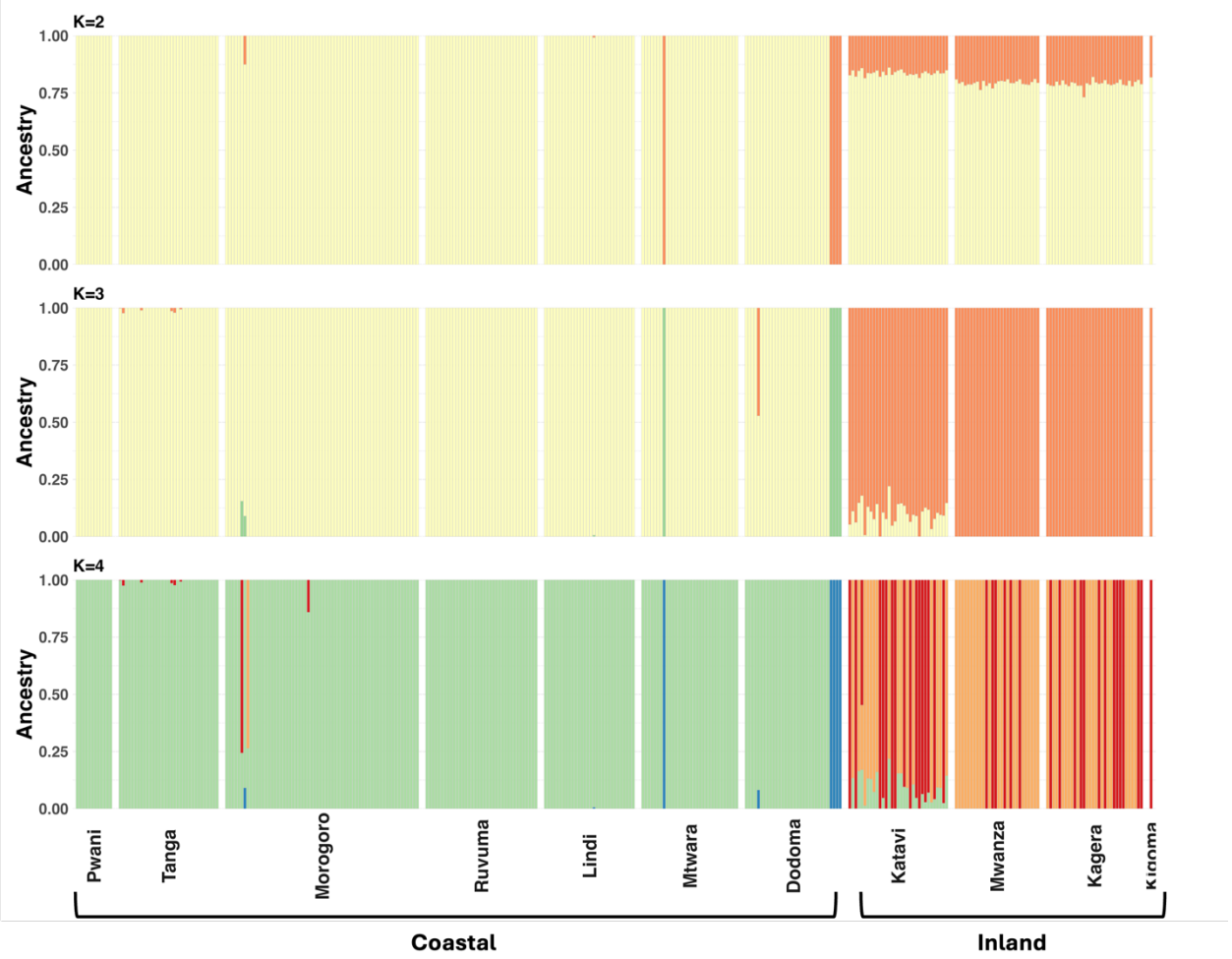


Figure 5-2: The *Admixture* proportions for *An. funestus* mosquitoes in Tanzania. An individual mosquito is represented as a vertical bar on the y-axis, and the bar's colour represents the proportion of the genome inherited from each of *K* = 2, 3 & 4 inferred ancestral populations. The x-axis represents the 11 regions where the samples originated.

5.4.3 Genome-wide Population Diversity

To determine genome-wide population diversity, we computed nucleotide diversity (π) (Figure **5-3A**) and Tajima's D (Tajima, 1989) summary statistics using the SNP dataset from chromosome 2RL (Figure **5-3B**). This was computed for populations with a minimum of 10 samples, hence Kigoma region in inland Tanzania was left out. The average nucleotide diversity (π) was higher in the

inland samples (π = 0.01208) compared to the coastal populations (π = 0.01084) (Figure **5-3A**). Similarly, the average values for Tajima's D were lower in the inland samples (-1.21527) compared to the coastal populations (-0.47317) (Figure **5-3B**). This suggested a greater excess of rare variants in inland *An. funestus* populations, in the northwestern regions (Kagera, Katavi, and Mwanza) indicating a recent positive selection or population expansion.

We determined the signatures of recent inbreeding among individuals in the populations by estimating genome-wide runs of homozygosity (ROH) (Figure **5-3 C&D**). Runs of homozygosity (ROH) are stretches of the genome where identical haplotypes are inherited from each parent due to inbreeding or a common ancestry (Ceballos et al., 2018). The inland populations (Kagera, Katavi, Kigoma, and Mwanza) had lower ROH segments and a lower fraction of their genomes under ROH suggesting outbred populations, while the coastal mosquitoes (Dodoma, Morogoro, Pwani, Ruvuma, Tanga, Lindi, and Mtwara) had higher ROH segments and a higher fraction of their genomes under ROH suggesting a history of inbreeding or a population bottleneck (Figure **5-3C&D**).

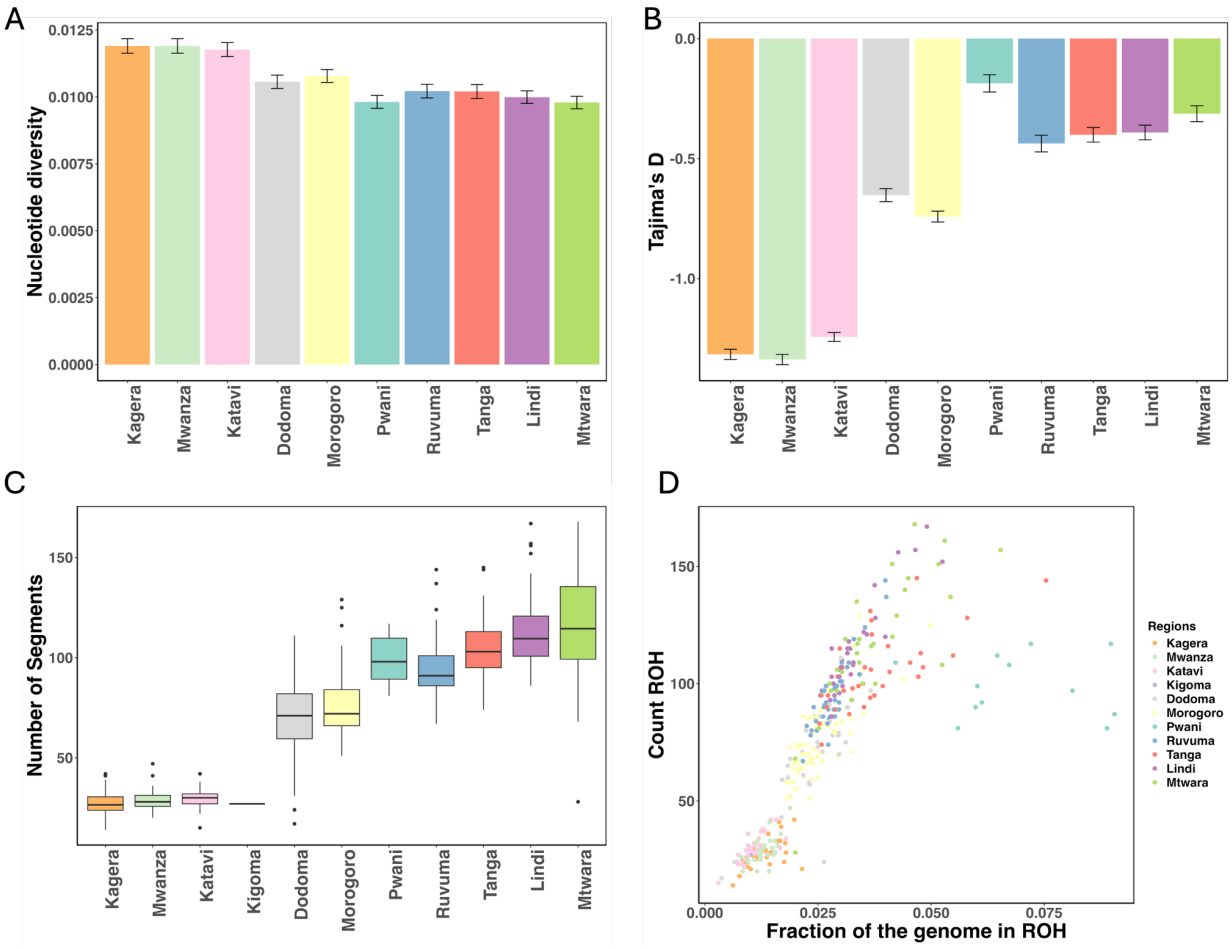


Figure 5-3: Genetic diversity in *An. funestus* populations in Tanzania. (A) Nucleotide diversity ($\theta\pi$) was calculated using SNPs from chromosome 2RL. (B) Tajima's D was calculated using SNPs from chromosome 2RL. (C) Boxplot showing the average number of segments of runs of homozygosity (ROH) per population across Tanzania. (D) Scatterplot showing the number of ROH (y-axis) and fraction of the genome in ROH (x-axis) in individual mosquitoes. Each marker represents a mosquito and is coloured by location (region).

5.4.4 Genome-wide signatures of selection

We estimated population differentiation (*Fst*) and detected regions under possible recent positive selection (H12) across the whole genome (chromosome arms 2RL, 3RL, and X) (Figure **5-4A**). Following the clustering in Figure **5-1C&D**, the analysis cohorts for pairwise *Fst* estimation and H12 were categorised into inland (found in samples from Katavi, Kigoma, Kagera, and Mwanza), coastal A (Tanga), coastal B (Morogoro, Pwani, and Dodoma), and coastal C (Ruvuma, Lindi, and Mtwara).

Genome-wide *Fst* measures the relative extent to which allele frequencies differ between groups of individuals and provides information about the degree of differentiation between the sampled populations. *Fst* was generally higher

between the inland and coastal A/B/C populations, with very high local peaks (*Fst* ~0.8) between inland and coastal A/B/C around the genomic regions containing insecticide resistance genes, *Cyp6* gene cluster on chromosome 2RL, and *Cyp9k1* on chromosome X, indicating strong differentiation at these regions (Figure **5-4A**). Peaks at the *Cyp6* cluster, and *Cyp9k1* were apparent between coastal A and coastal B/C, though much smaller than the peaks between inland and coastal clusters in general (Figure **5-4A**). To detect regions of the genome that have undergone a recent selective sweep, we used a statistical measure of haplotype homozygosity (H12) (Garud et al., 2015). Genome-wide selection scans (GWSS) with the H12 statistic revealed a clear signal of elevated H12 at the *Cyp6* cluster across all cohorts (Figure **5-4B**). *Cyp9k1* had a clear H12 signal in the inland genetic cluster, but possible peaks of elevated H12 near *Cyp9k1* in the three coastal clusters were unclear against the relatively higher genome-wide H12 background (Figure **5-4B**).

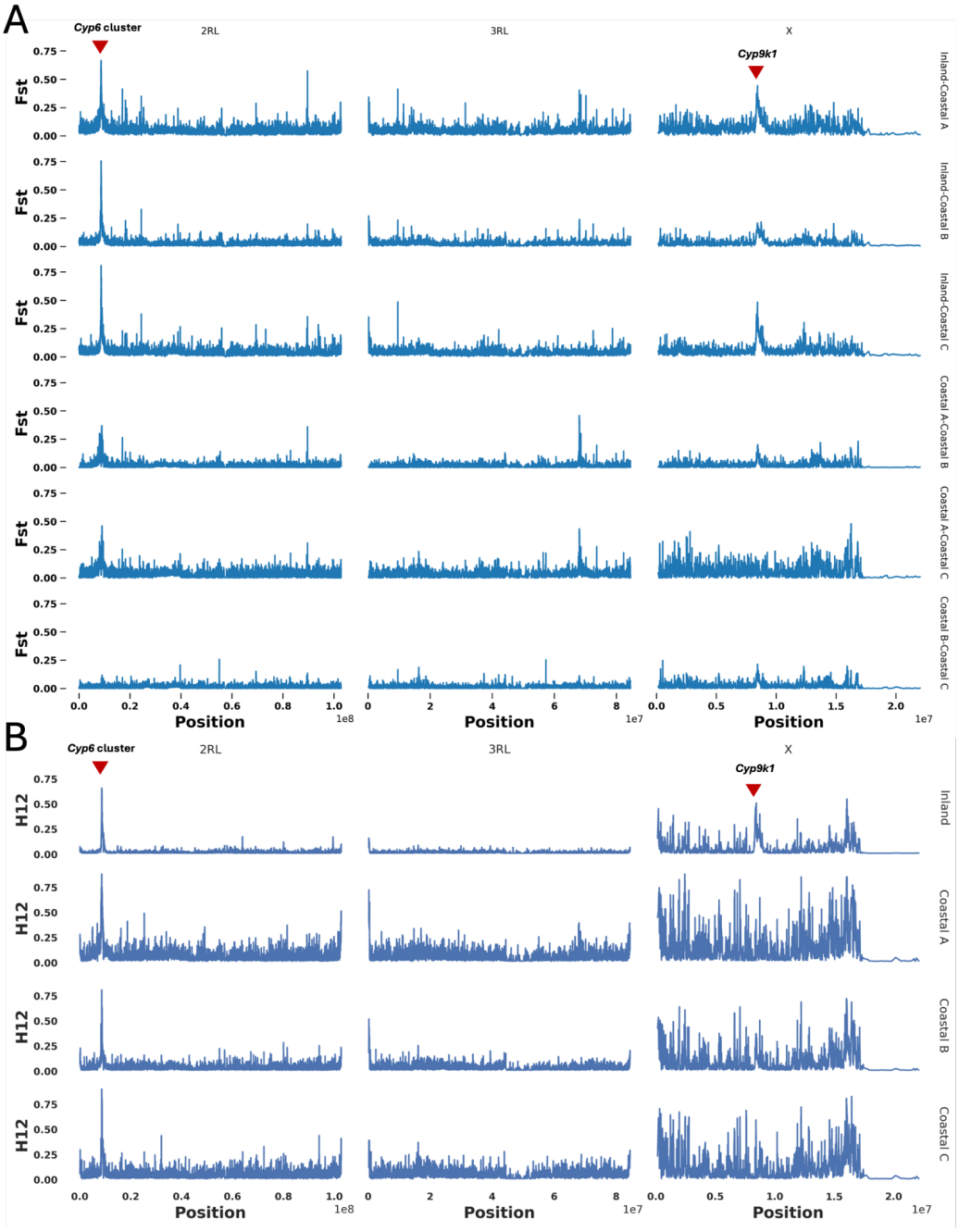


Figure 5-4: Genome-wide signatures of selection. (A) Genetic differentiation (F_{ST}) at genomic windows along chromosomes 2RL, 3RL, and X. The X-axis indicates the position (in base pairs (bp)), and the Y-axis indicates the fixation index (F_{ST}). (B) The measure of haplotype homozygosity (*H12*) genome-wide selection scans at genomic windows along chromosomes 2RL, 3RL, and X. The X-axis indicates the position (in base pairs (bp)), and the Y-axis indicates the selection statistic *H12*. The *H12* values range between 0 and 1, where zero indicates high haplotype diversity within a genomic window, and values closer to one indicate a strong positive selection of dominant haplotypes.

5.4.5 Haplotype clustering around the *Cyp6p* gene family and *Cyp9k1* genes

To elucidate the haplotype structure in *An. funestus* around known insecticide resistance genes with evidence for recent positive selection, we constructed a haplotype clustering dendrogram from haplotypes in all 334 individual mosquitoes for the genes *Cyp6p9* (LOC125764713_t1, 2RL:8,685,464-8,690,407) and *Cyp9k1* (LOC125764232_t1, X:8339269-8341975) (**Figure 5-5A&B**). The clustering dendrogram for the *Cyp6p9* gene family disclosed two major clades **(Figure 5-5A).** The first clade contained a swept haplotype present in populations from the inland cluster (Katavi, Kigoma, Kagera, and Mwanza). The second clade was subdivided into three subclades containing haplotypes present in populations from coastal populations from the south and east of Tanzania **(Figure 5-5A).** The *Cyp9k1* dendrogram revealed three major clades **(Figure 5-5B).** The first clade contained a haplotype present only in samples from Mwanza in the inland populations. The second clade contained haplotypes that did not have geographic segregation based on sampling location **(Figure 5-5B).**

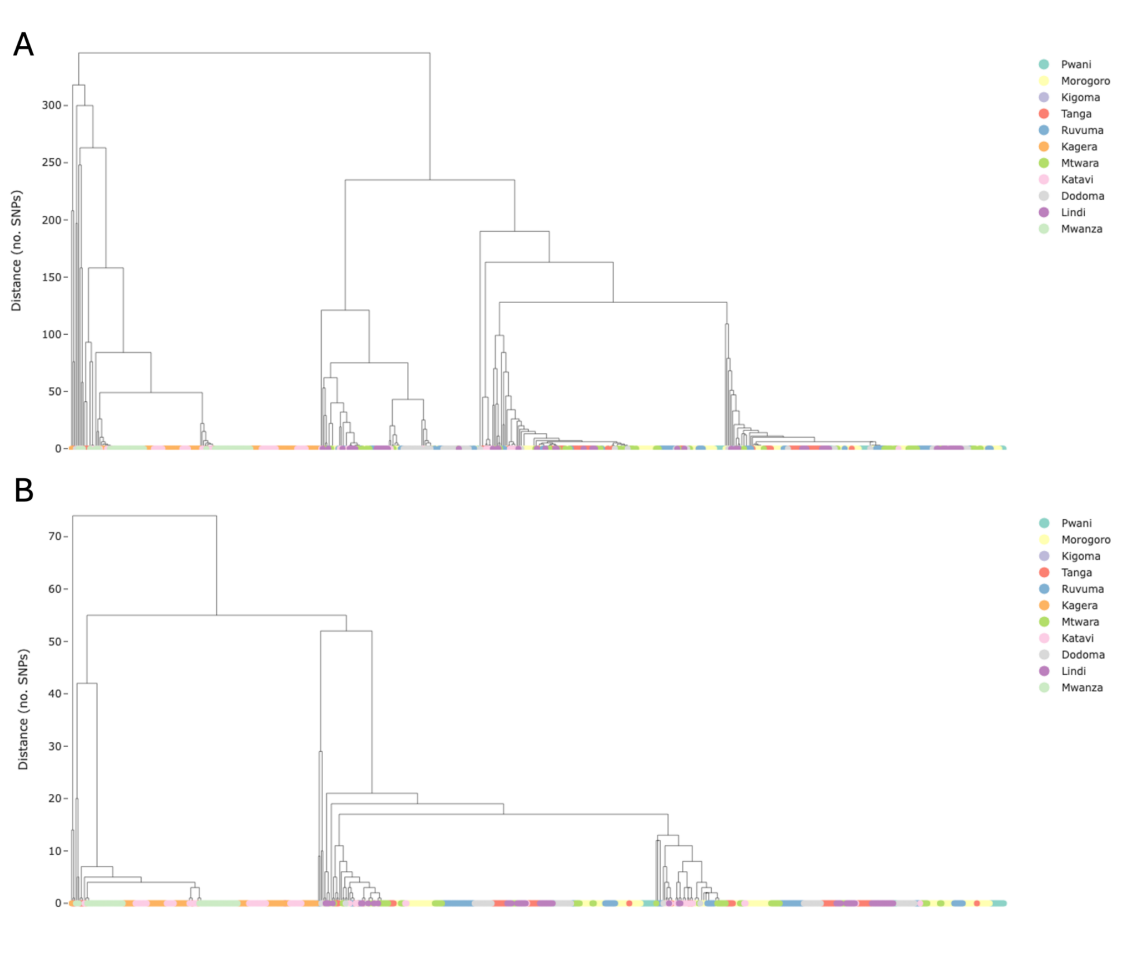


Figure 5-5: (A) Haplotype clustering over the *Cyp6p* gene family. (B) Haplotype clustering over *Cyp9k1* gene. The clusters are based on genetic distance as the number of SNPs and clusters with zero genetic distances indicate a selective sweep.

5.4.6 Amino acid variation around the Cyp6p9 and Cyp9k1 genes

We further investigated amino acid variation at the *Cyp6* gene cluster (2RL-chromosome) and *Cyp9k1* genes (X-chromosome) by plotting non-synonymous amino acid variation with 5% minimum allele frequencies, to identify potential nucleotide substitutions. We discovered 117 non-synonymous variants across the two genes. The analysis of the *Cyp6* gene cluster was performed on transcript LOC125764713_t1 (*Cyp6p* gene) containing the *Cyp6p9a* and *Cyp6p9b* which are crucial for insecticide detoxification in *An. funestus* in the east and southern Africa (Weedall et al., 2019, Mugenzi et al., 2019). The *Cyp6p* gene had 13 non-synonymous amino acid variations with notably high frequencies of V103I (86-100%) and L122F (87-100%) across many populations (Figure **5-6A**). However, both variants were absent in two inland populations (Kagera and Mwanza) and only at 32% frequencies in Katavi (Figure **5-6A**). Three additional variants, Y168H, V392F,

and V359I, were present in the inland populations at greater than 40% frequencies but absent across the coastal populations (Figure **5-6A**). Further variants were detected in the *Cyp6p* gene across the country without a discernible geographical pattern (Figure **5-6A**).

The *Cyp9k1* gene, transcript LOC125764232_t1, had 11 amino acid variations with variable frequencies of G454A across all populations (Figure **5-6B**). The allele frequencies of G454A were at near fixation (98%) in samples collected from the inland cohort (Katavi, Kagera, and Mwanza) (Figure **5-6B**). Two coastal B populations, Dodoma and Morogoro (2021), also had at least 80% frequencies of G454A, with the coastal A (Tanga) and C (Ruvuma, Mtwara and Lindi) having between 10 - 36% (Figure **5-6B**). Notably, in Morogoro, the frequencies of G454A mutation increased over 4 years peaking at 87% in 2023 but this trend was insignificant (P = 0.06627, Figure **5-6B**).

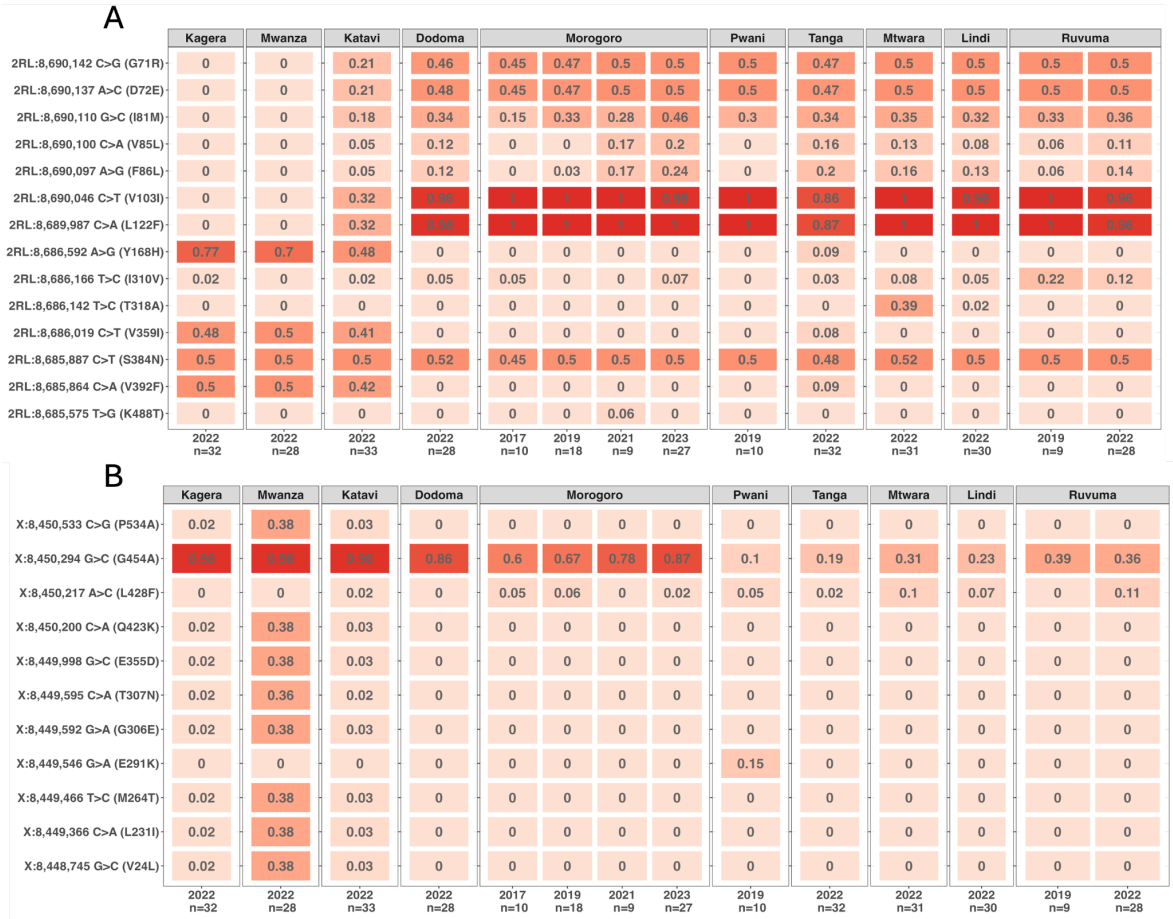


Figure 5-6: (A) Heatmap of *Cyp6p* gene allele frequencies. (B) Heatmap of *Cyp9k1* gene allele frequencies. On both plots, the Y-axis labels indicate mutation effect, chromosome position, and nucleotide change. X-axis labels indicate the collection date and heatmap intensity indicates frequency where darker = higher, with frequency labelled in each heatmap facet.

5.5 Discussion

The African continent has witnessed a significant reduction in the malaria burden over the past 20 years (Bhatt et al., 2015). However, progress has recently stagnated exposing the limits of the current insecticide-based vector control tools. A crucial component of getting progress back on track is having continuous and robust genomic surveillance data on the main vectors that can provide early warning of new evolutionary adaptations in response to the deployment of next-generation ITN and IRS products and detecting genetic discontinuities important in estimating the viability of transgenic approaches for mosquito control. To this end, we used whole-genome sequence analysis of 334 individual mosquitoes to decipher the population genetic structure and diversity of the major malaria vector *An. funestus* across Tanzania. Overall, our findings demonstrate the existence of two genetically differentiated *An. funestus* populations in Tanzania; an inland population (comprising Katavi, Kagera, Kigoma, and Mwanza) and coastal populations (found in the lower altitude regions including Pwani, Morogoro, Tanga, Ruvuma, Mtwara, Dodoma, and Lindi).

The genome-wide selection scans suggest that the population partitioning could be due to a combination of differentiation at insecticide resistance genes and demographic divergence reflecting diverse historical and contemporary dynamics. Recent population bottlenecks and inbreeding significantly reduces the frequency of rare genetic variants and overall genetic diversity. Based on the diversity estimators, the inland *An. funestus* populations had a greater excess of rare variants indicating a recent positive selection or population expansion in comparison to the coastal populations. The fact that the strongest differentiation between the two genetic populations was around regions of the genome containing insecticide-resistance genes suggests a strong evolutionary response emanating from agricultural or public health insecticidal vector control such as IRS or ITNs. The question however remains of what could be causing such rapid population disconnection in Tanzania.

In our findings, the inland and coastal populations are coincidentally geographically located on either side of the eastern arm of the Rift Valley making

it a plausible explanation for the differentiation. Major geographical features such as the Great Rift Valley have been postulated as a gene flow barrier across the east and southern African regions (Michel et al., 2005, Lehmann et al., 1999). In Malawi, *An. funestus* populations from the north were genetically differentiated from the south with the Rift Valley hypothesised as the potential driver of this separation (Barnes et al., 2017a). A similar effect of the Rift Valley on *An. funestus* populations have been found in Kenya (Ogola et al., 2019). Ecological barriers to gene flow such as the Congo Basin tropical rainforest have been demonstrated to guide population structuring in *An. gambiae* across Africa (Pinto et al., 2013). Tanzania's climate is largely equatorial with an arid desert cutting across the central part of the country (Beck et al., 2023). Such harsh conditions across central Tanzania are potentially less favourable to the proliferation of *An. funestus*. Indeed, vector surveillance data in Tanzania reveals that *An. arabiensis* is the predominant mosquito species in the arid desert region, while *An. funestus* and *An. gambiae* are more prevalent in the northwestern and southeastern zones of the country (Mwalimu et al., 2024). The potential impact of such geographic and climatic stratifications on local vector adaptation and genetic differentiation in Tanzania should be investigated.

We also uncovered positively selected haplotypes around the genomic regions containing insecticide resistance genes, *Cyp6p* and *Cyp9k1*. The allele variations in these swept haplotypes had discernible geographical patterns further reflecting gene flow barriers between the coastal and inland genetic populations. For instance, analysis of the *Cyp6* gene demonstrated that V103I and L122F mutations were widespread and at high frequencies in the coastal *An. funestus* populations while the inland populations had Y168H, V392F, and V359I. However, the impact of these mutations on insecticide resistance phenotypes in this vector remains unclear. The selective sweeps in the *Cyp6p* gene coincided with the patterns of distribution of key metabolic resistance genes in Tanzania with *Cyp6p9a* and *Cyp6p9b* observed as fixed in the coastal populations of the south and east but either at low frequencies or undetectable in the inland populations of north and west Tanzania (Odero et al., 2024b). The allelic variations around genomic regions containing crucial metabolic resistance genes in *An. funestus* suggests underlying differences in the molecular basis of insecticide resistance across Tanzania. The cytochrome P450 G454A-*Cyp9k1* mutation was near fixation in the inland

populations but present at low frequencies in the coastal populations. This mutation has been demonstrated to confer phenotypic resistance to type I (permethrin) and II (deltamethrin) pyrethroids, commonly used on ITNs (Hearn et al., 2022). Continentally, G454A-*Cyp9k1* is widespread in central Africa (Cameroon) and east Africa including Uganda (Djoko Tagne et al., 2024). We speculate that the presence of G454A-*Cyp9k1* at near fixation in inland Tanzania indicates that these populations are more connected to the larger African continent and coastal connected more to the southern African region where this mutation is either absent or at low frequencies (Djoko Tagne et al., 2024).

Our findings provide essential information when planning the implementation of transgenic vector control strategies such as gene drives and sterile insect release in Tanzania. The divergence between the inland and coastal *An. funestus* populations with limited gene flow would require multiple strategic releases across the potential genetic barriers. However, in this study, the sampling locations were not uniformly spread across the country leaving an area of ~600 km between Dodoma in central Tanzania and Mwanza in the northwest. Such large geographical distances can contribute to structuring through isolation by distance (IBD). Follow-up studies should focus on sampling and sequencing from these areas with a collective outcome of a finer-scale genetic structure of this important malaria vector. Our analysis could also have included *An. funestus* samples from countries neighbouring Tanzania to put the genetic variations and structuring into a broader perspective. Such analysis would for instance make it possible to discern the genetic connectedness between the coastal Tanzania populations and the greater southern African *An. funestus* populations and how this influences genetic diversity.

5.6 Conclusion

This study provides a comprehensive genetic diversity analysis of *An. funestus*, the main malaria vector in Tanzania. We demonstrate that the vector is differentiated into two genetically distinct populations with historical and contemporary gene flow patterns. We also reveal directional sweeps of haplotypes especially in the *Cyp6* gene cluster and geographic allelic variations in the genomic regions around insecticide resistance genes *Cyp6p* and *Cyp9k1*. The genomic

dataset generated in this study, accessible through the European Nucleotide Archive under study number PRJEB2141, will be crucial to expanding capabilities for molecular surveillance of insecticide resistance in *An. funestus* Tanzania.

6 Bionomics of Genetically Distinct *Anopheles funestus* Populations in Tanzania

6.1 Abstract

During a large-scale field study on genetic variations in *Anopheles funestus* populations across Tanzania, a supplementary investigation was conducted to explore behavioural and ecological differences in these mosquitoes. The primary objective was to examine variations in key vector bionomics, as well as phenotypic and transmission traits that might be linked to the observed genetic differences between inland populations and those from coastal, lower-altitude regions. Between November 2021 and December 2022, mosquitoes were collected from nine districts covering diverse ecological settings. CDC light traps and mouth aspirators were used for indoor collections and miniaturized double-net traps were used to compare indoor and outdoor-biting patterns. Mosquitoes were sorted by taxa and sibling species of *An. funestus* s.l distinguished by PCR. Blood meal sources were identified by PCR based on mammalian cytochrome-b primers, and *Plasmodium falciparum* infections detected using ELISA. Mosquito body sizes were estimated from wing lengths, and blood-fed mosquitoes were maintained until oviposition, and the resulting egg counts were correlated to body size. Age structure was determined by dissecting a subset of mosquitoes and examining their ovaries for parity and number of gonotrophic cycles. These entomological indicators were analysed and interpreted in the context of geographic and genetic populations. In both inland and coastal populations of *An. funestus*, mosquitoes fed predominantly on human blood compared to other animals (human blood index: 69.8-71.3%). There were also no significant differences in age structure between these populations. The prevalence of *P. falciparum* infections was higher in coastal populations, driven by particularly elevated rates in southeastern districts, which had ten times higher prevalence than other districts. On the contrary, the inland *An. funestus* were significantly larger (mean wing size: 2.87 (95%CI 2.84 – 2.9) inland vs. 2.69 (95%CI 2.67 – 2.71) coastal) and produced larger egg batches compared to their coastal counterparts (egg counts: 110.84 (95%CI 103.96 – 117.72) inland vs. 87.7 (95%CI 84.27 – 91.12) coastal). While the biting behaviour of *An. funestus* (primarily indoors), level of anthropophily (human blood indices consistently above 69%) and age structure were similar across populations,

differences were noted in the prevalence of *Plasmodium* infections, mosquito sizes, and fecundity between the inland and coastal genetic populations. Further research is needed to determine whether these phenotypic and demographic differences are a consequence of, or contribute to, the observed genetic separation between the inland and coastal populations of *An. funestus*.

6.2 Introduction

Anopheles funestus is a prolific malaria vector malaria vector across the East and Southern Africa region (Msugupakulya et al., 2023). In parts of Tanzania, this species contributes highly to the entomological inoculation rate (EIR) and mediates 9 out of every 10 transmissions (Mwalimu et al., 2024, Mapua et al., 2022). Despite the obvious public health importance, the vector has for years been neglected with most research focussing on malaria vectors within the *An. gambiae* complex, leaving most aspects of its biology unclarified. In attempt to fill this gap, our recent insecticide resistance surveillance analysis in the vector found no differences in pyrethroid and organophosphate phenotypic insecticide resistance profiles across Tanzania (Odero et al., 2024b). However, the variations in the underlying resistance mechanisms were quite stark (Odero et al., 2024b). For instance, the main genes thought to confer insecticide resistance in this species, *Cyp6p9a* and *Cyp6p9b,* evinced a south-north spread suggestive of a gene-flow barrier (Odero et al., 2024b). Population-wide genetic analysis of *An. funestus* in Tanzania also revealed two genetically distinct clusters, an inland population occupying the higher altitude regions across the broader east-African plateau, and coastal populations occupying the lower-altitude sites on the southern and eastern zones [**see Chapter 5 of this thesis**]. The populations displayed variations in diversity measures, had divergent demographic histories, and variations in frequencies of similarly crucial resistance genes such as *Cyp9k1* [**see Chapter 5 of this thesis**]. Although genetic variability in disease vectors may influence the selection and effectiveness of vector control interventions, the underlying drivers are not well understood. It also remains unclear whether the genetic differences in *Anopheles funestus* are a result of, or a cause for, variations in their bionomics and ecology.

Available evidence suggests that genetic variabilities in mosquito vector species can result in some innate differences that impact disease transmission dynamics (Hemming-Schroeder et al., 2020). For instance, variations in signals of putative genetic signatures associated with temperature and vegetation cover allow *Aedes aegypti* populations to adapt to either wet or dry environmental conditions (Bennett et al., 2021). *Anopheles gambiae, An. coluzzii,* and *An. funestus* s.s malaria vectors are considered to predominantly blood-feed on humans (anthropophilic) and rest inside houses (endophilic) (Kreppel et al., 2020, Akogbeto et al., 2018, Githeko et al., 1996), whereas *An. arabiensis* is a more generalist vector, biting humans but also readily feeding on domestic animals (zoophilic) and resting both outdoors (exophilic) and indoors (Sherrard-Smith et al., 2019). This generalist behaviour in *An. arabiensis* enables it to survive indoor-based interventions and is genetically linked to genetic chromosomal inversions (Main et al., 2016). In some settings, *An. funestus* has been found resting and feeding outdoors (Sherrard-Smith et al., 2019) and sometimes during daytime (Sougoufara et al., 2014), possibly as an adaptation to avoid indoor interventions. Another important ecological link to genetic population diversity is habitat availability. *Anopheles gambiae* and *An. arabiensis* mostly occupying small temporary aquatic habitats (Minakawa et al., 2012). Contrastingly, *An. funestus* typically occupy habitats characterised by slow-moving permanent to semi-permanent river streams usually covered by vegetation canopy (Nambunga et al., 2020), making river networks a potentially crucial aspect in the ecology and distribution of this vector.

This study therefore aimed to test for variations in key vector bionomic, phenotypic and transmission traits between the different geographic populations across Tanzania. In addition to the comparisons between regions, we also examined whether these variables differed alongside the inland and coastal genetic clusters as determined by the population genetic analysis [**see Chapter 5 of this thesis**].

6.3 Methods

6.3.1 Study area and mosquito sampling

Mosquitoes were collected from November 2021 to December 2022 in purposively selected villages, with high densities of *An. funestus,* in nine administrative districts of Tanzania **(Figure 6-1)**. The selection of the villages was guided by local public health officers and vector surveillance officials with knowledge of the places likely to have *An. funestus*. The collections were done as part of a large-scale field surveillance on insecticide resistance and genetic diversity of *An. funestus* in Tanzania (see Chapter 3&5 of this thesis. The locations represent a comprehensive geographical and ecological cross-section of the country and were selected for their high malaria prevalence and confirmed vector presence *An. funestus* (Tanzania-MoH, 2022).

Following the consent of household heads, adult mosquitoes were sampled in and outside houses using a combination of trapping methods. Indoor sampling used CDC-miniature light traps (CDC-LT) and mechanical mouth aspiration, while miniaturized double-net trap (i.e. the DN-Mini) (Limwagu et al., 2024) was used for both in and outdoor collections. The collected mosquitoes were first sorted by taxa using taxonomic keys (Coetzee, 2020, Gillies and Coetzee, 1987), and the *Anopheline* mosquitoes were further sorted based on physiological features and abdominal status (blood-fed, gravid, and unfed). The non-*Anopheline* bycatch mosquito numbers were recorded and discarded. The mosquitoes were stored individually in 1.5mL tubes with silica gel desiccant stored in a cool, dry place and moved to the laboratory for downstream processing. For every household where mosquito sampling was done, we recorded the number of humans sleeping in the house and the house's physical characteristics (i.e. roofing, floor and wall materials, presence of eaves openings, and windows). We also recorded the presence of any domesticated animals (cattle, pigs, sheep, goat, dog, and chicken) and the house's proximity to water bodies that could act as mosquito breeding places.

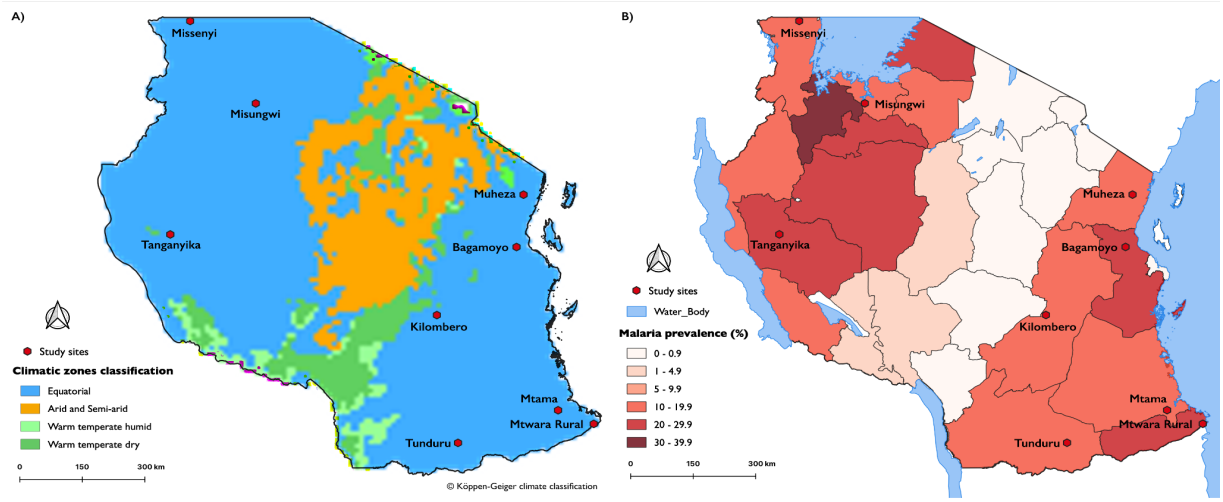


Figure 6-1: Study site selection in Tanzania. (A) Map of Tanzania showing study districts (red dots) selected based on sentinel data confirming the presence of the *Anopheles funestus* group. (B) Distribution of malaria prevalence by district: moderate (10-19.9%) and high (>20%) based on the 2022 Tanzanian Ministry of Health School Malaria and Nutrition Survey (SMNS).

6.3.2 Molecular identification of mosquito species

To establish the genetic identity of the mosquitoes, DNA was isolated from either mosquito legs or wings by direct boiling in Tris-EDTA (TE) buffer. Briefly, the legs/wings were boiled in 20µl TE buffer at 95°C for 15 minutes on a vortex heat block. The lysate was centrifuged at 8000rpm for 2 minutes and the supernatant (containing the DNA) was carefully separated and used as a template in the PCR. The molecular species identification followed a PCR-based protocol utilizing species-specific primers targeting the non-coding internally transcribed spacer region (ITS2) between the 5.8S and 28S ribosomal DNA sequence to distinguish between at least seven members of *An. funestus* group (Cohuet et al., 2003, Koekemoer et al., 2002b).

6.3.3 Molecular identification of blood-meal sources (vertebrate hosts)

To understand the host feeding patterns in the mosquitoes, we isolated DNA from the abdomens of all blood-fed mosquitoes using DNAzol™ Reagent (ThermoFisher Scientific). The resulting DNA was PCR-screened with cytochrome B primers targeting human, pig, bovine, chicken, goat and dog blood following a protocol developed by Kent *et.al* (Kent and Norris, 2005). In all PCR runs, we included

positive controls obtained by extracting DNA from laboratory colony mosquito blood-fed upon target vertebrates.

6.3.4 Detection of Plasmodium falciparum sporozoites

We used enzyme-linked immunosorbent assay (ELISA) as the method for parasite screening. The head thoraces from all *Anopheles* mosquitoes were individually processed and screened for *Plasmodium falciparum* circumsporozoite (CS) protein following a sandwich ELISA protocol (Wirtz et al., 1989). To avoid false positives due to cross-reacting antigens (Wirtz et al., 1989), the ELISA lysates were heated for 10 minutes at 100°C, denaturing the heat-unstable cross-reactive proteins (Wirtz et al., 1989).

6.3.5 Assessment of fecundity, age structure and body sizes

The biological ages of field-collected mosquitoes were estimated by ovarian dissection for evidence of previous egg-laying (Detinova, 1962). Ovaries were dissected from randomly selected, unfed females following the Detinova method (Detinova, 1962). The ovarian tracheoles were examined and recorded as parous (laid eggs at least once and enlarged tracheoles) or nulliparous (no prior blood meal and coiled tracheolar skeins) (Hugo et al., 2008). The Polovodova method was then used to estimate the age of parous mosquitoes by counting the number of dilatations in the ovarioles, with each dilatation representing a gonotrophic cycle following oviposition (Hugo et al., 2008). Fecundity was assessed by isolating engorged wild *An. funestus* females in individual plastic cups lined with moistened filter paper to induce oviposition. After oviposition, the eggs were counted under a microscope to quantify egg batch sizes, providing a measure of reproductive output.

In both parity and fecundity experiments, mosquito body size was estimated using wing length as a proxy. Wings were carefully removed from the right side of each mosquito and placed on a glass slide. Using a dissecting microscope fitted with an ocular micrometre, wing lengths were measured from the alula to the apical margin of the wing. These wing measurements provided an estimate of mosquito body size, allowing for correlations between body size, parity, and fecundity.

6.3.6 Data analysis

The statistical analysis aimed to compare the entomological parameters including host blood-feeding behaviour, *P. falciparum* infection rates, parity, fecundity, and body sizes between districts or by combining them into inland and coastal genetic populations. All analyses were conducted using R statistical software (version 4.1.1) with generalised linear models within the *lme4* package. A binomial model was used to analyse the relationship between the dependent variables, human blood index (HBI) or *P. falciparum* infection, and the predictor variables (Table **6-1**). In the HBI model, the response variable, host blood type, was categorised into 1 as 'human' and 0 as other vertebrate host types.

Similarly, for the *P. falciparum* infection model, the mosquitoes were analysed individually with 1 as 'infected' and 0 as 'uninfected'. The models had the explanatory variables as; district or genetic location (inland, coastal), the trap location (in/outdoor), the number of humans sleeping in a house, the trapping method (CDC, mouth aspiration, double-net mini), house eave status (blocked, open, partially open), the door status (closed, open), roofing material (iron, thatch), window (blocked, open, partially open), walls type (brick, mud, plastered), floor type (mud, plastered), habitat type within 100m of the house (dam, ditch, pond, river, swamp, no habitat), animal categories (cattle, chicken, goat, pig, and no animal), and chicken indoor keeping (yes/no).
The relationship between a mosquito gonotrophic cycle number and the predictor variables; trap type (aspiration, CDC light trap, and double-net mini), trap location (in/outdoor), mosquito size, and district/genetic location (inland, coastal), was fitted using a Poisson distribution. A linear regression model was used to analyse the relationship between fecundity (number of eggs laid by a mosquito) and the predictor variables; trap type (aspiration, CDC light trap, and double-net mini), trap location (in/outdoor), mosquito size (wing length), and district/genetic location (inland, coastal).

The Variance Inflation Factor (VIF) for the predictor variables in each model was calculated using *car* package in R. Predictor variables with a VIF ≥ 5 suggested a high correlation and multicollinearity and were removed from the model before model simplification. *Drop1* command was used to examine the models and

determine the variable to remove in a stepwise format starting with the most complex model. The likelihood ratio test (LRT) was then used to compare two nested models. The R command *ggemmeans* was used to get estimates of the best-fit model, and *gglot2* was used to plot the predictions.

6.4 Results

A total of 6685 *Anopheles* mosquitoes were collected across the nine districts in nine regions in mainland Tanzania. Of these, 91.9% (n=6141) belonged to the *Anopheles funestus* group, 7.6% (n=508) *An. gambiae* complex, and 0.5% (n=36) *An. coustani*. The *Anopheles funestus* group mosquitoes were present in all nine districts and were molecularly identified as 92.4% (n=5674) *An. funestus*, 1.9% (n=114) *An. leesoni*, 0.3% (n=19) *An. funestus*/*An. leesoni* hybrid, 0.15% (n=9) *An. rivulorum*, and 5.3% (n=325) not determined due to non-amplification by PCR (Figure **6-2**).

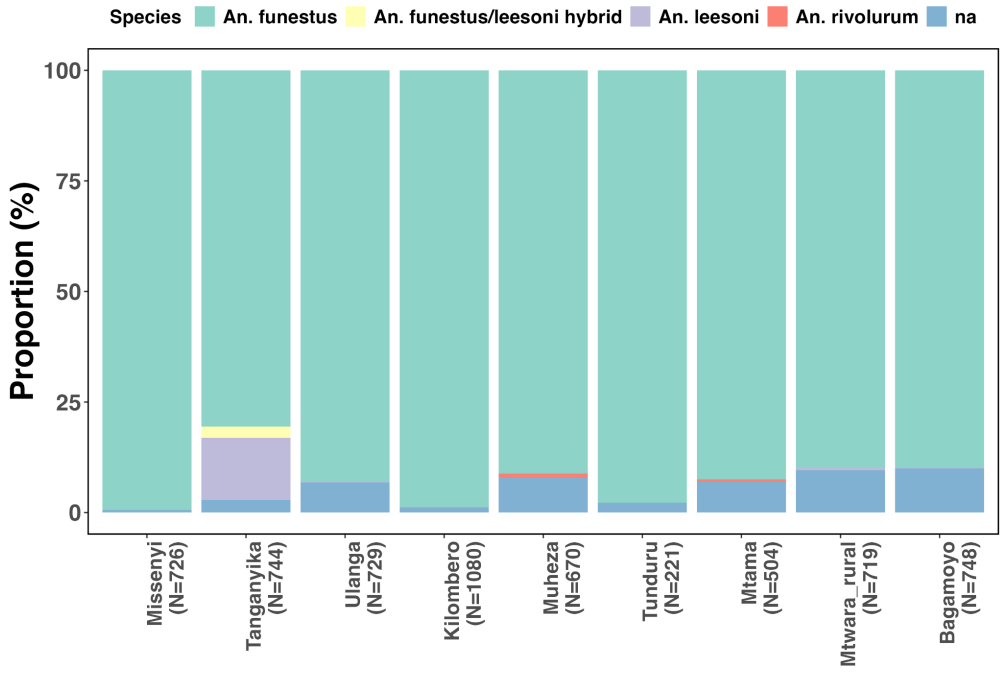


Figure 6-2: Distribution of *Anopheles funestus* group species across nine Tanzanian districts.

All the 6141 *An. funestus* group mosquitoes were individually analysed for *P. falciparum* malaria parasite infections. Of these, 54.7% (n = 3362) were collected indoors and 45.3% (n = 2779) outdoors (Table **6-1**). 1773 *An. funestus* group mosquitoes were collected blood fed of which 52.9% (n = 938) were collected

indoors and 47.1% (n = 835) outdoors. Another 908 mosquitoes were dissected for age structure analysis with 52.6% (n = 478) collected indoors and the rest collected outdoors. Fecundity parameters were measured in 446 mosquitoes, 51.3% (n = 229) collected indoors and the rest outdoors (Table **6-1**).

Table 6-1: The number of *An. funestus* group samples analysed for the various entomological parameters and in the modelling. The sample sizes are categorised into district/genetic location (inland, coastal) and sampling location (either in or outdoor). Pf = *Plasmodium falciparum*.

		Entomological parameter estimated							
		Human blood index (*An. funestus s.s*)		Pf infection (*An. funestus s.s*)		Age structure (*An. funestus* group)		Fecundity (*An. funestus* group)	
District	Genetic location	Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor
Missenyi	Inland	172	159	379	347	50	50	30	25
Tanganyika	Inland	183	163	397	347	50	50	25	25
Ulanga	Coastal	211	168	437	292	50	20	25	25
Kilombero	Coastal	83	81	682	398	112	80	25	17
Muheza	Coastal	122	184	291	375	50	50	25	25
Tunduru	Coastal	45	24	172	49	43	46	25	25
Mtama	Coastal			236	268	41	40	25	25
Mtwara Rural	Coastal	122	56	403	320	40	50	24	25
Bagamoyo	Coastal			365	383	42	44	25	25
Total		938	835	3362	2779	478	430	229	217

6.4.1 Host feeding patterns of the *Anopheles funestus* populations

A total of 1773 mosquitoes from *An. funestus* group (AFG) were collected blood-fed, as confirmed by visual observation of their abdomen, of which 52.9% (n = 938) were collected indoors and 47.1% (n = 835) outdoors (Table **6-1**). 96% (n=1699) of

these mosquitoes were molecularly confirmed as *An. funestus*. The majority of the 1699 blood-fed *An. funestus* fed on humans 71% (n=1201), less than 1% fed on either dog or goat and 29% (n=490) were unidentified blood meals (**Figure 6-3A).** The association between human blood index (HBI) and the multiple predictor variables were modelled using generalised linear models. The final model for the human blood index included animal keeping (cattle, chicken, goat, pig, no animal; deviance = 17.3967, p = 0.001618), trap location (indoor or outdoor: deviance = -6.9047, p = 0.008597) and number of house occupants (deviance = 7.4945, and p = 0.006189) as significantly associated variables. House occupants were negatively associated with HBI **(**Table **6-2)**, HBI was higher in outdoor collected mosquitoes, while cattle keeping had a notable but insignificant effect of reducing HBI compared to other animal categories (mean = 0.945, 95%CI = 0.871 - 1) **(Figure 6-3B).** No variation in HBI across districts was found (Table **6-2**).

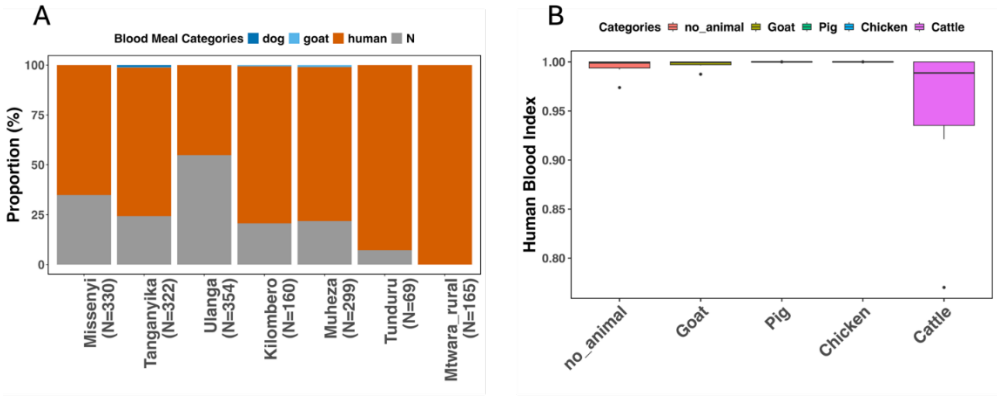


Figure 6-3: Host blood feeding patterns and human blood index (HBI) of *Anopheles funestus*. (A) Blood meal sources for the *An. funestus* (B) Impact of alternative host availability on HBI.

Table 6-2: Significance of explanatory variables included in the model for assessing variations in *An. funestus* human blood index and *P. falciparum* infection. The missing values in the HBI column are variables removed from the model due to high multicollinearity values. n/a indicates variables that were not tested due to collinearity.

	Human blood index (HBI)		***P. falciparum* infection**	
Explanatory variable (categories)	**Deviance**	**P-value**	**Deviance**	**P-value**
Floor (mud, plastered)	n/a	n/a	-0.070733	0.7903
Animal (cattle, chicken, goat, pig, no animal)	**17.3967**	**0.001618** **	-0.74797	0.9453
Walls (brick, mud, plastered)	n/a	n/a	1.0061	0.3158
Roof (iron, thatch)	n/a	n/a	0.24402	0.6213
Window (blocked, open, partially open)	n/a	n/a	-1.9478	0.3776
Door (closed, open)	n/a	n/a	0.78227	0.3764
Trap location (indoor, outdoor)	-**6.9047**	**0.008597** **	-1.043	0.3071
Bloodmeal (human, other animals)	n/a	n/a	1.458	0.2273
Chicken-in (yes, no)	n/a	n/a	-2.1564	0.142
Eaves (blocked, open, partially open)	-0.69227	0.4054	1.5661	0.2108
Habitat (dam, ditch, pond, river, swamp, no habitat)	4.5424	0.2085	-6.379	0.09456
Number of house occupants	**7.4945**	**0.006189 ** **	1.8342	0.1756
Trap (aspiration, CDC light trap, double-net mini)	0.55043	0.7594	**-6.366**	**0.04146 ***
District	-2.1911	0.90131	**157.63**	**<0.00001 *****

6.4.2 *Plasmodium* parasite infection in *Anopheles funestus*

A total of 6141 mosquitoes from *An. funestus* group were analysed individually for *P. falciparum* sporozoite detection. The average sporozoite infection rate for the nine districts was 2.4% (n=147). *P. falciparum* infection was significantly associated with the trapping method (deviance = -6.366, p = 0.04146), and district (deviance = 157.63, p <0.0001) (Table **6-2**). There was a higher average infection

rate in the south and east Tanzanian districts – the coastal region - (Ulanga, Kilombero, Muheza, Bagamoyo, Tunduru, Mtama, and Mtwara Rural) compared to the northwest – the inland region - (Missenyi and Tanganyika) (Figure **6-4A**). Notably, districts in the Kilombero Valley, Ulanga and Kilombero recorded the highest *P. falciparum* infection rate of 10.4% (CI 8.2 – 13.1) and 2.7%% (CI 1.8 – 3.9) respectively [(Figure **6-4A**). The other notable *P. falciparum* infection rates were from Muheza 1.9% (CI 1.1 – 3.3) and Bagamoyo 1.7% (CI 0.9 - 3) districts in coastal Tanzania. All other districts recorded ⩽1% infection rates in the *An. funestus* mosquitoes (Figure **6-4A**).

When comparing *P. falciparum* infection rates in the various trapping methods, mouth aspiration on average consistently collected higher numbers of infected mosquitoes (1.9% CI 1.3 - 3), followed by the double-net (1.4% CI 0.9 – 1.9) and CDC-light traps (0.9% CI 0.5 – 1.6) (Figure **6-4B**). Mosquito parasite infection was not associated with the increasing number of house occupants; deviance = 1.834, p=0.1756, mosquito habitat around the household; deviance = -6.379, p=0.0946, keeping chicken indoors; deviance -2.1564, p=0.142, other multiple other house characteristics (Table **6-2**).

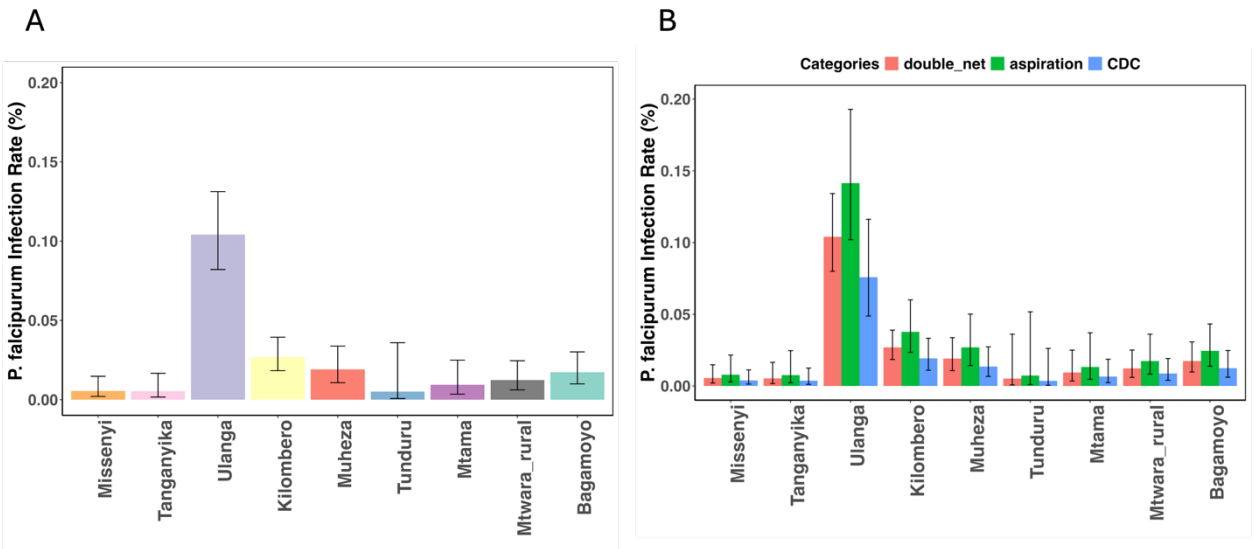


Figure 6-4: *Plasmodium falciparum* infection rates in *Anopheles funestus*. (A) Distribution of *P. falciparum* infection rates across ten Tanzanian districts. (B) Comparison of mean *P. falciparum* infection rates by district and mosquito trapping method. The error bars indicate the lower and upper confidence intervals around the predicted mean values.

6.4.3 Body size and fecundity of *An. funestus* across Tanzania

A total of 446 mosquitoes from *An. funestus* group from nine districts oviposited, and their wing size length was measured as a proxy to body size. Of these 51.3% (n = 229) collected indoors and the rest outdoors (Table **6-1**). Fecundity was significantly associated with wing length – a proxy for mosquito body size (deviance = -24384, p = <0.0001) and district (deviance = 92228, p = <0.0001) (Table **6-3**). On average, mosquitoes from Tanganyika, northwest Tanzania, were larger and laid more eggs compared to the other districts (wing size = 2.94, 95% CI 2.90 - 2.97) (Figure **6-5**A&B).

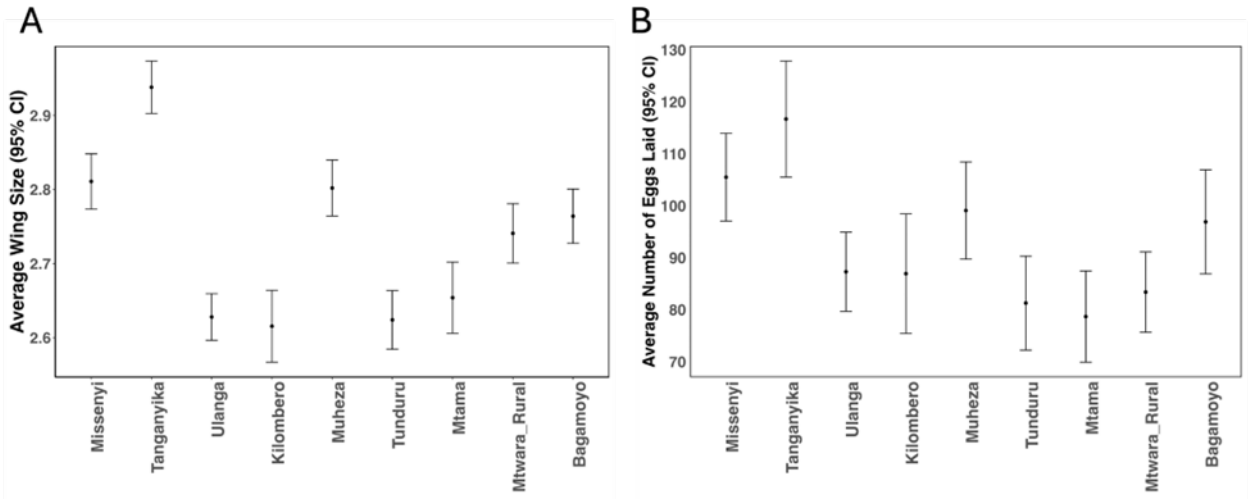


Figure 6-5: (A) Average wing size across ten Tanzanian districts. (B) Average number of eggs laid per female across ten Tanzanian districts. The error bars on pane A and B are lower and upper confidence intervals around the average values for mosquito size and number of eggs laid per district.

There was a linear relationship between the number of eggs laid (fecundity) with the mosquito size in all districts (Figure **6-6**)

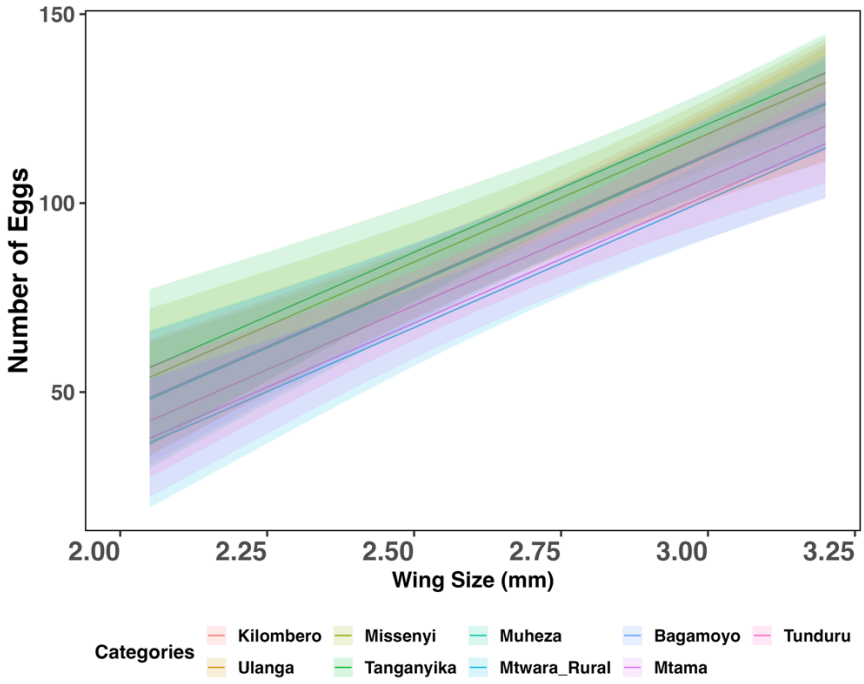


Figure 6-6: Correlation between wing size and egg count, with 95% confidence intervals.

Table 6-3: Significance of explanatory variables included in the model for assessing variations in *An. funestus* fecundity and gonotrophic cycle.

	Fecundity		**Gonotrophic cycle**	
Explanatory variable	**Deviance**	**P-value**	**Deviance**	**P-value**
Trap (aspiration, CDC light trap, double-net mini)	-2894.1	0.2571	-0.45164	0.7979
Trap location (indoor, outdoor)	1547.3	0.2284	-1.36691	0.2423
Wing size	**-24384**	**<0.0001*** **	0.18093	0.6706
District	**92228**	**<0.0001*** **	**-17.755**	**0.03812 ***

6.4.4 Age structure of *Anopheles funestus* populations

A total of 908 mosquitoes from the *An. funestus* group from nine districts were dissected, their ovaries examined. Of these 52.6% (n = 478) collected indoors and the rest outdoors (Table **6-1**). The number of gonotrophic cycles were significantly associated with districts (deviance = -17.755, p = 0.03812) (Table **6-3**). Ulanga district in southeastern Tanzania had the highest mean gonotrophic cycle values

(mean = 0.9, 95%CI = 0.703 - 1.152) and the least Bagamoyo (mean = 0.465, 95%CI = 0.341 - 0.634) **(Figure 6-7**). The age structure was not associated with the trapping method (deviance = -0.45164, p=0.798) or trap location (deviance = -1.36691, p=0.2423) (Table **6-3**).

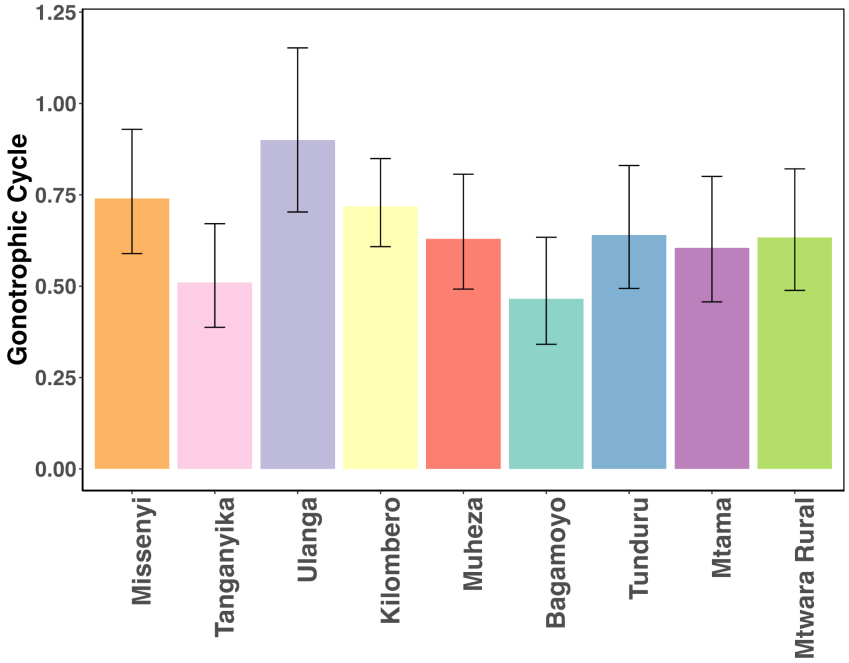


Figure 6-7: The gonotrophic of *Anopheles funestus* across nine Tanzanian districts. Northwest Tanzania includes the Missenyi and Tanganyika districts; southeast Tanzania includes Ulanga, Kilombero, Muheza, Bagamoyo, Tunduru, Mtama, and Mtwara Rural districts.

6.4.5 Variations of the entomological variables across the inland-coastal genetic populations

In addition to the between-district analysis, we also examined whether key vector bionomic, phenotypic and transmission traits varied along the inland-coastal genetic clusters as determined in the population genetic analysis [**see Chapter 5 of this thesis**]. Inland Tanzania populations include Missenyi and Tanganyika districts and coastal include Ulanga, Kilombero, Muheza, Bagamoyo, Tunduru, Mtama, and Mtwara Rural districts. A total 1699 *An. funestus* mosquitoes were analysed for host blood meal of which 38.4% (n = 652) were from inland and 61.6% (n = 1047) were coastal populations. The frequencies of vertebrate blood meals in the inland mosquitoes were 69.8% human, 0.46% dog, 0.15% goat, and 29.6 PCR non-amplification (Figure **6-8A**). The frequencies of vertebrate blood meals in the coastal mosquitoes were 71.3% human, 0.4% goat, and 28.4% PCR non-

amplification (Figure **6-8A**). A total of 6141 mosquitoes were analysed for malaria parasite infection, of these, 76.1% (n = 4671) were collected coastal and the remainder inland (Table **6-1**). *Plasmodium falciparum* infections were significantly higher in the coastal mosquitoes (2.4%, CI 1.6 – 3.54) compared to the inland populations (0.4%, CI 0.1 – 0.91) (Figure **6-8B**). 908 mosquitoes were dissected for age structure analysis with 78% (n = 708) were coastal and the rest inland genetic populations. Fecundity parameters were measured in 446 mosquitoes, 76.5% (n = 341) were coastal indoors and the rest inland.

Mosquito sizes were significantly larger in the inland (2.87, CI 2.84 – 2.9) compared to coastal (2.69, CI 2.67 – 2.9) (Figure **6-8C**). Similarly, the average number of eggs laid were significantly larger in the inland (110.84, CI 103.94 – 117.72) compared to coastal (87.7, CI 84.27 – 91.14) (Figure **6-8D**). However, there were no differences in the average gonotrophic cycles and parity of the inland coastal mosquitoes (Figure **6-8E&F**).

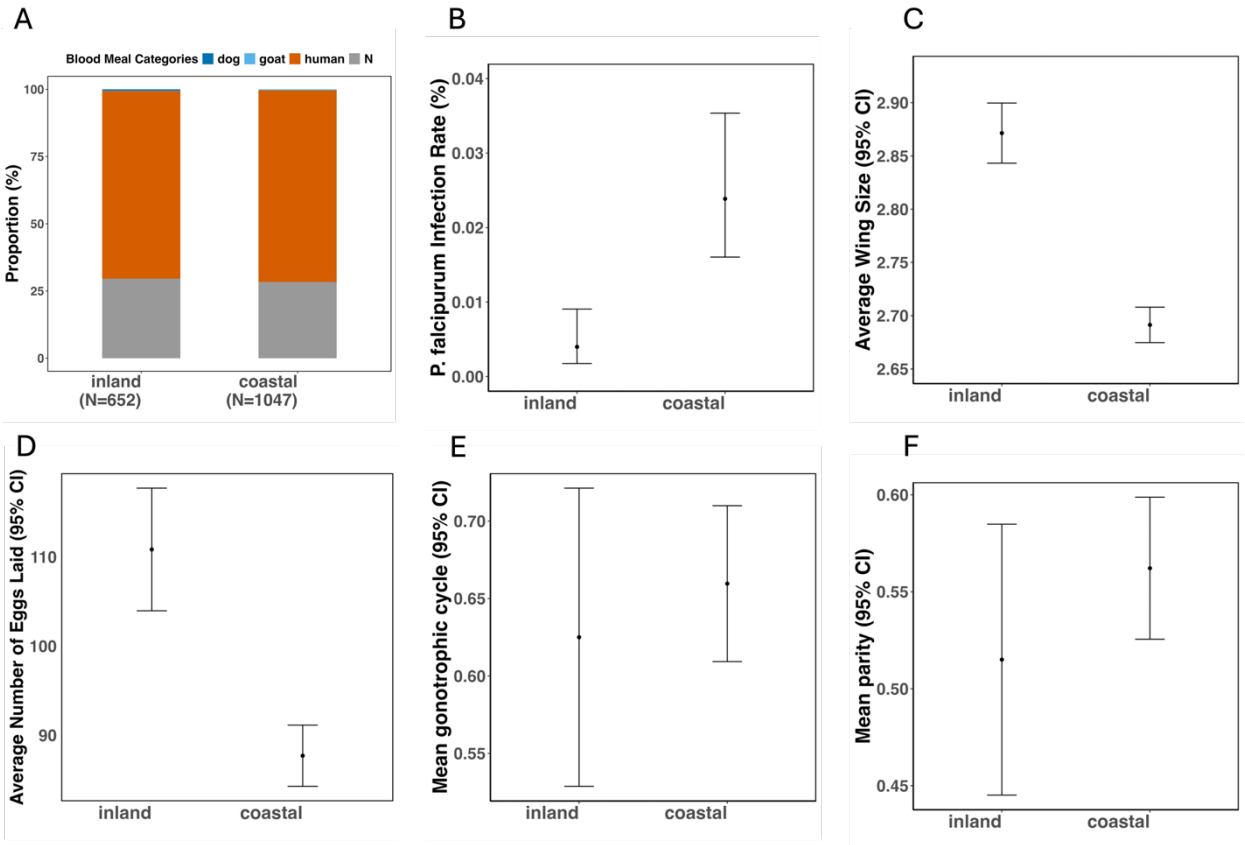


Figure 6-8: The variations of *An. funestus* entomological indicators across the inland and coastal genetic populations. The panel has variations in host blood feeding pasterns (A), *P. falciparum* infection rates (B), mosquito sizes (C), average number of eggs laid (D), mean gonotrophic cycle (E), and mean parity

(F). The error bars in B, C, D, E & F indicate confidence around the mean estimates. The inland populations included Missenyi and Tanganyika, while coastal were Kilombero, Ulanga, Muheza, Mtwara Rural, Bagamoyo, Mtama, and Tunduru.

6.5 Discussion

This study aimed to understand whether key vector bionomic, phenotypic and transmission traits vary between a breadth of geographic (district) populations across Tanzania. The study also examined if these entomological parameters varied between the inland and coastal genetic clusters discovered in the population genetic analysis of the species in Tanzania. Despite the pronounced genetic distinction between inland and coastal populations, *An. funestus* across the two genetic and geographical populations fed more on human blood, with alternative host availability (chicken, cattle, goat, and pig) exerting minimal influence on reducing human blood index (HBI). This preference for human blood in *An. funestus* has been previously observed in multiple other studies (Takken and Verhulst, 2013). The majority of *An. gambiae* and *An. funestus* in Zambia were shown to have strong anthropophilic behaviour compared to feeding on other vertebrates (Das et al., 2017). In western Kenya, *An. funestus* shows a strong preference for human blood feeding in contrast to *An. arabiensis* which fed more on cow/bovine (McCann et al., 2014a). Human host availability (higher number of people sleeping in a house) hurts HBI as did the availability of alternative host (cattle). Local host availability is a crucial determinant in *Anopheles* mosquito blood feeding with hosts that are easier to locate fed upon frequently (Killeen et al., 2001). Some studies have reported zoophagic behaviour in *An. funestus* that is influenced by the abundance of alternative hosts (Takken and Verhulst, 2013). The domestication of cattle for instance has been associated with low HBI (Finney et al., 2021) and significantly low malaria infections (Mayagaya et al., 2015). Increased coverage of ITNs and IRS can confer a shift in feeding behaviours of less opportunistic vectors. The average countrywide bed net coverage in mainland Tanzania is ~65% (Tanzania-MoH, 2022). Indeed, the upscaling of vector control interventions has led to *An. funestus* shifting to outdoor and early biting influencing HBI and malaria transmission (Kreppel et al., 2020). Taken together, the high human blood feeding in *An. funestus* warrants consideration of additional control interventions.

Understanding local-level heterogeneity of malaria mosquito infections is crucial for devising spatially targeted vector control interventions. Malaria parasite infections in the mosquitoes were significantly higher in the coastal genetic populations compared to inland. The infections were notably higher in two districts, Kilombero and Ulanga, within the Kilombero Valley. Previously, *An. funestus* parasite infection has been estimated at 3% in the inland Tanzania districts (Misungwi, not considered in this study) (Matowo et al., 2021), and up to 4.6% in some villages in the Kilombero Valley (Kaindoa et al., 2017). The markedly high parasite infection rates in a village in Ulanga district could be due to potential heterogeneity in infections. Over the years, malaria has generally declined significantly in the Kilombero Valley (Finda et al., 2018). However, pockets of villages within the valley remain with incredibly high malaria transmission intensities [**Mshani et.al Unpublished**]. Coincidentally, from our age structure analysis, the specific village in Ulanga district had older mosquitoes, which have a higher propensity to parasite infection and transmission.

The higher infection rates in coastal Tanzania compared to the inland genetic populations could be due to differences in mosquito response to insecticide-based interventions. *Anopheles funestus* is resistant to most public health insecticides including pyrethroids used on ITNs but with no discernible differences in phenotypic resistance levels between northwest and southeast established in Chapter 3 of this thesis (Odero et al., 2024b). However, there are differences in the mechanisms underlying the resistance in these populations (see Chapter 3 of this thesis). Coincidentally, the coastal Tanzania populations with higher *P. falciparum* infections were at near fixation for the resistance alleles of the main resistance genes, *Cyp6p9a/b* (Odero et al., 2024b). Further studies should investigate if such patterns in resistance genotype translate to differences in the infection levels Targeted vector interventions taking advantage of the ecology of this *An. funestus* is important, even as its role in malaria transmission across Tanzania becomes more apparent.

Mosquito body sizes were markedly varied between the inland and coastal genetic populations with inland mosquitoes significantly larger than coastal, and a positive correlation between mosquito size and egg batch size in all locations. There was

however no difference in either parity or gonotrophic cycles across either districts or genetic populations. The body size of an *Anopheline* mosquito influences its fitness traits such as host-seeking and ability to produce progeny (Takken et al., 1998). Mosquito size also impacts other important aspects of malaria vector control such as response to insecticide exposure (Owusu et al., 2017) and laboratory husbandry (Ngowo et al., 2021). The cause and effect of such differences in inland-coastal *An. funestus* phenotypes and demographic traits should be investigated especially in the context of genetic-based control approaches. Several other environmental factors beyond the scope of this study such as temperature, relative humidity, and precipitation can affect mosquito growth and physiology and warrant investigation.

Despite generally achieving the stated objectives, this study also had some limitations. First, the sampling locations and period were targeted at areas where *An. funestus* was more likely to be sampled. This led to very few collections of the other important malaria vectors in the *An. gambiae* complex and a loss of opportunity for any comparative analysis. Second, the sampling of the blood-fed and resting mosquitoes was mainly done indoors by mechanical mouth aspirators and more likely to be human-fed. Expanding the collection to animal sheds could provide more diverse blood sources. Importantly, this highlights the challenges of field estimation of the feeding preference of malaria vectors. Lastly, the low to no PCR amplification rates of mosquito blood meals in some districts, especially in Mtama and Bagamoyo, affected the estimation of vertebrate feeding preference. This could have been caused by opting for silica gel desiccation as a preservation method. Future research should consider using filter paper preservation as it maintains vertebrate DNA integrity for longer (Reeves et al., 2016).

6.6 Conclusion

This study found *An. funestus* across the genetic and geographic populations in Tanzania to prefer human feeding compared to other blood meals. There were in between population differences in mosquito *P. falciparum* infections with coastal genetic populations carrying significantly higher infections compared to the inland. The inland mosquito populations were larger in size and laid significantly

more eggs on average compared to coastal. Both populations however had similar age structure profiles about parity and gonotrophic cycle. Further research is needed to determine whether these phenotypic and demographic differences are a consequence of, or contribute to, the observed genetic separation between the inland and coastal populations of *An. funestus*.

7 General discussions: Towards a deeper understanding of *Anopheles funestus* population biology for improved vector control

7.1 Key findings

The overarching aim of this thesis was to provide a comprehensive understanding of the population biology, insecticide resistance, and population genetic structure of the major malaria vector *An. funestus* in mainland Tanzania. Despite the apparent importance of *An. funestus* in sustaining malaria transmission across most parts of Africa, research on this vector has historically lagged those of other malaria vectors in the *An. gambiae* complex, leaving many aspects of its biology uncertain (Coetzee and Koekemoer, 2013). To bridge this gap, I began by highlighting genetic advances and research opportunities crucial for the pragmatic control of *An. funestus* mosquitoes (Chapter 2, (Odero et al., 2023)). Some of the gaps and recommendations highlighted in this chapter include a call for a holistic research approach in the development and deployment of genetic control technologies, including *An. funestus*, an improved understanding of the vector's range-wide genetic diversity, and improved insecticide resistance monitoring data.

A key aspect of *An. funestus* biology relevant for malaria control is a robust baseline understanding of the phenotypic insecticide resistance status. Resistance bioassay data on this vector is very limited across Africa, with assumptions of responses to control interventions similar to other malaria vectors despite obvious differences in their behaviours (Kreppel et al., 2020, Tirados et al., 2006, Githeko et al., 1996). To close this gap, I provided the first large-scale phenotypic and molecular survey of insecticide resistance in Tanzania's *An. funestus,* a significant step towards evidence-based decision-making for improved vector control in the country (Chapter 3, (Odero et al., 2024b)). Despite the observed widespread pyrethroid (permethrin and deltamethrin) resistance I detected, the populations across the country remained susceptible to the organophosphate, pirimiphos-methyl, suggesting that this chemical, which is already widely used in agriculture, may be one of the few remaining options suitable for chemical vector control of this species.

In addition to the phenotypic aspects of vector resistance, an understanding of the genetic basis underlying the resistance through molecular surveillance is crucial. The patterns of these genes in populations can also help to infer contemporary populations' structuring and gene flow. The analysis revealed distinct geographical patterns, with *CYP6P9a*-R and *CYP6P9b*-R alleles, and the SV6.5kb structural variant absent or undetectable in the northwest but prevalent in all other sites, while SV4.3kb was prevalent in the northwestern and western regions but absent elsewhere (Chapter 3, (Odero et al., 2024b)). The distribution patterns of the metabolic resistance genes did not reflect the phenotypic resistance, but the finding was further supported by a wider population genomic analysis on the vector (Chapter 5). Since this was the first large-scale survey of resistance in Tanzania's *An. funestus*, I concluded this chapter by recommending regular updates of this data with greater geographical and temporal coverage.

In Chapter 4, I carried out a more in-depth analysis of the genetic basis of resistance mechanisms by extending from the analysis of candidate genes (Chapter 3) to whole genome sequence analysis. An unexpected finding in Chapter 3 was the unique phenotypic resistance of one *An. funestus* population (from Morogoro). Sequencing analysis unexpectedly discovered two knockdown resistance (*kdr*) mutations in this population, L976F (L995F in *An. gambiae*) and P1842S that have never before been reported for this species. These mutations were in the voltage-gated sodium channel gene (*Vgsc*), which is the insecticide binding site in mosquitoes. The localised and possibly contemporary evolution of *kdr* reported here contradicts previous research where *kdr*-driven resistance phenotypes appeared and spread rapidly across Africa after the introduction of organochlorine dichloro-diphenyl-trichloroethane (DDT) spraying for insect control in the mid-19th century (Pinto et al., 2007). I found the *kdr*- L976F to be strongly associated with DDT resistance but not with pyrethroids. Since this was the first observation of *kdr* in *An. funestus*, I conclude the chapter by proposing further research to explore its origins and potential impact on current control.

In Chapter 5, I used population genetic analysis, PCA on inversion-free regions of chromosome 2, *Admixture* analysis of discrete ancestral populations, genetic diversity tests (Nucleotide diversity and Tajima's D, and ROH to infer recent inbreeding), and population differentiation tests (*Fst* and selection scans) to highlight two genetically differentiated inland and coastal *An. funestus*

populations in Tanzania. The populations were potentially separated by an area of unsuitable climate and a geographically coincident with the eastern arm of the Great Rift Valley. Further, they showed demographic differences and divergence, likely due to differentiation at insecticide resistance genes reflecting diverse historical and contemporary dynamics. I concluded this chapter by recommending that follow-up research to examine the epidemiological relevance of the distinction and if these populations have different malaria transmission abilities.

Finally, in chapter 6, I set out to explore if there could be variations in key vector bionomics, as well as phenotypic and transmission traits that might be linked to the observed genetic differences between inland populations and those from coastal, lower-altitude regions. While the biting behaviour of *An. funestus*, level of anthropophily and age structure were similar across populations, differences were noted in the prevalence of *Plasmodium* infections, mosquito sizes, and fecundity between the inland and coastal genetic populations. Further research is needed to determine whether these phenotypic and demographic differences are a consequence of, or contribute to, the observed genetic separation between the inland and coastal populations of *An. funestus*.

Overall, this thesis provides key insights into the population diversity and molecular ecology of *An. funestus* mosquitoes in Tanzania that are relevant for disease control, vector surveillance, and the development of genetic vector control technologies. The whole genome sequence dataset developed in this thesis, publicly available at the European Nucleotide Archive (study number PRJEB2141), represents the largest data on a disease vector in Tanzania and provides a crucial resource in the continued monitoring of the vector.

7.2 Practical implications of findings

7.2.1 Insecticide-based vector control in Tanzania

Vector control has for decades been the most impactful malaria control approach. The sustainability of continued overreliance on insecticides as the active ingredient on these tools is however threatened by the evolving resistance in key vectors. The insecticide resistance surveillance findings from this thesis highlight two key developments important for disease vector control. Firstly, the

widespread pyrethroid resistance in *An. funestus* mosquitoes reported here, and in other vector species, present a grave challenge to malaria control (Ranson and Lissenden, 2016a). The observed susceptibility restoration in the populations if pre-exposed to a synergist, piperonyl-butoxide (PBO), adds to the body of evidence on the effectiveness of using PBO-pyrethroid bed nets to combat resistance (Gleave et al., 2021). Following recommendations from the WHO, many countries, including Tanzania, are now adopting PBO-pyrethroid nets in response to increasing vector resistance (Prevention, 2022). Secondly, despite finding evidence of vector resistance to other insecticide classes, carbamates (bendiocarb) and organochlorines (DDT), *An. funestus* remained widely susceptible to organophosphates (pirimiphos-methyl). Similarly, the other major malaria vector in Tanzania, *An. gambiae*, also remain susceptible to pirimiphos-methyl and PBO-pyrethroids (Tungu et al., 2023, Kisinza et al., 2017). This presents a unique opportunity to respond to the worsening vector resistance in Tanzania through a combination or rotation of PBO-pyrethroid nets and indoor residual spraying using pirimiphos-methyl. However, if adopted by countries, there are concerns about the field retention of PBO on nets that might require more frequent distribution than the 3-year recommended period. Additionally, the unintended and potentially antagonistic consequences of pirimiphos-methyl and pyrethroid modes of action should be closely monitored. For instance, recent evidence has found that combining the two approaches in the same locality increases mosquito survival compared to when they are deployed individually (Syme et al., 2022). This could be because both insecticides act on the cytochrome P450 system; but while pirimiphos-methyl first requires the activation of this system before inhibiting acetylcholinesterase, PBO acts by inhibiting the P450s (Yunta et al., 2019), thus could work antagonistically.

7.2.2 The use of genetically modified mosquitoes for vector control

Genetic control of mosquitoes is a promising high-impact approach to malaria control. In Chapter 2, I highlighted some of the advances in the genetic control of mosquitoes, including the recent development of gene-drive cassettes for *An. funestus* (Quinn et al., 2021). This approach can however only be successful and sustainable if the desirable genetically engineered trait in the released laboratory mosquitoes can be introgressed and spread in the extant population by mating

(Bier, 2021). As such, gene drive success is limited to how populations intermix in nature, making a robust understanding of vector population genetics a prerequisite on the pathway to gene drive deployment (James et al., 2018). My results revealed two genetically distinct *An. funestus* population in Tanzania with varying demographic histories, and geographic distributions. Population separation can be driven by multiple factors including physical barriers such as the Rift Valley and the Congo forest ecosystem as seen in *An. gambiae* (Pinto et al., 2013, Lehmann et al., 1999). Additionally, the coastal populations subcluster into groups, thus considering the proximity of their geographical collection points, additional barriers to gene flow might exist in the country. Highlighting such genetic discontinuities in vector target populations will be key when planning gene drive deployment strategies. For instance, following the genetic structuring findings in this thesis, it is highly likely that any gene drive deployments in Tanzania will require strategic releases at multiple points. The deployment of gene drive constructs should also account for the dynamics in local adaptations and historical traits including genetic and ecological differences (Eckhoff et al., 2017). We reported a location in Tanzania (Morogoro in the Kilombero Valley) where *kdr* mutations have evolved and locally spread to high frequencies (Chapter 4). Knockdown resistance alleles are selected for and rapidly spread through population populations through either gene flow (Pinto et al., 2007) or multiple independent mutation events (Clarkson et al., 2021). If the former is true, it remains unclear why *kdr* has not spread to other *An. funestus* in the coastal genetic population of Tanzania despite relative proximity to the discovery point and no evidence of a gene flow barrier. The underlying factors that could generate such localised dynamics within the target mosquito population should be considered during the development of gene drives.

The prospect of using gene drive for mosquito control is a relatively new endeavour with no data yet available on the potential epidemiological implications of this approach, or its impact on the environment. Most studies therefore rely on mathematical models to simulate the different field scenarios. However, accurate and realistic model parameterization requires field data on the local vector's ecology under question (Nolan, 2021, Eckhoff et al., 2017). Current malaria gene drive models are mostly based upon *An. gambaie* and *An. coluzzi* and are as such only valid for these vectors (Eckhoff et al., 2017, North et al., 2013). This thesis provides data on three aspects of *An. funestus* ecology that

can be utilised together with other available eco-epidemiological data to parameterization species-specific gene drive models. (i) a country-wide genetic variation and evidence of genetically based population structure, (ii) variations in insecticide resistance genes, including the newly discovered *kdr*, that can be used as a proxy to gene flow patterns, and (iii) host blood feeding and other aspects of *An. funestus* life history traits.

7.2.3 Using insects to monitor eco-remediation efforts of environmental contamination

Pesticides have a wide array of uses across the globe with obvious benefits including in agriculture and public health control of diseases. However, pesticide exposure and pollution can have devastating consequences on the environment and human health. For example, insecticide exposure is a key stressor affecting the population decline of pollinators, essential for ecosystem health and food production (Nicholson et al., 2023, Douglas et al., 2020). In the 1940's dichlorodiphenyltrichloroethane (DDT) was widely used as a mosquito control agent. However, DDT is also toxic to vertebrates and possesses high bio-accumulative and persistent properties (Mansouri et al., 2017). In *An. gambiae,* resistance to DDT has evolved in the form of knockdown resistance (*kdr)* mutations in the voltage-gated sodium channel (*Vgsc*) (Reimer et al., 2008, Ranson et al., 2000). In this thesis, the timing and location of the *kdr* discovery in *An. funestus* allows me to hypothesize that the occurrence of this mutation in the Morogoro *An. funestus* population could have been in response to DDT stockpiling or illegal agricultural use in Tanzania. Efforts by the Tanzanian government with support from the World Bank to destroy old DDT stockpiles are argued here to have led to significant yearly reductions in *kdr* frequencies, indicating potential alleviation of contamination impact on the environment. Consequently, this presents a unique example of the potential of using mosquito vectors to monitor the environmental impacts of pesticide pollution and the progress of remediation efforts.

7.3 Limitations and future research directions

7.3.1 Investigating the underlying drivers of genetic differentiation and barriers to gene flow in Tanzania

In chapter 5 of this thesis, I established *An. funestus* in Tanzania to be genetically separated into inland and coastal populations. The two populations are coincidentally geographically located on either side of the eastern arm of the Rift Valley making it a plausible explanation for the differentiation. Major geographical features such as the Great Rift Valley have been postulated as a gene flow barrier across the east and southern African regions (Lehmann et al., 1999). Ecological barriers to gene flow such as the Congo Basin tropical rainforest have also been argued to cause genetic differentiation in malaria vectors. In the case of Tanzania, multiple other potential genetic discontinuities such as climatic variations and topography also exist. The potential impact of these factors on local vector adaptation and genetic differentiation should be investigated. Here, our sampling design made it difficult to assess this. For instance, we had no data collection points around the centre of the country which is mostly arid-semiarid. A proposed methodology to achieve this would be to conduct fines scale population sampling across the country considering these discontinuities. An analysis of this combined with landscape genetics techniques would give a vector migration surface and landscape connectivity measures capable of revealing where active gene flow barrier occurs (Manel and Holderegger, 2013).

7.3.2 Investigating the origin and the spread of knockdown resistance in the malaria vector Anopheles funestus in Tanzania

The strong selection pressures generated from insecticide use in agriculture and public health have resulted in multiple independent evolutions of various mechanisms that confer insecticide resistance phenotypes in numerous insect species. One of the earliest described resistance mechanisms was the emergence of knock-down resistance (*kdr*), mediated by mutations in the target site of pyrethroid and organochlorine insecticides (Reimer et al., 2008, Ranson et al., 2000), located in the voltage-gated sodium channel gene (*Vgsc*), an essential component of the insect nervous system. These *kdr*-driven resistance phenotypes appeared rapidly after the introduction of organochlorine dichloro-diphenyl-

trichloroethane (DDT) spraying for insect control in the mid-19th century (Pinto et al., 2007). In the malaria vectors *An. gambiae* and *An. coluzzii*, two common *kdr* mutations L1014F and L1014S on the *Vgsc* gene emerged around this period and quickly spread across Africa, resulting in the loss of pyrethroid bed net efficacy (Clarkson et al., 2021, Pinto et al., 2007).

Though extensively used for vector control in the past, DDT is no longer WHO-approved and banned in Tanzania. It is therefore unclear whether the *kdr* emergence in Tanzania is due to the legacy effect of previous DDT use and stockpiling or due to illegal use in agriculture (see Chapter 4 of this thesis). Additionally, since the insecticide-phenotype association analysis in this chapter included only type II pyrethroids (deltamethrin) and on a relatively small sample size, additional data are urgently required to assess the potential impacts of *kdr* on other pyrethroids. The discovery of *kdr* in *An. funestus* can pose a serious risk to current and future malaria control. Future studies should focus on; i) investigating the frequency of *kdr* mutations in *An. funestus* collected around documented DDT contamination sites, ii) investigate the phenotypic association between *kdr* and vector control insecticides, and iii) explore the potential of *kdr* to spread across *An. funestus* ecological ranges in Tanzania and beyond. A priority step towards achieving these goals will be to develop an allele specific PCR assay for the detection of *kdr* in wild mosquitoes.

7.3.3 Evolutionary trajectories of the inland and coastal genetic populations and impact on malaria transmission dynamics

In this thesis, I provide evidence that inland and coastal *An. funestus* potentially have diverse evolutionary trajectories. For instance, the G451A-*Cyp9k1* mutation is at a near fixation state in mosquitoes in the inland populations but at low frequencies in most populations in the coastal cluster (Chapter 5). A similar pattern is observed where *Cyp6p9a/b* resistant alleles are at fixation in the coastal populations but are at either low frequencies or absent in the inland populations (Chapter 3). The analysis of copy number variations (CNV), known to play a role in insecticide resistance, would help decipher these evolutionary trajectories. In *An. gambiae*, CNV analysis has revealed a region of the genome with increased copy number, especially in the *Cyp6p* and *Cyp6aa1* gene clusters conferring pyrethroid resistance (Lucas et al., 2023, Njoroge et al., 2022). Furthermore, the

appearance of initial mutations around these gene clusters has been shown to rapidly lead to the selection of multiple resistance haplotypes that quickly spread across populations (Njoroge et al., 2022). The imminent availability of CNV datasets on *An. funestus* will help establish if this observation is similar.

7.3.4 Field tracking of molecular markers of insecticide resistance

A significant constraint of this study was the potentially low statistical power to discern the associations between specific gene variants and phenotypic resistance (Chapter 3). The study design and sample sizes used in this thesis did not permit an exploration of the cumulative or epistatic effects exerted by multiple insecticide resistance gene mutations within the same individual mosquito. It is conceivable that a combination of mutations across multiple genes investigated, rather than the impact of each mutation in isolation, could more accurately account for the variations observed in the phenotypic resistance. Furthermore, the resistance landscape in western Tanzania (Tanganyika) where all five resistance genes (*Cyp6p9a/b*, *L119F-GSTe2*, SV4.3kb, and SV6.5kb) were present warrants further investigation. These complexities underscore the need for future studies to consider larger sample sizes and more sophisticated statistical and genetic analyses that can capture these nuances.

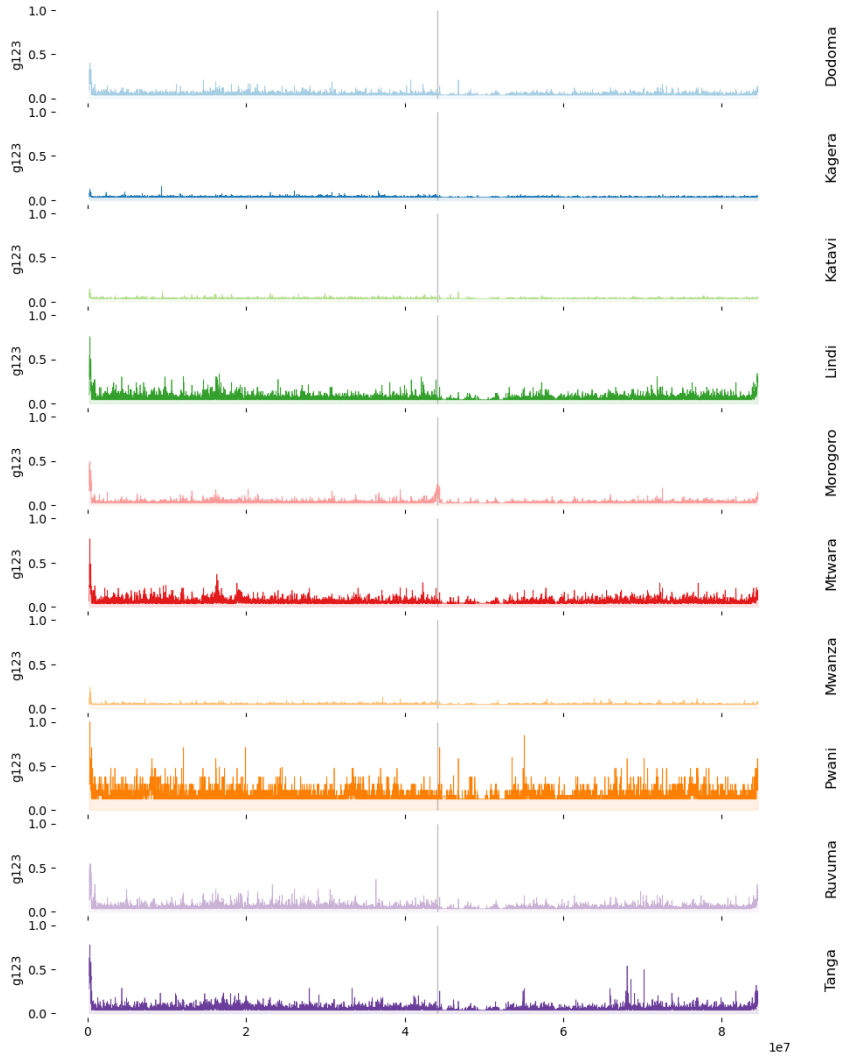
7.4 Conclusion

In conclusion, this thesis provides a comprehensive analysis of the population diversity, ecology, and insecticide resistance of *An. funestus* mosquitoes in Tanzania. The findings advance knowledge on the status of vector resistance to public health insecticides in Tanzania. The continued effectiveness of PBO-pyrethroid and pirimiphos-methyl against resistant *An. funestus* is important for sustainable malaria control in the country. It is important for future research to monitor the evolution of genetic patterns of insecticide resistance alleles highlighted in the populations and how they might change in response to the introduction of new vector control insecticides. Furthermore, I highlight two distinct populations of *An. funestus* with varying demographic histories. I reflect on future research directions that can build upon and advance the findings of this thesis. I have made all genomic datasets from this thesis publicly available for the wider community use. The resistance phenotype data have also been availed

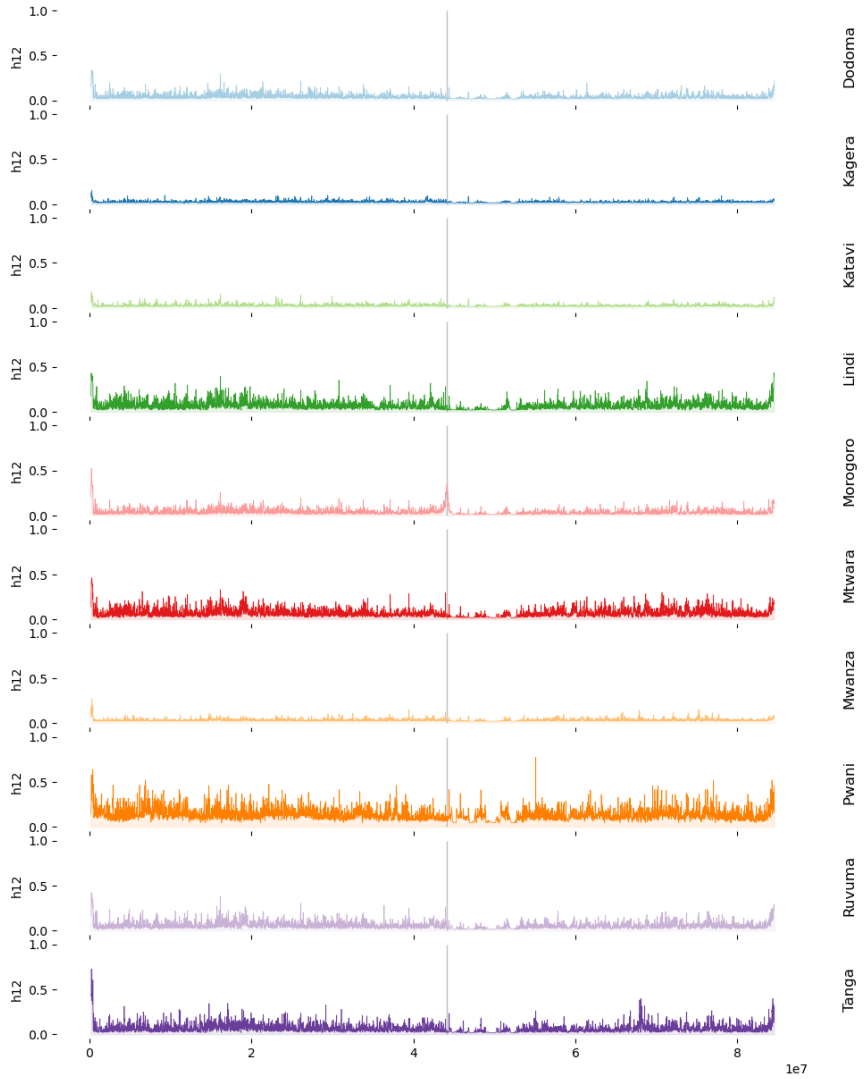
through an interactive online platform that will be continuously updated for use by malaria control programs.

Appendices

Chapter 4 - Supplementary figures

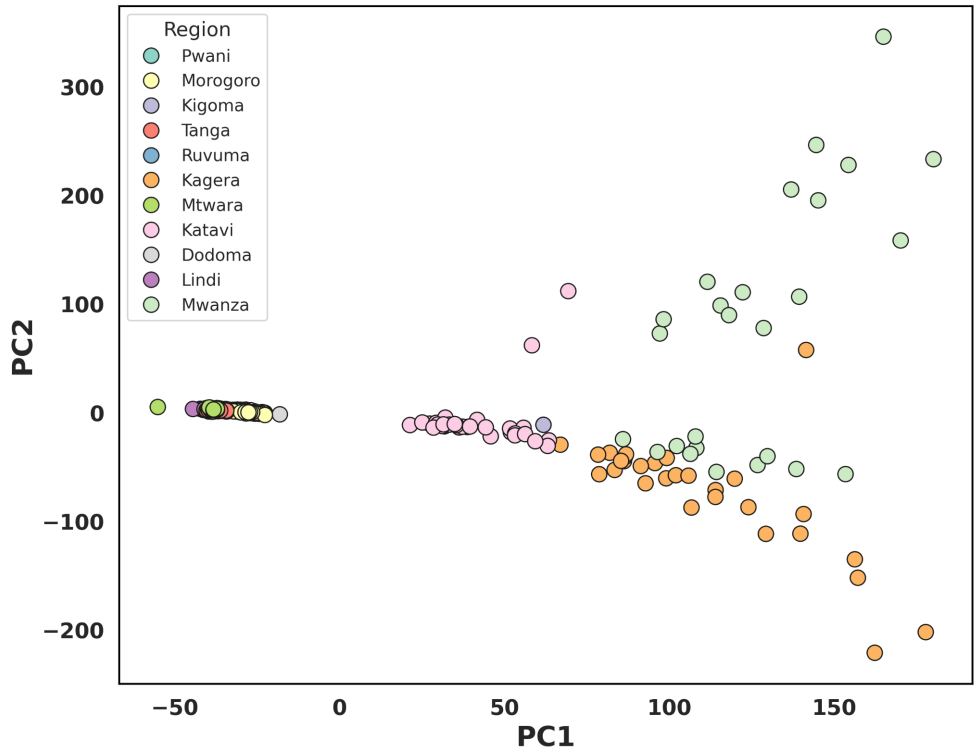


Supplementary Figure 7-1: G123 selection scans of *An. funestus* chromosome 3RL, coloured and windowed by sample collection region (where n>20 – see Supp Table 2). X-axis indicates the position (in base-pairs (bp)), Y-axis indicates the selection statistic G123. The Grey dotted line indicates the location of the *Vgsc* gene. Note Mwanza region is absent as there were too few samples (n<20) to perform a selection scan.

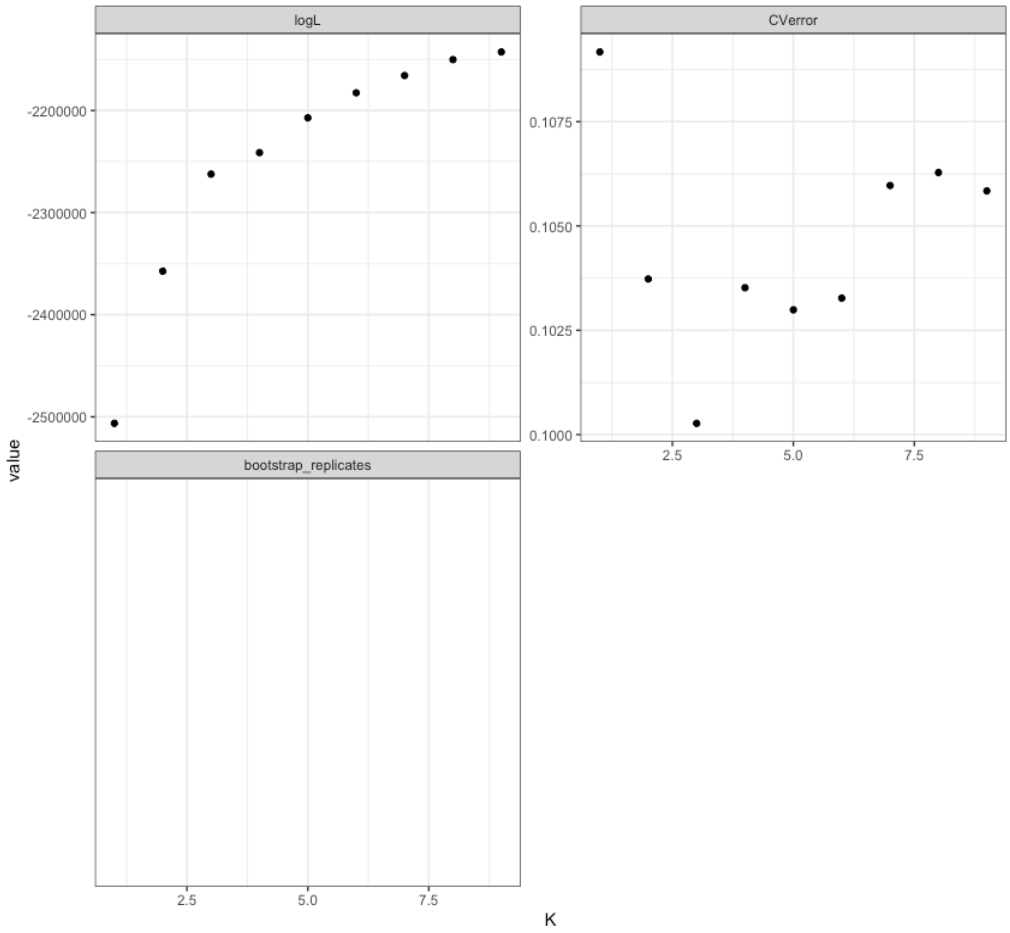


Supplementary Figure 7-2: H12 selection scans of *An. funestus* chromosome 3RL, coloured and windowed by sample collection region (where n>20 – see Supp Table 2). X-axis indicates the position (in base-pairs (bp)), Y-axis indicates the selection statistic H12. The Grey dotted line indicates the location of the *Vgsc* gene. Note Mwanza region is absent as there were too few samples (n<20) to perform a selection scan.

Chapter 5: Supplementary information



Supplementary Figure 7-3: The population structure of *An. funestus* in Tanzania. PCA plot of the first two principal components from the X chromosome. The colours denote the sampling location (regions).



Supplementary Figure 7-4: Validation of the best *Admixture* value of *K* with the lowest cross-validation error (CVE).

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