MAKERERE  **UNIVERSITY**

MATHEMATICAL MODELING AND SIMULATION OF MALARIA VECTOR PROPAGATION: A TOOL FOR EVALUATION OF NOVEL CONTROL APPROACHES

BY

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DECLARATION

I, **Rita Mwima**, declare that this PhD thesis is my original work and has never been submitted to any university or institution of higher learning for the award of a degree. The literature mentioned in this thesis is appropriately referenced.

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DEDICATION

To my husband, Noel Thomas Kalunda, my mother Mrs. Winnie Kabasa, and my father Professor John David Kabasa.

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LIST OF ABBREVIATION

ABC	Approximate Bayesian Computation
ACTs	Artemisinin-based Combination Therapies
An.	*Anopheles*
ANOSPP	**ANO**pheles **SP**ecies and **P**lasmodium
bp	base pair
Bti	*Bacillus thuringiensis israelensis*
Cas9	CRISPR associated protein 9
CBT	Catch Basin Traps
CHC	Cuticular Hydrocarbon
CRISPR	Clustered Regularly Interspersed Palindromic Repeats
DNA	DeoxyriboNucleic Acid
DS	Dry season
EDS	Early Dry Season
EDTA	Ethylenediaminetetraacetic Acid
ERS	Early Rainy Season
F_{ST}	Fixation Index
GMMs	Genetically Modified Mosquitoes
gRNA	guide Ribonucleic Acid
HEGs	Homing Endonuclease Genes
HLC	Human Landing Catches
IBD	Isolation-By-Distance
IPT	Intermittent Preventive Therapy
IRS	Indoor Residual Spraying
IVM	Integrated Vector Management

LD	Linkage Disequilibrium
LDM	Long Distance migration
LDS	Late Dry Season
LLINs	Long-Lasting Insecticide Nets
LRS	Late Rainy Season
MAF	Minor Allele Frequency
malERA	Malaria Eradication Research Agenda
MCMC	Markov Chain Monte Carlo
MEDEA	Maternal Effect Dominant Embryonic Arrest
MRR	Mark Release Recapture
Ne	Effective population size
PCA	Principle Component Analysis
PCR	Polymerase Chain Reaction
pH	potential of Hydrogen
PSC	Pyrethroid Spray Catches
RS	Rainy season
SE	Standard Error
SIT	Sterile insect Technique
SNPs	Single Nucleotide Polymorphism
TALENs	Transcription Activator-Like Effector Nucleases
Tes	Transposable Elements
Tris-HLC	Tris(hydroxymethyl)aminomethane hydrochloride
VCF	Variant Call Format
WF	Wright Fisher
WHO	World Health Organisation

ZFNs	Zinc Finger Nucleases

ABSTRACT

Background: Malaria transmission is sustained by highly adaptable *Anopheles* mosquitoes that persist across dry seasons and rebound in large numbers when the rains return. Despite major progress in vector control, knowledge gaps remain regarding the survival mechanisms that sustain mosquito populations during dry seasons. This study investigated the evolutionary and ecological mechanisms enabling such persistence and spread. It contributes critical insights for evaluating the effectiveness and sustainability of current and emerging vector control tools, including gene drive technologies.

Methods: To investigate the dry season persistence mechanisms of malaria mosquitoes, existing studies on survival strategies were critically evaluated to identify strengths, weaknesses, and knowledge gaps. A novel population genetic modeling framework was developed to estimate the proportion of aestivating adults, mosquitoes typically difficult to sample due to unknown habitats, and was initially applied to the *Anopheles coluzzii* dataset from Mali. This model was subsequently extended to jointly estimate both aestivation and long-distance migration and then applied to temporal genetic data from Eastern Uganda. Additionally, to assess the potential impact of novel vector control strategies such as gene drive, the population genetic structure and demographic history of *Anopheles gambiae* and *Anopheles arabiensis* were characterized using amplicon sequencing data collected from three island and three mainland sites in Uganda.

Results: Malaria mosquito populations persist through the dry season and rapidly rebound at the onset of the rainy season via four key mechanisms: aestivation, local refugia, local migration, and long-distance migration. Application of the developed population genetic model to temporal data from Mali successfully estimated the proportion of aestivating adults. When extended to incorporate both aestivation and long-distance migration and applied to temporal data from Eastern Uganda, the model revealed that the Sahelian region

exhibits stronger seasonality compared to Eastern Uganda. Furthermore, genomic analysis of amplicon sequencing data from island and mainland sites in Uganda showed pronounced spatial population structure, with island populations showing greater genetic differentiation not only from mainland populations but also among individual island sites. This strong within-island differentiation highlights their potential suitability for contained gene drive field trials.

Conclusion: This study aimed to determine the role of seasonal and evolutionary dynamics in mosquito survival by demonstrating that population genetics models can effectively estimate proportions of aestivating mosquitoes typically difficult to sample in the field. Moreover, this modeling framework is adaptable to quantify the relative contributions of multiple mosquito survival mechanisms within diverse ecological contexts. These insights are critical for optimizing the design of field trials for novel vector control strategies, such as gene drive, by elucidating population connectivity and seasonal persistence patterns. Additionally, the findings contribute to improved insecticide resistance management by illuminating how seasonal dynamics and vector life-history strategies influence the strength of selection and the spread of adaptive alleles, thereby supporting the responsible and sustainable deployment of genetic control technologies.

Keywords: *Gene drive systems, insecticide resistance, field trial sites, gene flow, mosquito persistence mechanisms, malaria vectors.*

CHAPTER ONE - INTRODUCTION

1.1 Background

Malaria is an infectious disease caused by protozoan parasites belonging to the genus *Plasmodium*, which are transmitted by blood-feeding mosquitoes of the genus *Anopheles*. Most malaria cases occur in sub-Saharan Africa, with about 246 million cases (94%) and 569,000 deaths (95%) reported annually (WHO, 2024). Uganda bears a predominantly large burden from malaria that stems from high disease transmission intensity, inadequate health care resources, a weak health system, and the impact of control interventions which have increased resistance of parasites to drugs and mosquitoes to insecticides among others (Yeka *et al*., 2012).

In sub-Saharan Africa, the *Anopheles gambiae* complex and the *Anopheles funestus* complex are the most important vectors of malaria, and transmit *Plasmodium falciparum*, the most important malaria parasite (Akogbéto et al., 2018; Bhatt, et al., 2015; Yeka et al., 2012). The *An. gambiae* complex comprises at least seven sibling species that are morphologically indistinguishable but vary in their vectorial ability and ecological niche (Ebenezer et al., 2014; Simard et al., 2009; White et al., 2011). *Anopheles gambiae* and *An. arabiensis* are the most efficient and broadly distributed vectors of *Falciparum* malaria in sub-Saharan Africa (Coetzee et al., 2000).

Efforts to reduce the malaria burden globally include vector control which involves the use of both long-lasting insecticide-treated nets (LLINs) and indoor residual insecticide spraying (IRS), effective malaria case management (treatment with artemisinin-based combination therapies (ACTs)), preventive therapies (intermittent preventive therapy (IPT)), and stakeholder commitment (Bhatt, et al., 2015; Gaye, 2019; WHO, 2024). There has been enormous progress over the years, but malaria remains a burden globally

especially in the African region because of the exceptionally robust vector dynamics of *Anophelines* (Bhatt et al., 2015; Lehmann et al., 2010).

Vector control through chemical interventions, particularly long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS), has historically formed a central component of malaria control strategies in sub-Saharan Africa. Although malaria vaccines have now been introduced, their effectiveness is partial and context dependent, not able to fully interrupt malaria transmission (Galactionova et al., 2021). Alongside the emergence of antimalarial drug resistance and the high cost and limited availability of effective treatments, these challenges highlight the continued importance of vector control interventions (Karunamoorthi, 2011a; Sougoufara et al., 2020a), that extend from protection at the household level to entire communities (Killeen et al., 2006, 2007). For example, LLINs are noted to have contributed about 68% and IRS about 10% to the general decrease in malaria cases in the early 2000s, but their efficiency has generally stagnated in recent years (Bhatt, et al., 2015). These interventions on their own are therefore insufficient to achieve malaria elimination, especially in the most endemic settings where transmission rates are very high (Bugoro et al., 2011; Gillies & Smith, 1960; Reddy et al., 2011; Russell et al., 2011; Van Bortel et al., 2010). Furthermore, these interventions mainly target indoor feeding and (or) resting vectors, instead of other aspects of their biology like outdoor feeding and resting, oviposition site preference and mating behavior (The malERA Consultative Group on Vector Control, 2011a; World Health Organisation, 2015).

Given that the major malaria vector control approaches are based on mosquitoes that feed on humans indoors at night (Kreppel et al., 2020), these methods could be compromised by changes in feeding and resting behaviors, plus alterations in the vector community that can facilitate residual malaria transmission despite the decline in primary vectors (Lwetoijera

et al., 2014; Sougoufara et al., 2016). Changes in mosquito behavior, therefore, pose new challenges to malaria control using conventional methods and call for developing new tools that will be used alongside those already in use to offer an appropriate platform for the efficient delivery of interventions (Muganga, 2011).

Genetically modified mosquitoes (GMMs) constitute a new set of tools that could either replace malaria vector populations with introduced genes for refractoriness that limit malaria transmission or disrupt fertility gene(s), thus lowering mosquito numbers to achieve vector population suppression (Eckhoff et al., 2016). Proof-of-concept mosquito transformation studies in laboratories are promising and some candidate stratified mosquito population loci for modification have been identified (Bernardini et al., 2014; Galizi et al., 2014; Gantz & Bier, 2015; Hammond et al., 2016; Windbichler et al., 2008).

Also, molecular tools for vector transformation and engineering for malaria gene drive mosquitoes have been developed (Hammond et al., 2016; Windbichler et al., 2011). However, to evaluate these genetic modification-related methods in the natural environment, there is need to understand the malaria vector propagation dynamics such as how *An. gambiae s.s* populations re-establish at the beginning of the rainy season (Adamou et al., 2011; Lehmann et al., 2010, 2017).

This requires isolated and well-characterized study sites with known parameters such as mosquito species composition, diversity, density, propagation, biting behavior, genetics, and parasite infection rates. By using mathematical models and simulations, we can replicate various intervention combinations in different study locations using several parameters to aid in precise predictions about where and how the deployment of the novel malaria vector control approaches will be done (Eckhoff et al., 2016; Lambert et al., 2018).

1.2 Statement of the Problem

There is evidence from several laboratory studies to prove that GMMs are effective tools for malaria vector control (Bernardini, 2014; Galizi, 2014; Hammond and Galizi, 2017; Windbichler et al., 2008). However, before implementing them in the natural environment, there is a need to make predictions on their deployment and effectiveness in the natural environment (A. P. Eckhoff et al., 2016). These predictions are using mathematical models and simulations which offer viable strategies on when and how to deploy GMMs for their maximum success in highly seasonal environments (Lambert et al., 2018).

Before these predictions are made, there is a need to understand the local mosquito population dynamics in the natural environment. Despite alternative explanations, one of the long-standing mysteries in mosquito ecology is the source of malaria mosquito populations that re-establish at the start of a rainy season (Adamou et al., 2011a; Lehmann et al., 2010; Lehmann, Weetman, Diana L Huestis, et al., 2017). Whether populations survive the long dry season by aestivation or are re-established by migrants from near or distant locations with larval sites available all year round remains unclear (Donnelly et al., 2002; Lehmann, Weetman, Diana L Huestis, et al., 2017).

1.3 Objectives of the Research

1.3.1 Main Objective

To characterize mosquito population dynamics at selected mainland and island field sites in Uganda for evaluation and prediction of the effectiveness of gene drive as a novel malaria vector control tool.

To investigate how seasonal variation in malaria mosquito populations influences population genetic dynamics and the effectiveness of novel vector control approaches at selected sites in Uganda and the Sahelian regions using mathematical models and simulations.

1.3.2 Specific Objectives

i. To determine the potential persistence mechanisms of the major *Anopheles gambiae* species complex malaria vectors in sub-Saharan Africa

ii. To simulate the population genetics of partial aestivation with application to *Anopheles coluzzii* from selected sites in Mali

iii. To investigate the potential seasonal rebound mechanisms of *Anopheles gambiae* in Eastern Uganda by simulating the population genetics of aestivation and long-distance migration

iv. To evaluate the population genetic structure and demographic history of *An. gambiae* and *An. arabiensis* at three mainland and three island sites in Uganda

1.4 Research Questions

1.4.1 Overall Research Question

How do seasonal population dynamics influence genetic variation and the effectiveness of novel vector control interventions in malaria mosquito populations?

1.5 Study Hypotheses

Hypothesis 1: Seasonal population bottlenecks significantly reduce effective population size and increase the strength of genetic drift in malaria mosquito populations.

Hypothesis 2: Strong seasonal fluctuations in population size increase stochastic variation in allele frequencies, affecting the persistence and spread of alleles relevant to vector control.

1.6 Significance of the Research

This study investigated the mechanisms driving malaria mosquito population rebounds in the *An. gambiae* complex at selected sites in Uganda and the Sahelian region. By identifying the susceptible phases of malaria vector populations, the study provided critical

data to predict the effectiveness of novel malaria vector control strategies, and understanding these seasonal dynamics provided essential parameters for mathematical models and simulations used to evaluate and optimize next-generation vector control approaches.

1.8 Justification for the Study

This study is justified by the need to evaluate the potential efficacy of novel malaria vector control approaches, such as gene drive-based *An. gambiae* population suppression, at three mainland and three island sites in Uganda. As a baseline study, it aims to characterize these sites and investigate mosquito persistence mechanisms during the dry season to inform the design of future field trials. Implementing an effective genetic-based control strategy has the potential to provide long-term vector suppression without repeated intervention campaigns, thereby reducing malaria transmission irrespective of vector feeding behavior, bionomics, or insecticide resistance.

CHAPTER TWO – LITERATURE REVIEW

2.1 Malaria Overview

2.1.1 The Parasite

Five species *of* the protozoan parasite in the genus *Plasmodium, P. falciparum, P. vivax, P. ovale, P. malariae,* and *P. knowlesi* are known to affect humans (Siciliano et al., 2015) and two of these species *(P. falciparum and P. vivax*) pose the greatest threat (Wesolowski et al., 2015). Research shows that *P. falciparum* is the most lethal and prevalent species in the tropics and sub-tropics (Arrow et al., 2004; Kotepui et al., 2020). The malaria parasite has a very complicated lifecycle (Aly et al., 2009; Siciliano et al., 2015), which could be attributed to the parasite's ability to change its cellular and molecular makeup and develop in intracellular and extracellular niches in the mammalian host and mosquito vector (Aly et al., 2009).

The malaria parasite lifecycle involves cyclical infection of both humans and female *Anopheles* mosquitoes, such that, a female mosquito will only acquire the malaria parasite by biting an infected person (Bousema & Drakeley, 2011; Crutcher et al., 1996). The parasite then goes through a cycle of growth and multiplication in the mosquito, 10-18 days later, a form of the parasite known as a sporozoite migrates to the mosquito's salivary glands and will be ready to be transmitted to another person (Garcia et al., 2006; Gerald et al., 2011). The biology of the *P. falciparum* allows it to survive and exploit various environments which include the liver and blood cells of humans, as well as the gut, vascular system and salivary glands of mosquitoes (Arrow et al., 2004; Muhammad & Muhammad, 2023; Venugopal et al., 2020).

The parasite enters the human body through the bite of an infected mosquito, immediately colonizes and multiplies in the liver cells, and then moves to the red blood cells of the blood

(Arrow et al., 2004). While in the blood, the parasite multiplies into other pathogen forms that continue to affect the red blood cells (Dhangadamajhi et al., 2010; Mohandas & An, 2012). Blood-stage parasites are responsible for malaria symptoms, and much of the disease pathogenesis results from malaria-infected cells blocking small capillaries (Arrow et al., 2004; Crutcher & Hoffman, 1996; Mawson, 2013). Some *Plasmodium* cells undergo meiosis, resulting in male and female gametes that may be taken up by a blood-feeding mosquito (Dahalan et al., 2019; Henry et al., 2019).

These gametes are then released into the mosquito gut, where mating occurs to form a zygote that grows and multiplies (Godfray & Charles, 2013; Nilsson et al., 2015). Ten to eighteen days later, another form of the parasite known as a sporozoite migrates into the mosquito's salivary glands (Crutcher & Hoffman, 1996; Godfray & Charles, 2013; Mawson, 2013). When this mosquito bites another human, it injects its saliva which contains anticoagulants, along with malaria sporozoites which then travel to the liver, initiating a new infection cycle (Arora et al., 2023; Godfray & Charles, 2013). This life cycle is depicted in Figure 2.1.

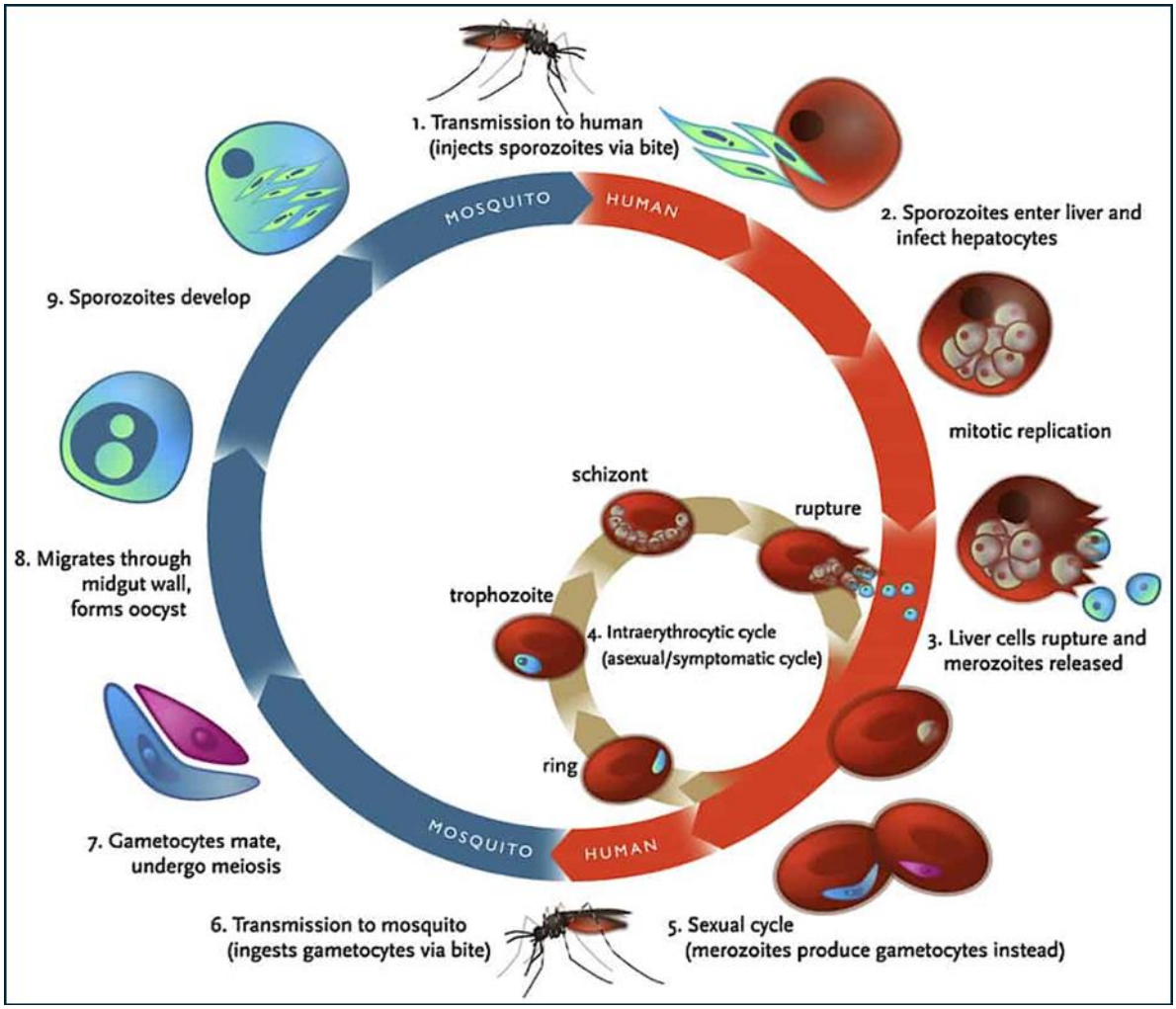

Figure ***2.1*** *Life cycle of the malaria parasite (Klein, 2013)*

2.1.2 Life Cycle of the Malaria Vector

Like all mosquito species, the malaria vector undergoes four distinct stages (Fig. 2.2): egg, larva, pupa, and adult (Clements, 1992, 1999). This complete metamorphosis is critical to the mosquito's development and environmental adaptation. Female mosquitoes lay small, buoyant eggs on stagnant or slow-moving water surfaces, which then hatch into larvae within 2–3 days under favorable ecological conditions (Clements, 1992). Female mosquitoes are compelled to feed on blood frequently, which maintains a high rate of laying eggs, hence increasing the vectorial capacity for malaria transmission considerably (Brackney et al., 2021; Clements, 1992; Shaw et al., 2020; Wu et al., 2020). The larval stage, lasting 4-10 days, is aquatic and depends on factors such as temperature and food

availability since larvae are active feeders (Clements, 1992, 1999). The pupa stage lasts 1-4 days; here, no feeding happens, but it is responsive to environmental stimuli such as light and movement (Clements, 1992; Williams & Pinto, 2012). The adult mosquitoes emerge from the pupa stage, followed by the feeding of male and female adult mosquitoes with nectar and other sugar sources as the primary source of energy (Clements, 1992; Foster, 1995).

However, female mosquitoes uniquely require blood meals for the development of their eggs, after which they rest for 2–3 days to digest the blood and convert it into nutrients for egg production (Clements, 1992, 1999; Shaw et al., 2020; Wu et al., 2020). Once these eggs are ready, the female seeks suitable aquatic habitats to lay her eggs, thus completing the reproductive cycle (Childs et al., 2016; Clements, 1992). This life cycle is particularly influenced by temperature and humidity, known to either accelerate or delay development (Beck-Johnson et al., 2017; Christiansen-Jucht et al., 2014; Ciota et al., 2014), even though the aquatic nature of the egg, larva, and pupa stages emphasizes the importance of water sources in mosquito population dynamics (Forsyth et al., 2020).

When malaria parasites are ingested during a blood meal, they undergo further development in the mosquito's stomach and go through several stages in the next 10 to 20 days, eventually multiplying and penetrating all parts of the mosquito’s body (Bloland & Williams, 2003). The development of these parasites is also temperature-dependent, given that a decrease in ambient temperature increases the time required for the parasite to develop (Bloland & Williams, 2003). With time, these parasites end up in the mosquito salivary glands, where they could be transmitted to humans when the mosquito takes another blood meal (Bloland & Williams, 2003).

Generally, understanding the stages of the mosquito life cycle (Figure 2.2) is critical for designing effective vector control strategies that target aquatic habitats or interrupt reproductive cycles (Forsyth et al., 2020).

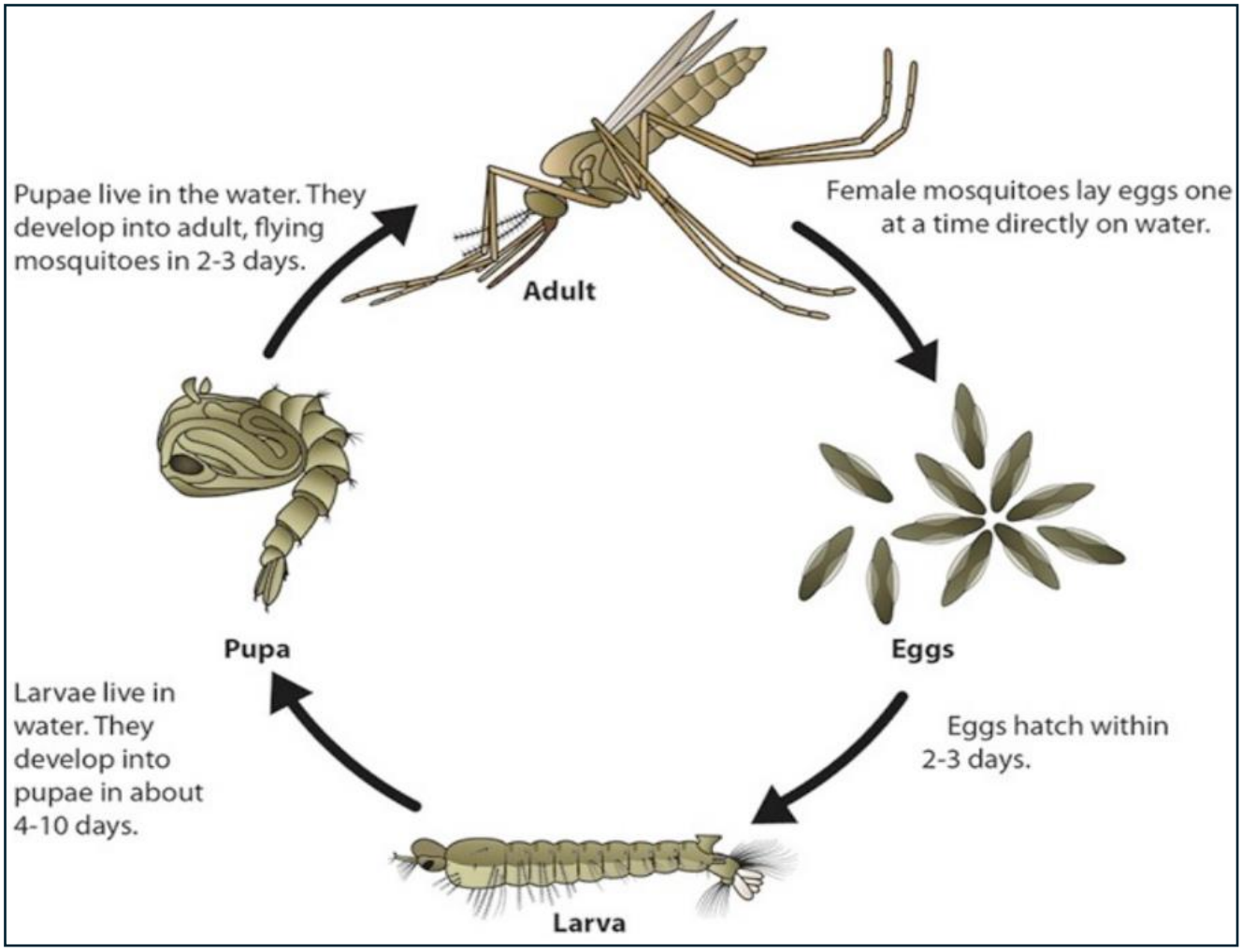

Figure 2.2 *Life cycle of the malaria mosquito (Department of Health et al., 2023)*

2.1.3 Ecology of the Female *Anopheles* Mosquito

The female mosquitoes of the genus *Anopheles* are responsible for the spread of malaria between humans (Crutcher & Hoffman, 1996; Nilsson et al., 2015). These mosquitoes take blood meals to enable egg production, and this is the link between them and humans in the parasite life cycle (Arrow et al., 2004; Crutcher & Hoffman, 1996). Understanding the ecology of the malaria vector is a requirement for eradication to be achieved and essential to sustaining long-term success (Ferguson et al., 2010; Kahamba et al., 2022; Rabinovich

et al., 2017; The malERA Consultative Group on Vector Control, 2011b). Knowing the different aspects of the malaria vector's ecology is important for taking into consideration new and complementary targets for integrated vector control (Beier et al., 2008; Ferguson et al., 2010; Wilson et al., 2020).

Previously, vector biologists focused on evaluating specific vector control approaches and less on their ecology (Ferguson et al., 2010; Sougoufara et al., 2020a; Wilson et al., 2020). However, even though the benefits from the existing vector control approaches were significant, elimination is still elusive (Govella & Ferguson, 2012; Killeen et al., 2013; Wilson et al., 2020). This could be because less emphasis has been placed on vector ecology, and yet alterations in ecological factors can affect the disease transmission potential of mosquitoes (Afrane et al., 2012).

In sub-Saharan Africa, the *An. gambiae* and the *An. funestus* complexes are the most important vectors of malaria and transmit *P. falciparum*, the most important malaria parasite (Yeka et al., 2012). The *An. gambiae* complex comprises at least seven sibling species, namely, *An. gambiae*, *An. arabiensis*, *An. melas*, *An. merus*, *An. bwambae* and *An. quadriannulatus*, one unnamed species, and several emerging species that are morphologically indistinguishable but exhibit distinct genetic and ecological differences (Favia et al., 1997; Gillies et al., 1987; Hunt et al., 1998).

The most efficient, broadly distributed, and dominant malaria vectors in sub-Saharan Africa are *An. gambiae*, *An. coluzzii*, *An. arabiensis*, and *An. funestus* (Harbach, 2004; Sinka et al., 2012; Wiebe et al., 2017). *Anopheles gambiae* and *An. coluzzii* were once considered as one species until recently. They remain as part of the *An. gambiae* species complex alongside *An. arabiensis*, hence are morphologically inseparable (Lehmann, et al.,

2014), while *An. funestus* belongs to the *Anopheles funestus* species complex group (Afrane et al., 2012; Coetzee et al., 2000, 2013).

These species inhabit diverse environments that include areas where the water that is required for larval development is absent for more than four months (Jawara et al., 2008; Lehmann et al., 2010; Omer et al., 1968; Yaro et al., 2012). *Anopheles arabiensis* lives in dry savannah environments but occupies similar larval habitats to *An. gambiae* (Tandina et al., 2018) thus, occurs in sympatry (Coetzee et al., 2000) with their relative abundance dependent on local ecological conditions (Hay et al., 2000a). It is said that "*An. gambiae* is predominantly anthropophagic and endophilic, and together with its longevity, has a higher vectorial capacity than other species of the *An. gambiae* complex" (Hay et al., 2000a).

The *An. gambiae* species complex is the major malaria vector characterized by endophagy (preferences for obtaining blood meals indoors), anthropophily (blood meals from humans), and endophily (indoor resting following blood meals) (Touré et al., 1994). Its distribution spans most of sub-Saharan Africa and can survive under a wide range of ecological, geographical, and seasonal conditions (Hay et al., 2000a). *Anopheles coluzzii* has high ecological plasticity; thus, it can exploit different habitats (Kamdem et al., 2012; Lehmann & Diabate, 2008) and has an opportunistic host-seeking behavior (Lefèvre et al., 2009a).

However, *An. arabiensis* is known for its ecophenotypic plasticity and is predominantly exophagic (feeds outdoors) and exophilic (rests outdoors) (Hay et al., 2000a). Because of its ability to develop in residual pools of water in dry riverbeds, it can survive arid conditions and in turn, rapidly become abundant at the onset of rains (Hay et al., 2000a).

The African continent accounts for the highest burden of malaria globally (WHO, 2023), partly because of how efficient and effective the *An. gambiae* complex species is as a

malaria vector (Cohuet et al., 2010; Coluzzi, 1999). Uganda, which is among the top five countries with the highest malaria transmission rates globally, also attributes the high number of malaria cases to the high occurrence of the *An. gambiae* complex (Tokarz & Novak, 2018). *An. gambiae* and *An. arabiensis* are the most efficient and broadly distributed vectors of *P. falciparum* malaria in sub-Saharan Africa (Coetzee et al., 2000).

2.2 Epidemiology of Malaria

2.2.1 Background

Malaria is a vector-borne parasitic infectious disease that is transmitted from person to person by a bite of a female *Anopheles* mosquito and is caused by a protozoan parasite of the genus *Plasmodium* (Ashley et al., 2018; Cox, 2010; Djihinto et al., 2022; Lempang et al., 2023; Nkumama et al., 2017). Malaria remains a serious public health problem among various human populations that live in different malaria-transmission settings in sub-Saharan Africa and is the main cause of early childhood mortality (Braack et al., 2015; Kweka et al., 2013; WHO, 2023).

2.2.2 Global Burden and Distribution of Malaria

In 2023, 263 million malaria cases and 597, 000 deaths were reported globally, compared to 249 million cases and 608000 deaths in 2022 and 244 million cases and 610000 deaths in 2021 (WHO, 2024). Between 2000 and 2015, substantial progress was made in reducing the global malaria burden (Fig. 2.3), and this was indicated by declining trends in both malaria incidence and mortality (Bhatt, et al., 2015; WHO, 2023, 2024). Following 2015, there was a slower rate of improvement in the subsequent years, with stagnation, which now highlights ongoing challenges in malaria control and elimination, emphasizing the need for renewed global efforts to deal with the emerging barriers and sustain the gains made over the years in reducing the disease burden (Bhatt, et al., 2015; Epstein et al., 2022).

Figure 2.3 Comparison of previous and current malaria incidence trends. Panel (a) shows that there was a gradual decline in malaria incidence from 79 cases per 1000 people at risk in 2000 to 60.4 in 2023, and this reduction was notable between 2000 and 2015, after which the rate of decline slowed, with little change in incidence from 2015 onwards (WHO, 2024). Similarly, in Panel (b), there was a consistent decline in malaria mortality, dropping from 28.5 deaths per 100,000 people at risk in 2000 to 13.7 in 2023, and the steepest reduction occurred between 2000 and 2015, with the pace of decline leveling off thereafter.

Sub-Saharan Africa accounts for about 94% of global malaria cases and deaths (Fig. 2.4), with children under five and pregnant women being the most vulnerable groups, indicating the disparate impact of this preventable and treatable disease (WHO, 2024). Some key

drivers of this high burden include the wide distribution of *P. falciparum*, the most lethal malaria parasite species, and the abundance of efficient mosquito vectors like *An. gambiae* (Brown et al., 2024; WHO, 2023, 2024). In addition, there are socioeconomic challenges in the way of substantial poverty, poor health infrastructures, and low coverage with proven preventive measures such as insecticide-treated nets and indoor residual spraying (Degarege et al., 2019; WHO, 2023).

Malaria reduces productivity, places substantial financial strain on households for prevention and treatment and hampers educational attainment due to illness-related absenteeism (Andrade et al., 2022; Malaria and the UN Sustainable Development Goals (SDGs) 2030, 2018; Smith, 2024). On a macroeconomic scale, malaria's impact is estimated to reduce GDP growth rates significantly, perpetuating cycles of poverty in affected nations (Datta & Reimer, 2013; Gallup & Sachs, 2000; Laxminarayan, 2003; Patouillard et al., 2023; Sarma et al., 2019).

Addressing malaria in sub-Saharan Africa therefore requires intensified investment in healthcare infrastructure, innovative vector control strategies, and community-level interventions to reduce the disease's impact sustainably (Okumu et al., 2022; WHO, 2023).

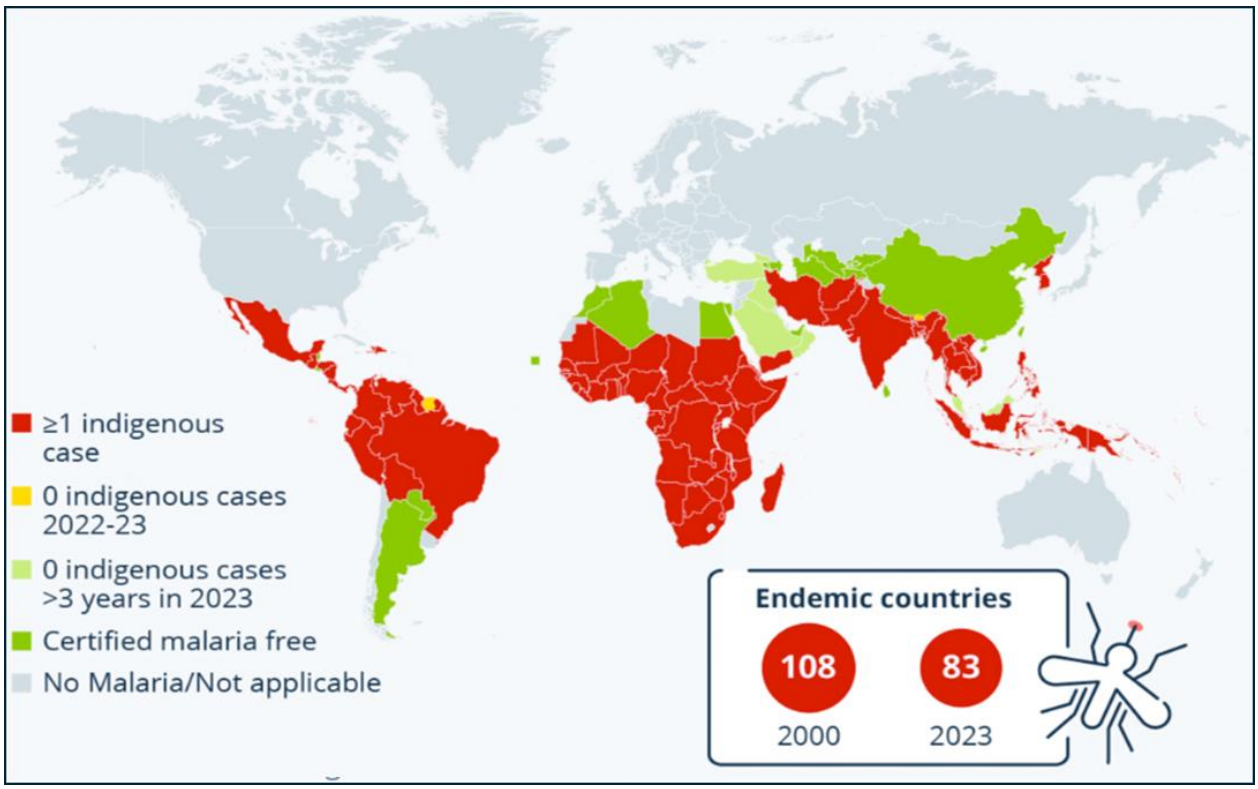

Figure 2.4 *The global map showing malaria distribution (WHO, 2024)*

2.2.3 The Burden of Malaria in Uganda

Uganda is one of the countries with the highest recorded malaria transmission intensities worldwide, and according to the 2024 World Malaria Report, about 12.6 million malaria cases were reported, putting the country among the top five affected globally (WHO, 2024). The country faces stable, recurrent malaria transmission (US. President's Malaria Initiative, 2019; WHO, 2024) and its epidemiology varies widely from the highlands with a low disease prevalence to the large areas with a lot of agricultural settlements where prevalence is high (Okello et al., 2006).

Malaria is the leading cause of death among children less than five years of age and the major cause of complications in pregnant women (Ministry of Health, 2011; Murphy et al., 2001). Other groups at risk of acquiring malaria include among others; HIV/AIDs patients, international travelers from non-endemic areas, and immigrants from endemic areas living in non-endemic regions but returning to their home countries to visit friends and relatives

(Angelo et al., 2017; Arinaitwe et al., 2020; Center for Disease Control, 1979; Lynch et al., 2015; Mischlinger et al., 2020; Okwa, 2012). The country still faces challenges in controlling malaria and this is attributed to very high malaria transmission intensity, inadequate health care resources, weak health care systems, poor understanding of malaria epidemiology and its impact on control approaches used, increasing drug resistance to parasites, increased vector resistance to insecticides, inappropriate case management, and inadequate preparedness and response to malaria epidemics (Atukunda et al., 2021; Kibira et al., 2021; Kiguba et al., 2021; Mpimbaza et al., 2022; Nuwa et al., 2022; Yeka et al., 2012).

Current malaria treatment is focused on a combination of drug therapies known as Artemisinin-based Combined Therapies (ACTs) which are the most effective first-line drugs and have a rapid therapeutic response to the disease (Conrad et al., 2023; Forstinus et al., 2015; Mpimbaza et al., 2022; WHO, 2023). This combination of drugs was introduced because of the increased resistance of malaria parasites to conventional drug therapies like chloroquine, sulfadoxine-pyrimethamine (SP) and amodiaquine (Asua et al., 2019; Byakika-Kibwika et al., 2017; Conrad et al., 2023; Ebong et al., 2021; Forstinus et al., 2015; Kamya et al., 2002; Nsobya et al., 2010; Yeka et al., 2016).

2.2.4 Malaria transmission dynamics

Given that there are different *Anopheles* species, their behavior can differ in terms of breeding or larval habitats, feeding preferences, and resting habits (Bloland & Williams, 2003; Foster & Walker, 2002; Service, 1993). These differences can affect malaria epidemiology, and the choice of malaria control strategy used, therefore, malaria control strategies are aimed at vector control (Bloland & Williams, 2003). Malaria transmission stops when the time required for infective sporozoites to develop exceeds the mosquito lifespan (Gilles, 1993).

Malaria transmission is mainly influenced by (1) vector species, which vary in behavior, distribution, and vectorial capacity; (2) the *Plasmodium* parasites that cause the disease (3) environmental factors, including transmission seasonality and climate change that have a great impact on mosquito breeding and survival; and (4) human host factors, such as agricultural practices, deforestation, and urbanization known for creating conducive habitats for mosquitoes to breed and disperse (Hemingway et al., 2016; Kweka et al., 2011; Rodríguez-Rodríguez et al., 2021; Sadoine et al., 2018). Malaria epidemiology varies widely across regions and is influenced by climate, individuals' immunity, and intervention measures (Leal Filho et al., 2023; Zhou et al., 2024). The populations that are disproportionately affected and at a higher risk of acquiring malaria include children under five years of age whose immune systems make them susceptible to severe malaria, pregnant women at risk of maternal anemia, low birth weight, and stillbirth, and travelers and migrants traveling to endemic areas (Bloland & Williams, 2003; Demsash et al., 2024; Mbishi et al., 2024; McKinney et al., 2020; Okoyo et al., 2021; WHO, 2024).

2.2.5 Control and Prevention Strategies

Malaria control majorly focuses on preventing and reducing disease transmission through vector control (using ITNs and IRS), chemoprevention using antimalarial drugs for intermittent preventive treatment in pregnancy and among children in high transmission areas, case management that involves prompt diagnosis and treatment with artemisinin-based combination therapies (ACTs) essential for reducing disease severity and preventing transmission, vaccination which is just being rolled out and environmental management to eliminate mosquito breeding sites (Killeen et al., 2017; Littmann et al., 2024; Musoke et al., 2023; WHO, 2024; Winskill et al., 2019).

Despite significant progress, several challenges, such as insecticide and drug resistance, health system weaknesses, and climate change, which alters transmission dynamics, hinder malaria elimination (Bhatt, et al., 2015; Haldar et al., 2017; Killeen et al., 2017; F. Okumu et al., 2022; The malERA Refresh Consultative Panel on Insecticide and Drug Resistance, 2017). Overall, a complex interplay of biological, environmental, and social factors shapes malaria epidemiology, and while significant progress has been made over the years in reducing the global burden, continued efforts and innovative approaches are required to overcome the existing challenges to move towards the ultimate goal of malaria elimination (Bhatt, et al., 2015; Jones et al., 2021; Killeen et al., 2017; Rabinovich et al., 2017; The malERA Consultative Group on Vector Control, 2011b; The malERA Refresh Consultative Panel on Combination Interventions and Modelling, 2017; WHO, 2024).

2.3 Malaria Vectors in Africa

Human malaria is transmitted by female mosquitoes that belong to the genus *Anopheles*, which consists of over 400 species in the world, with about 60 that are important malaria vectors (Beier et al., 1999; Sinka et al., 2012; WHO, 2023). However, species of the genus *Anopheles* could be important malaria vectors in one area and of little or no effect in another (Coluzzi, 1984; Sinka et al., 2010; WHO, 2023). In Africa, the main groups of malaria vectors are *An. gambiae*, *An. funestus*, *An. nili* and *An. moucheti* (Aboud et al., 2014a). Each of these groups comprises genetically distinct species that are similar in their morphology but differ in the traits responsible for malaria transmission (Harbach, 2004; Sinka et al., 2012). *Anopheles gambiae* and *Anopheles funestus* complexes are the most significant in transmitting malaria (Yeka et al., 2012). The *An. gambiae* complex is the most important of these groups and comprises eight closely related species that are spread throughout sub-Saharan Africa and its islands (Coetzee et al., 2013). In this complex, *An. gambiae s.s*, *An. coluzzii* and *An. arabiensis* are the most efficient and dominant vectors of

malaria (Coetzee et al., 2013), with a distribution that ranges from tropical Africa to the Sahel in West Africa (Afrane et al., 2012).

Anopheles mosquitoes are poikilotherms in nature, and their lives depend on ambient temperatures (Afrane et al., 2012; Beck-Johnson et al., 2013, 2017; Mordecai et al., 2019). Their bionomics vary according to sub-species and comprise of biting rates, duration of their gonotrophic cycles, fecundity, survival and development of immature mosquitoes and adult stages (Afrane et al., 2012; Agyekum et al., 2021; Zahar, 1983). *An. coluzzii* populations are said to persist throughout the dry season by aestivation, while *An. gambiae s.s.* and *An. arabiensis* disappear during the dry season and rebound a few weeks into the rainy season via migration (Adamou et al., 2011a; A. Dao et al., 2014; Lehmann et al., 2010), a sign that temperature and moisture play an important role in determining their density (Depinay et al., 2004a). *An. gambiae s.s* is predominant in the humid Savanna, while *An. coluzzii* in arid Savanna (Coulibaly et al., 2016). *Anopheles arabiensis* live in dry savannah environments but occupy larval habitats that are similar to those of the *An. gambiae s.s* (Tandina et al., 2018b), the reason they are said to occur in sympatry (Coetzee et al., 2000), with their relative abundance dependent on local ecological conditions (Hay et al., 2000). The *An. gambiae s.s* is predominantly anthropophagic and endophilic and, together with its great longevity, has a higher vectorial capacity than other species of the gambiae complex (Hay et al., 2000a).

Anopheles gambiae s.s. is the major malaria vector characterised by specific man-biting and indoor resting habits (Touré et al., 1994). Its distribution spans most of sub-Saharan Africa and can survive under various ecological, geographical, and seasonal conditions (Touré et al., 1994). *Anopheles arabiensis*, on the other hand, is known for its ecophenotypic plasticity and is predominantly exophagic (feeds outdoors) and exophilic

(rests outdoors) (Hay et al., 2000a). It is occasionally abundant in arid areas because of its ability to breed in residual pools of water in dry riverbeds and rapidly increase at the onset of rains (Hay et al., 2000a). *Anopheles coluzzii* has a high ecological plasticity, from which it can exploit different habitats (Kamdem et al., 2012; Lehmann & Diabate, 2008), and has an opportunistic host-seeking behavior (Lefèvre et al., 2009a).

2.4 Malaria vector control

Malaria vector control refers to measures against mosquitoes that limit their ability to transmit malaria (World Health Organization, 2021). The primary goal of malaria vector control is to reduce the vectorial capacity of local mosquito populations to a level that keeps the malaria reproduction rate (R_0) below 1, where R_0 represents the number of new human malaria cases generated by each existing case within the population (Smith et al., 2007; Smith & McKenzie, 2004).

Vectorial capacity, the ability of the malaria vector to transmit disease, is defined as the number of new malaria infections that the malaria mosquito population would induce per case per day at a given place and time, if the human population remains fully susceptible to malaria (World Health Organization, 2021).

The formula for vectorial capacity is; $C = \frac{ma^2P^n}{-log_eP}$ Where, C stands for vectorial capacity, ma stands for the number of bites per human per night by the available vectors, a; feeding frequency X human blood index, P; survival probability and n, the incubation period of the parasite in the mosquito (Gari & Lindtjørn, 2018). This formula shows that vector control measures that kill the vector or shorten its survival time significantly limit its ability to transmit malaria (Gari & Lindtjørn, 2018).

For malaria transmission to occur, *Plasmodium*, which is the causative agent of malaria, entirely depends on completing a complex cycle in the responsible vecto*r* (Ghosh et al., 2000). Therefore, efforts toward eliminating the vector or interfering with its ability to support the parasite cycle will stop malaria transmission (Wang & Jacobs-Lorena, 2013). Vector control is, therefore, the gold standard and at the heart of malaria control targeting indoor biting mosquitoes, especially in highly endemic countries due to the lack of reliable vaccines, drug resistance, and unaffordable antimalarials (Benelli, 2015; Chanda et al., 2017; Hemingway et al., 2016; Karunamoorthi, 2011b).

Vector control is significant in managing vector populations and, in turn, interrupting disease transmission through two main strategies: insecticide-treated mosquito nets (ITNs) and indoor residual spraying (IRS), known to be highly effective, resulting in significant declines in malaria morbidity and mortality, whether used singly or in combination (Bhattarai et al., 2007; Curtis & Mnzava, 2000; Enayati & Hemingway, 2010; Kleinschmidt et al., 2009; WHO, 2023). The IRS is a vector control tool that involves applying an insecticide to the walls of houses, killing mosquitoes that land and rest on them, while ITNs are defined as bed nets that are treated with insecticides to prevent mosquitoes from biting people (Karunamoorthi, 2011b; Pryce et al., 2022).

These tools have proven to be highly effective in controlling the malaria vectors that are adapted to biting and resting indoors (Bhatt, et al., 2015; Epstein et al., 2022; Patouillard et al., 2017; Scott & Takken, 2012). They have also been the central pillars of malaria control since the 2000s and have resulted in a decline in vector abundance, parity, and infection rates in *Anopheles* populations (Bhatt et al., 2015; O'Meara et al., 2010; Sougoufara et al., 2017). From 2000 to 2015, concerted malaria control efforts reduced global malaria mortality and incidence, with most of this progress attributed to the successful scale-up of

malaria vector control (Alonso & Noor, 2017; WHO, 2023). During this time, malaria control became part of the political agenda of several of the world's wealthiest countries, resulting in an effective flow of funds from various organizations whose investments scaled up global production, procurement, distribution, and use of ITNs and IRS (O'Meara et al., 2010).

Recent trends however, show that the number of malaria cases has consistently increased since 2016 and that the spread of insecticide resistance and change in vector behavior in key vector populations threatens the effectiveness of vector control approaches in use (Lobo et al., 2018; Ranson, 2017; Sokhna et al., 2013; Takken et al., 2024). This shift is attributed to insecticide resistance and behavioral changes in both *Anopheles* mosquitoes and human populations (Killeen, 2014; Killeen et al., 2016; Russell et al., 2013). The malaria vector has become more exophilic (preferring to rest and feed outdoors) rather than endophilic (indoor-feeding), and as a result, reducing malaria prevalence further has become more challenging (Debebe et al., 2018; Le Sueur et al., 1996; Oxborough, 2016; Sherrard-Smith et al., 2018; Sougoufara et al., 2017). Consequently, ITNs and IRS tools have proved ineffective in malaria transmission disruption when used in isolation, emphasizing the need for interventions targeting outdoor feeding or resting mosquitoes to be used in tandem with the already existing ones, to curb malaria transmission (Dambach, 2018; Lobo et al., 2018; Oxborough, 2016; Sougoufara et al., 2017; WHO, 2023).

2.5 Novel malaria vector control approaches

The fight against malaria is increasingly hampered by the evolution of resistance in both the parasite and vectors, and the partial efficacy of current control methods (Hemingway, 2014; Mushtaq et al., 2024; Plowe, 2022; Thu et al., 2017; WHO, 2023). It is, therefore, important that new and more durable methods of malaria vector control be developed, and

such novel approaches seek to reduce transmission either by directly targeting the mosquito vectors or by modifying their ability to transmit the disease (James & Santos, 2023; Mushtaq et al., 2024; USAID, 2022; WHO, 2023).

2.5.1 Biological Control with Predators

Biological control using natural predators like fish, for example, *Gambusia affinis* (mosquito fish), bacteria like *Bacillus thuringiensis israelensis*, or fungi like *Metarhizium anisopliae*, is one of the most promising eco-friendly approaches against malaria vectors, and has been tested for their capabilities to feed on mosquito larvae at their aquatic breeding sites (Benelli et al., 2016; Buxton et al., 2020; Eba et al., 2021; Gowelo et al., 2020; Jayapriya & Gricilda Shoba, 2014; Kahamba et al., 2022; Singh et al., 2023). These predators either consume mosquito larvae or produce biological agents that impede their development, thereby reducing mosquito populations (Benelli et al., 2016; Dalal et al., 2020; Gardner et al., 2018; Ong'Wen et al., 2020).

These fish predators are particularly mosquito fish, which have been used to effectively control mosquito larvae in ponds and rice paddies in several small-scale trials (Alomar & Alto, 2021; Azevedo-Santos et al., 2017; Louca et al., 2009; Polverino et al., 2019; Priyadarshana & Slade, 2023). Their efficiency rides on the fact that they can actively search for and consume larvae (Alomar & Alto, 2021; Azevedo-Santos et al., 2017; Louca et al., 2009; Polverino et al., 2019). Microbial control agents such as *Bacillus thuringiensis israelensis* (Bti) produce toxins specific only to the larvae stage of mosquitoes, which minimizes their impact on other aquatic organisms (Boyce et al., 2013; Empey et al., 2021; Melo et al., 2016, 2016; Priyadarshana & Slade, 2023), while fungi like *Metarhizium anisopliae* attack adult mosquitoes and reduce their capacity to survive and reproduce (Alves et al., 2002; Mnyone et al., 2011; Pereira et al., 2009; Vivekanandhan et al., 2020).

Even though these methods have not been widely adopted compared to chemical insecticides, they have become increasingly integrated into broader vector management strategies and boast the dual advantage of reducing reliance on chemical interventions to which mosquitoes can develop resistance, and also have minimal environmental impact when correctly applied (Arias-Castro et al., 2020; Benelli et al., 2016; Demirak & Canpolat, 2022; Rayalu et al., 2024). When put together under integrated vector management with conventional methods like insecticide-treated nets and habitat modification, biological controls ensure sustainable, effective control of mosquito-borne diseases (Jones et al., 2021; Mutero et al., 2012; Rayalu et al., 2024; Walker & Lynch, 2007).

However, there are challenges regarding scale-up, especially in ensuring their efficacy in diverse ecological settings and community acceptance (Oliver et al., 2021; Roiz et al., 2018; Zhang et al., 2024). Other means to enhance biological controls involve selective breeding for more efficient predator species, enhancing formulation and delivery of microbial agents, and integrating such methods into local and regional malaria control programs, such that, as the use of these methods expands, they hold a good prospect of lowering the burden of malaria (Hemingway, 2014; Jones et al., 2021; Kahamba et al., 2022).

2.5.2 Wolbachia

Wolbachia, an endosymbiotic bacterium, presents a novel, environmentally harmless method for controlling mosquito populations and mitigating the spread of vector-borne diseases (Gomes & Barillas-Mury, 2018; McGraw & O'Neill, 2013). When artificially introduced into these vectors, *Wolbachia* can manipulate their reproductive processes to suppress population growth and, consequently disease transmission, or can directly reduce vector competence by interfering with the vector's ability to support pathogen development (Gomes & Barillas-Mury, 2018; McGraw & O'Neill, 2013; Ross et al., 2020).

One of the main tactics used by Wolbachia involves introducing it into male mosquitoes. When these infected males mate with uninfected females, they induce a phenomenon called cytoplasmic incompatibility, which causes the females' eggs to be non-viable (sterile) (Walker et al., 2009; Zabalou et al., 2004; Zheng et al., 2011). This method has been efficient in reducing populations of *Aedes aegypti*, a major vector for Dengue and Zika viruses, and yet, if both males and females are infected with Wolbachia, maternal transmission of the bacteria ensures that it is maintained in the population and reduces the vectorial capacity of the mosquitoes to transmit pathogens such as the malaria parasite (Asad et al., 2018; Carrington et al., 2017; Ross et al., 2016; Ye et al., 2015).

While Wolbachia has primarily been applied to controlling *Aedes aegypti* and related species to reduce diseases such as dengue, Zika, and chikungunya, recent studies have focused on exploring the feasibility of adapting Wolbachia-based approaches for *Anopheles* mosquitoes in sub-Saharan Africa (Asad et al., 2018; Gomes & Barillas-Mury, 2018; Lim et al., 2024; McGraw & O'Neill, 2013). Although no field trials have yet been conducted, several laboratory studies and surveys have demonstrated natural Wolbachia infections in wild *Anopheles* populations and experimental trans infections under controlled conditions, indicating potential for future application despite the unique reproductive and ecological challenges posed by Anopheles compared to Aedes mosquitoes (Asad et al., 2018; Gomes & Barillas-Mury, 2018; Lim et al., 2024; McGraw & O'Neill, 2013).

Challenges such as (1) mass rearing and release, which include developing methods for mass-producing Wolbachia-infected mosquitoes and efficiently releasing them in affected areas, is resource-intensive; (2) the long-term sustainability of Wolbachia in wild populations which needs to be further researched, (3) broad-range studies that are required to ensure safety in various ecosystems, (4) modification of strategy to effectively spread

into natural populations of malaria vectors, given that it has widely been implemented in *Aedes* mosquitoes (Lim et al., 2024; Mancini et al., 2020; Pagendam et al., 2020; Pinto et al., 2021; Sawadogo et al., 2022; Werren, 1997).

2.5.3 Treated ovitraps and mosquito lures

Ovitraps are containers designed to attract and capture gravid mosquitoes by mimicking their natural breeding sites, something achieved by offering conducive conditions for egg-laying and capturing or killing the larvae or adults approaching the mosquito trap (Ahmad-Azri et al., 2019; Hapairai et al., 2021; Mackay et al., 2013; Talbalaghi et al., 2020; Wong et al., 2011). Their primary advantage is the ability to target mosquitoes during their reproductive stage, preventing new generations from hatching and establishing a population (Hapairai et al., 2021; Talbalaghi et al., 2020; Tsunoda et al., 2021).

Recent developments in ovitrap technologies have been geared towards making them effective through the inclusion of insecticide-treated surfaces; such insecticide-treated ovitraps kill mosquitoes by landing or other types of contact with the trap, particularly before developing into the adult stages from eggs and larvae (Hapairai et al., 2021; Juan et al., 2013; Mackay et al., 2013; Perich et al., 2003). Field trials have shown that these traps can reduce mosquito populations in a localized area, making them crucial for controlling mosquito-borne diseases (Dixon et al., 2024; Reza et al., 2016; Tsunoda et al., 2021). They are also very effective in managing mosquito populations if deployed tactically, especially in urban and semi-urban environments (Dixon et al., 2024; Reza et al., 2016; Tsunoda et al., 2021). To make ovitraps even more attractive, research has combined mosquito attractants, like fake human scents, into the traps to simulate the presence of a host, which addition improves the capability of the traps to attract mosquitoes from greater distances, especially species like *Anopheles* mosquitoes, known to be attracted to human odors

(Cribellier et al., 2020; Okumu et al., 2010; Pombi et al., 2014; Wondwosen et al., 2021). If more mosquitoes are attracted to these traps, the chances of capturing and killing a greater portion of the mosquito population in a locality are greater (Mackay et al., 2013; Velo et al., 2016).

Ovitraps have been shown in a few field studies to lower the mosquito populations around the area in which they were placed (Hapairai et al., 2021; Jaffal et al., 2023; Long et al., 2015; Velo et al., 2016). By combining them with other vector control methods, such as insecticide-treated nets or indoor residual sprays, ovitraps can become an important part of integrated vector management (IVM), which combines multiple control methods to decrease mosquito populations and the spread of vector-borne diseases in an ecologically sustainable and economically effective manner (Aguilar-Durán et al., 2024; Dixon et al., 2024; Jaffal et al., 2023). Besides, insecticide-treated ovitraps are a promising and more effective way of mosquito control in general, but more precisely in an area where other conventional methods of control might either be less efficient or have very limited impact (Aguilar-Durán et al., 2024; Jaffal et al., 2023; Mackay et al., 2013; Reza et al., 2016; Sharp et al., 2019).

2.5.4 Attractive Targeted Sugar Baits (ATSBs) and Toxic Sugar Baits (TSBs)

Attractive Targeted Sugar Baits exploit mosquito sugar-feeding behavior to deliver oral toxicants to both male and female *Anopheles*, providing a control pathway independent of blood feeding and indoor exposure (Gary et al., 2009; Traore et al., 2020; Yalla et al., 2023). By targeting outdoor-feeding and resting mosquitoes, ATSBs address residual transmission that persists despite widespread use of insecticide-treated nets and indoor residual spraying (Fraser et al., 2021; Müller, Beier, Traore, Mahamadou B. Toure, et al., 2010; Sougoufara et al., 2020b; Zembere, 2024). Formulations typically combine an attractant, a sugar base,

and an orally active insecticide, enabling population-level impacts and potential mitigation of contact-based insecticide resistance (Lukenge et al., 2023; Müller, Beier, Traore, Mahamadou B. Toure, et al., 2010; Muyaga et al., 2023; Traore et al., 2020; Yalla et al., 2023). Field trials in Africa have demonstrated substantial reductions in mosquito density and transmission indicators (Chanda et al., 2023; Traore et al., 2020; Yalla et al., 2023), although challenges remain regarding environmental durability, competition with natural sugar sources, and non-target effects (Fiorenzano et al., 2017; Kyomuhangi et al., 2024; Marshall et al., 2013; Reegan et al., 2025). Within Integrated Vector Management, ATSBs are best positioned as complementary tools that enhance existing interventions, particularly in settings characterized by outdoor transmission and behavioral avoidance (Muyaga et al., 2023; Njoroge et al., 2023; Sougoufara et al., 2020b; Zembere, 2024).

2.5.5 Sterile Insect Technique (SIT)

The SIT involves releasing large numbers of sterile male mosquitoes into the wild to compete with wild males for mates, and when they successfully mate with wild females, no viable offspring is produced, resulting in a decline of the overall mosquito population (Culbert et al., 2020; Maïga et al., 2014; Munhenga et al., 2016; Oliva et al., 2012; Zhang et al., 2020). These mosquitoes are sterilized using irradiation or chemical treatments to render them incapable of producing offspring (Bouyer, 2024; FAO/IAEA, 2020).

Recent developments have substantially enhanced the prospects of the SIT method in the control of mosquitoes, and with innovations in the mass-rearing of mosquitoes to enable the production of millions of males, while modern techniques of sterilization, like irradiation or genetic modification, make sure the sterility of males without jeopardizing their competitiveness, this solves the problems affecting large-scale releases due to logistical and technical issues (Bourtzis & Vreysen, 2021; Gentile et al., 2015).

The encouraging outcomes from various pilot projects undertaken with *Anopheles* species, the major vectors of malaria, have generated interest in expanding SIT for malaria control in endemic countries (Alphey et al., 2010; Culbert et al., 2020; Gentile et al., 2015; Klassen, 2009; Maïga et al., 2014). Trials, for instance, done under controlled conditions and in limited field environments have returned measurable population reductions of mosquitoes, which further confirms the potential role of SIT as a complementary approach to integrated vector management methods (Gentile et al., 2015; Klassen, 2009; Maïga et al., 2014; Sun et al., 2022).

However, challenges like ensuring that sterile males can compete effectively with wild males in diverse ecological settings, sustaining the release of sufficient numbers over time, and the acceptance of SIT by local communities remain (Bouyer, 2024; Dobson, 2021; Klassen, 2009). In this regard, SIT is one component of integrated vector management that offers a targeted and environmentally friendly approach, which, when linked with other forms of controls such as insecticide-treated nets and habitat modification, maximizes the impact (Beier et al., 2018; Boëte et al., 2021; Khamis et al., 2018; Townson, 2009). Scaling up SIT in endemic regions could contribute significantly to malaria transmission and reduce the burden caused by other mosquito-borne diseases (Alphey et al., 2010; Bouyer, 2024; Dobson, 2021; Gentile et al., 2015; Klassen, 2009; Zhang et al., 2024).

2.5.6 Genetically modified mosquitoes (GMMs)

Among the novel vector control approaches, GMMs are one of the most promising, and these are genetic manipulations that spread rapidly through wild mosquito populations (Burt et al., 2018; Carballar-Lejarazú et al., 2020; Hosack et al., 2021; Pham et al., 2019). Such manipulations may involve rendering mosquitoes resistant to *Plasmodium* parasites, sterile, or biased in their inheritance to reduce populations of the insects (Bier, 2021;

Champer et al., 2017; Gantz et al., 2015a; Pham et al., 2019). Promising results have been obtained in recent field trials, while several gene-editing technologies, CRISPR-Cas9 among them, are being tested to interfere with mosquito reproduction or survival (Carballar-Lejarazú et al., 2020; Gantz et al., 2015; Pham et al., 2019). Although these technologies are promising, ecological impacts and ethical considerations remain key points to be considered before they are finally rolled out to be used on a large scale (Kormos et al., 2022; Kuzma, 2022; Pham et al., 2019).

2.6 Genetic modifications in malaria control

Advances in molecular biology through gene editing have significantly advanced malaria vector control by either prompting large-scale population replacement to reduce the inherent ability of individual mosquitoes to transmit the malaria pathogen or suppress the overall mosquito population (Alphey, 2014; Burt, 2014; Champer et al., 2016; Godfray et al., 2017). The altered traits generally reduce evolutionary fitness, and because they cannot spread over many generations, they are eliminated through selective pressures towards the loss of transgenes (Esvelt et al., 2014; Irvin et al., 2004; Lambrechts et al., 2008; Marrelli et al., 2006; Oye et al., 2014). These traits, therefore, require a mechanism to spread the desirable trait through the natural vector population at a rate greater than the Mendelian rate, regardless of whether they confer a fitness cost (Burt, 2003; Selvaraj et al., 2020; Wang et al., 2021). This mechanism is known as gene drive and is defined as selfish genetic elements that show super-Mendelian patterns of inheritance (Bier, 2021b; Burt, 2003a; National Academies of Sciences, Engineering, 2016), and its success is crucial for fixing transgenes into wild malaria mosquito populations (Garrood et al., 2022).

Selfish genetic elements are generally defined as genetic segments that can boost their transmission at the expense of other genes in the genome despite their negative effect on

organismal fitness (Burt & Trivers, 2006; Hurst & Werren, 2001; Mclaughlin & Malik, 2017; Werren et al., 1988). They use three main strategies to be disproportionately transmitted through several generations; namely, interference, through which they get ahead by disrupting the transmission of the alternative allele, over replication, in which they bias their transmission to the next generation by getting themselves replicated more than other genes in the same organisms and gonotaxis in which they move preferentially toward the germline and away from somatic cells whenever they can (Burt & Trivers, 2006).

Naturally occurring selfish genetic elements have for long been known to increase the likelihood of being inherited, which advantage allows them to spread through populations regardless of whether they reduce the fitness of an individual organism and can consequently be the basis for the spread of gene drives' ability to spread engineered traits through wild populations (Alphey, 2014; Burt & Trivers, 2006; Sinkins & Gould, 2006).

The types of gene drives include natural, endonuclease, and engineered gene drives (Esvelt et al., 2014). The ability for a standard gene drive to spread through a target population depends on homing efficiency, fitness cost, evolutionary stability, mating dynamics, generation time, and other characteristics specific to the target population (Esvelt et al., 2014; Marshall & Hay, 2012). Their ability to self-maintain and have an impact even beyond the release site makes gene drive systems appealing and able to provide the ultimate way to achieve the transgenic frequencies required for disease control without prohibitive release sizes (Marshall & Hay, 2012). Given that gene drive systems can cross borders even from isolated islands, there is a need to develop sensitive methods of monitoring population genetics, naturally self-exhausting drives, and strategies to respond to self-sustaining drives to remove all engineered genes from wild populations (Noble et al., 2018).

Selfish genetic elements known to yield effective gene drive systems are those that involve transposable elements (TEs), meiotic drive, homing endonucleases, and engineered underdominance and Wolbachia endosymbionts (Sinkins & Gould, 2006). Whether a gene drive spreads through a target population depends on molecular factors like homing efficiency, fitness cost, and evolutionary stability (Marshall & Hay, 2012), given that the rate of spread of gene drives majorly depends on the mating dynamics, generation time and other characteristics of the target population (Alphey et al., 2020; Beaghton et al., 2019; Dhole et al., 2024; Eckhoff et al., 2017; Marshall & Hay, 2012; North et al., 2020).

2.6.1 Gene drive systems

Gene drives are generally defined as selfish elements that are abundant in nature and are transmitted to offsprings at super-mendelian frequencies (>50%) (Burt, 2003a; Burt & Trivers, 2006; Dawkins, 1976). Gene drives are broadly classified into high-threshold and low-threshold drives depending on how easily they spread through a population (Curtis, 1968), with the former involving more than the number of native individuals, while the latter requires very low numbers to take over the population, given that they persist in the environment (Bier, 2021). High-threshold gene drive systems are particularly attractive for population replacement strategies because to achieve their objective, they require the modification to persist at high levels provided malaria continues being transmitted (James et al., 2018).

Low-threshold gene drive systems are deployed using two main strategies: population suppression and modification/replacement (Bier, 2021). Suppression drives force deleterious traits into a population, resulting in the population crashing or getting diminished (Bier, 2021). Modification drives, on the other hand, involve altering the vector to prevent it from transmitting the pathogen in consideration, thus, the vector continues

surviving, but disease transmission is blocked (Collins et al., 2019; James, 2005). Modification drives carry negligible fitness costs and, when released into the wild populations, are said to follow a simple logistic growth trajectory (Gantz & Bier, 2016). They maintain stability in a population for a long time while achieving local elimination of the pathogen and in the long run from neighboring or more distant regions to achieve continent-scale elimination of the parasite (Bier, 2021). This is what gives them an advantage over suppression drives, meaning that the latter may require repeated releases in the same location, thus impacting on cost and logistical barriers if multiple regions are to be considered (Eckhoff et al., 2016; North et al., 2019, 2020).

Gene drive systems consist of two approaches: self-limiting and self-sustaining systems (James & Santos, 2023). Self-limiting gene drive systems increase the frequency of the modification within the target population over some time after which the frequency declines, while self-sustaining gene drives such as those based on a homing mechanism pass the modification on at increasing frequency through successive generations such that it is maintained at a high frequency within the target population, and sometimes reaching fixation (Alphey et al., 2020; James & Santos, 2023; WHO, 2021). With one release, a desired trait can spread into all populations of the same species, something beneficial to malaria vector control (Noble et al., 2018).

Self-limiting strategies for vector control include approaches such as the sterile insect technique (SIT), which involves the mass release of sterilised mosquitoes to suppress local populations (Alphey, 2014; Burt, 2003; Gabrieli et al., 2014; Gould & Schliekelman, 2004; Knipling, 1959). While SIT does not employ gene drives, it shares the feature of being self-limiting, requiring repeated releases, particularly in areas with high vector densities. In contrast, self-sustaining gene drive systems can spread a trait through a population with

fewer releases, offering ease of production and durability of effect (James & Santos, 2023). However, their persistence in the environment raises ecological and regulatory concerns (James & Santos, 2023). It has therefore been suggested that intermediate self-limiting gene drives be tested first, to gain operational experience and inform risk assessment before deploying self-sustaining drives (James et al., 2018; WHO, 2021).

Population Suppression aims to reduce the size or reproductive capacity of mosquito populations to limit their ability to transmit malaria and uses techniques like CRISPR-Cas9 to create "self-limiting" drives that reduce mosquito fertility, preventing populations from sustaining high densities over time (James et al., 2018). (Alphey, 2014; Alphey et al., 2020; Garrood et al., 2022; James & Santos, 2023). It is based on inactivation or knockout of the genes involved in the target mosquito's survival or reproduction such as reducing fertility or biasing the sex ratio towards more males (James et al., 2018). Population Replacement, on the other hand, focuses on introducing modified genes into the mosquito population that makes them incapable of transmitting the malaria parasite without necessarily reducing population numbers (Edgington & Alphey, 2022; James & Santos, 2023; Leung et al., 2022; Marshall & Akbari, 2016), for example, inactivation of the gene(s) that facilitate parasite survival in the vector or that are required for the mosquito to transmit malaria (James et al., 2018). These "self-sustaining" drives ensure that the modified trait spreads through the population, ideally replacing susceptible individuals with genetically modified, disease-resistant ones (Champer et al., 2018; Noble et al., 2017, 2019).

Effective large-scale population replacement and suppression strategies require that the developed gene drive systems biologically spread crucial genes within populations, which can be done using several categories of naturally occurring selfish genetic elements that

show non-mendelian inheritance regardless of whether or not they provide benefit to the host organism (Ågren & Clark, 2018; Dawkins, 1976; Hurst & Werren, 2001).

The candidate gene drive systems will be evaluated in relation to the criteria of their potential for success, such as whether the drive mechanism is powerful enough to spread effector genes to fixation on a timescale that is relevant for ensuring disease control (Boë & Koella, 2003.; Hilbeck, 2001; James, 2005), whether the system is as resistant as possible to the potential loss of linkage between the drive mechanism and the effector gene to be driven and whether the gene drive system can spread new or modified effector genes to counteract loss of linkage, mutational inactivation of the drive system or evasion by the pathogen (Sinkins & Gould, 2006).

The likelihood of a pathogen or vector becoming resistant to a gene drive system could be significantly reduced by involving multiple independently acting effector genes in spreading simultaneously (Sinkins & Gould, 2006). This means that the ideal gene drive system should have the ability to spread large multi-gene constructs and at the same time function in several vector species, given that many tropical diseases have multiple vectors (Sinkins & Gould, 2006), and be as safe as possible with no significant risk of causing undesirable side effects in the target vector or cause ecological damage in the non-target species (Moreira et al., 2004). The type of gene to be spread is therefore equally important, given that transgenes that substantially decrease the engineered mosquito's fitness would be extremely difficult to spread through populations even with a powerful drive system (Sinkins & Gould, 2006).

For applications where confinement to the target area is preferred (population replacement), the desired trait can only spread to the target wild-type population when the gene drive element is introduced above a particular frequency, given that migration into

geographically non-target populations hardly occurs and is below the drive threshold (Altrock et al., 2010, 2011; Davis et al., 2001; Marshall & Hay, 2012). These threshold-dependant gene drive systems could be considered more controllable, but could also be temporarily Iimited, eventually declining in number and getting eliminated from the population due to fitness effects (Garrood et al., 2022; Noble et al., 2018). Such systems are useful for understanding the spread dynamics of gene drive systems during early testing phases, given that they are theoretically easily reversible (Garrood et al., 2022). If developed for malaria mosquitoes, threshold-dependent gene drives would, however, require longer-term repeated releases whenever they got below the drive threshold, resulting in increased costs due to the need to do mass production and releases (Garrood et al., 2022). The success of gene-drive mosquitoes will depend on their ability to have a multigenerational effect, which relies on measures of competitiveness, longevity, and the duration of the population effect to understand the scale of releases needed to achieve the intended outcome (Garrood et al., 2022).

2.6.2 Invasive gene drives

Gene drive technology, which enables the wide genetic modification of target populations, offers both great promise and complexity for malaria vector control. A key consideration when releasing these gene drives is their potential invasiveness the possibility that they could spread beyond the intended mosquito population into neighboring territories and even across international borders (Eckhoff et al., 2017; Greenbaum et al., 2021; Hosack et al., 2021; Long et al., 2020; Noble et al., 2019) This is what discussions of *"invasive gene drives"* typically refer to: gene drives designed to spread widely and at little fitness cost, allowing them to move unimpeded by the geographical and ecological barriers that would normally limit other forms of genetic modification (Noble et al., 2016).

Because the goal of gene drive technology is to introduce heritable factors into a population, any release must be carefully limited not only in space and time but also to specific target populations, such as Anopheles gambiae and closely related mosquito species in malaria-endemic areas (Burt, 2014; Burt et al., 2018; Eckhoff et al., 2017; Nolan, 2021). However, the free-flying nature of these mosquitoes and their overlapping habitat ranges could allow gene drive elements to spread unintentionally from the targeted to non-targeted areas, possibly across international borders, affecting mosquito populations in neighboring nations. For instance, releasing gene drive mosquitoes that are sterile or have reduced vector competence in one region might eventually alter populations beyond where they were first deployed, raising ecological and ethical concerns by potentially affecting local ecosystems and disease dynamics outside the jurisdiction of the releasing country (Eckhoff et al. 2017).

Importantly, gene drives are not all designed to be invasive. Several newer strategies aim to create localized or self-limiting gene drives**,** which would be less likely to spread uncontrollably. These include threshold-dependent systems, daisy-chain drives, and split drives, which are designed to lose their drive capability over generations or require a certain frequency to persist (Burt & Deredec, 2018; Greenbaum et al., 2021; Hammond, et al., 2021; Metzloff et al., 2022; Noble et al., 2019; Zapletal et al., 2021), Such non-invasive designs could help balance effectiveness with safety, reducing cross-border risks. Invasive gene drives, by contrast, include systems such as transposable elements (TEs), homing endonuclease genes (HEGs), and Wolbachia even though the key question is whether it will be possible to achieve this balancing act: creating gene drives that are effective but not too invasive, so that the benefits for malaria control can be enhanced without spilling over into areas where such changes have been rejected (Eckhoff et al., 2017; Long et al., 2020; North et al., 2019, 2020; Willis & Burt, 2021). Invasive gene drives include and are not

limited to, TEs, HEGs and *Wolbachia* (Marshall & Hay, 2012). The TEs spread into the neighboring populations whenever they are capable, HEGs, one of the most invasive gene drive systems, can be confined/localised if they are associated with a large dominant fitness cost, and *Wolbachia* could be confined for high fitness costs and/or low maternal transmission rates, however, it is predicted to spread widely (Marshall & Hay, 2012)

2.6.3 Driving elements

Many naturally occurring driving elements are essential to developing new genetic tools for controlling malaria vectors and can be used as building blocks to create synthetic gene drives. Among these are homing endonuclease genes (HEGs), transcription activator-like effector nucleases (TALENs), Zinc Finger nucleases (ZFNs), and clustered regularly interspersed palindromic repeats (CRISPR). Gene drive systems generally comprise of maternal effect dominant embryonic arrest system (*MEDEA*), homing-based drives that use homing endonuclease genes (HEGs), underdominance or heterozygote inferiority drives, sex-linked meiotic drives and heritable microorganisms (Mudziwapasi et al., 2021).

2.6.3.1 Maternal Effect Dominant Embryonic Arrest (MEDEA)

The MEDEA was originally discovered in the flour beetle '*Tribolium castaneum*' and works through a form of segregation distortion that favours the inheritance of specific genetic elements (Lorenzen et al., 2008). The MEDEA loci encode two components, namely, a maternal product that is lethal to developing embryos and a zygotic product that rescues only those embryos that inherit the MEDEA element (Macias et al., 2017). Only the progeny carrying the MEDEA allele survive to adulthood, and as a result, ensuring its propagation in the population (Grossniklaus et al., 1998; Ward et al., 2011).

The MEDEA provides a foundation for population replacement strategies, and its functionality relies on the interplay between maternal and zygotic gene products, which

can be tailored for use in vector control programs (Legros et al., 2013). The MEDEA-based systems offer a non-Mendelian inheritance mechanism capable of driving desirable traits, such as pathogen resistance or infertility, through wild mosquito populations, making them a promising strategy for reducing mosquito-borne disease burdens (Macias et al., 2017).

The MEDEA systems require careful design to balance efficacy and minimize ecological risks and ensuring stability and avoiding unintended consequences in target populations remain critical challenges (Buchman et al., 2018; Champer et al., 2016; Raban et al., 2020). The MEDEA drive systems are generally weaker than those based on homing or driving sex chromosomes, and the offspring of heterozygous females die if they do not inherit the MEDEA element (Burt et al., 2018; Mudziwapasi et al., 2021).

2.6.3.2 Zinc-finger nucleases (ZFNs)

The ZFNs are a type of synthetic protein used for precise genome editing (Bonawitz et al., 2019; Geurts et al., 2009; Meyer et al., 2010), and consist of two main components: (1) Zinc-finger DNA-binding domains that recognize and bind to specific sequences of DNA, in which each zinc-finger can be engineered to target a unique three-base pair DNA sequence, and multiple zinc-fingers can be linked together to recognize longer sequences, and (2) FokI cleavage domain, a DNA-cleaving enzyme derived from a bacterial restriction enzyme, which requires pairing to cut DNA (meaning two FokI domains must come together to make a double-strand break) (Santiago et al., 2008; Zeevi et al., 2008).

In practice, ZFNs involve two sets of zinc-finger proteins that are engineered with each binding to opposite sides of a target DNA sequence, a FokI cleavage domain that is attached to each zinc-finger, which then binds to their specific DNA sequence to create a double-strand break at the targeted location, which can be harnessed to introduce specific genetic modifications (Moehle et al., 2007; Santiago et al., 2008; Zeevi et al., 2008). The ZFNs

have high specificity and can be designed to target specific genes with minimal off-target effects that enable diverse genetic modifications and are applied in various forms of genetic engineering, like gene knockouts, gene insertions, and gene corrections (Geurts et al., 2009; Lloyd et al., 2005; Meyer et al., 2010; Moehle et al., 2007; Osakabe et al., 2010; Santiago et al., 2008).

Regarding malaria vector control, ZFNs can be applied in sterility induction to disrupt genes critical for mosquito reproduction, gene drive systems that either suppress mosquito populations or render them incapable of transmitting malaria, and in parasite resistance in which they are employed to insert or modify genes in mosquitoes to enhance their resistance to malaria parasites by reducing the overall transmission rate (Kim et al., 1996; Macias et al., 2017).

The ZFNs require expertise and significant time to design and optimize; they are expensive to develop and validate and have off-target effects that could lead to undesired genetic changes (Yee, 2016; Zettler et al., 2019). These technical and economic challenges have resulted in the limited use of ZFNs in malaria control, and thus, recent efforts in genome editing of malaria mosquitoes have shifted towards the use of CRISPR-Cas9, which offers greater ease of use and efficiency (Gantz et al., 2015; Li et al., 2017). However, they remain a valuable alternative for specific applications where their unique properties may be advantageous because, with continued advancements in genome editing and delivery systems, they could still play a role in integrated strategies for malaria mosquito control (Gantz et al., 2015; Li et al., 2017).

2.6.3.3 Transcription Activator-Like Effector Nucleases (TALENs)

The TALENs consist of engineered proteins that recognize specific DNA sequences through modular repeat domains and induce site-specific DNA cleavage (Deng et al., 2012;

Lei et al., 2012). They were among the early tools for precise genome editing in mosquitoes (Macias et al., 2017) and consist of a DNA-binding domain made of repeats that target specific sequences and a FokI nuclease domain that cuts DNA (Deng et al., 2012; Sun & Zhao, 2013).

The TALENs introduced a new level of specificity in genome editing and improved older transposon-based systems (Lei et al., 2012), they were also more accessible to researchers compared to earlier methods like zinc-finger nucleases (ZFNs), with modularity simplifying in-laboratory construction (Lei et al., 2012; Macias et al., 2017).

The TALENs were used to edit mosquito genomes by targeting genes related to vector competence and population suppression and played a critical role in advancing genetic tools for studying mosquito biology and developing control strategies (Basu et al., 2015; Smidler et al., 2013). The challenge of using TALENs is that their cloning process involves assembling long repeat sequences, and that they were relatively complex to design, less effective, and efficient in gene editing when compared to the CRISPR/Cas9 system (Pu et al., 2015; Reyon et al., 2012). The TALENs demonstrated the potential of targeted gene disruption for mosquito population modification or suppression, and their use laid the groundwork for the rapid adoption of CRISPR-based systems in vector biology (M. Li et al., 2017; Macias et al., 2017).

2.6.3.4 Transposable Elements (TEs)

The TEs are defined as selfish genetic elements that can replicate and transpose within the genome, resulting in their spread into a population from low initial frequencies (Charlesworth et al., 1994). The TEs will spread into a population given that its transposition rate overcompensates for its associated fitness cost and will reach an equilibrium copy number in the population due to several mechanisms that slow down the

rate of transposition with increasing copy number (Townsend & Hartl, 2000). The TEs are linked to a genetic payload known to increase the frequency of the TE and genetic payload in the genome of the target organisms and ultimately the whole population (Mudziwapasi et al., 2021). On the downside, TEs are unpredictable, often have transposition rates that are too low to be used, control over their integration sites is too low, and are difficult to mobilize after integration (Mudziwapasi et al., 2021; Tu & Li, 2013).

2.6.3.5 Sex-linked meiotic drives

Meiotic drive mechanisms distort the normal segregation of sex chromosomes during meiosis by involving genes that suppress or alter the function of the counterpart chromosome (Meiklejohn, 2016; Tao et al., 2007). Sex-linked meiotic drives can skew mosquito populations toward a specific sex, typically males, that do not transmit diseases, thereby reducing female populations and directly limiting the mosquitoes' ability to spread pathogens like malaria (Macias et al., 2017; Meade et al., 2020).

Meiotic drives can bias sex ratios non-Mendelianly (Hamilton, 1967; Knipling et al., 1968), are highly effective at rapidly altering population dynamics, do not require repeated releases of modified mosquitoes, and can be integrated with other genetic strategies for enhanced vector control (Macias et al., 2017). Resistance may, however, evolve if natural selection favors suppression of the drive mechanism, which calls for the need for a detailed understanding of mosquito genetics and careful deployment to prevent unintended ecological impacts (Beaghton et al., 2019; Hammond, et al., 2021; Meade et al., 2020; Price et al., 2020).

Sex-linked meiotic drives have a low resistance allele generation rate, can be reversed and may not be removable with the wild type (Champer et al., 2016). However, they could be inactivated by a suppressor using a second-generation drive reversal element (Mudziwapasi

et al., 2021). Given that they have a moderate rate of spread, they could result in species extinction, thus rendering them suitable for suppressing or eliminating the malaria mosquito species (Mudziwapasi et al., 2021).

2.6.3.6 Homing Endonuclease Genes (HEGs)

Homing is defined as a simple mechanism of achieving drive and can home into a critical gene whose interruption results in the suppression of a population as the gene drive spreads (Mudziwapasi et al., 2021). Homing endonucleases are, therefore, a class of selfish genetic elements that induce double-strand breaks in DNA at specific recognition sites to activate the cell's recombination repair mechanism using the homologous chromosome that carries the homing endonuclease gene (HEG) as a template, resulting in the spread of the heritable HEG throughout the population (Garrood et al., 2022; Goddard et al., 2001). The simplest use for homing is to produce a population-wide gene knockout, given that if the knockout phenotype is recessive and if the homing reaction is confined to the germline, the homing endonuclease that causes lethality or sterility could increase in frequency in the target population, resulting in population suppression (Austin Burt et al., 2018; Mudziwapasi et al., 2021).

The HEGs are spread by expressing an endonuclease that creates a double-stranded break on versions of the homologous chromosome that lack the HEG at the position where it occurs (Marshall & Hay, 2012). Homologous DNA repair is then said to copy the HEG to the cut chromosome increasing its representation in the subsequent generations (Marshall & Hay, 2012). A HEG will spread provided its homing rate overcompensates for its fitness cost; nevertheless, there is a range of parameter space for which the HEG will display threshold behavior, becoming either fixed or lost depending on its initial frequency (Marshall & Hay, 2012).

The HEGs are said to have provided the first practical tools for building and testing synthetic gene-drive systems in strains of Drosophila flies and *Anopheles* mosquitoes, which are designed to carry a HEG recognition site (Chan et al., 2013; Rong & Golic, 2003; Windbichler, et al., 2011). They are an example of low-threshold selfish genetic elements found in microorganisms and are said to encode highly sequence-specific endonucleases known to cut a naïve homologous chromosome at the site where they are inserted into the genome and copied into the DNA breaks, they create (Bier, 2021).

To use HEGs in natural mosquito populations requires that their specificity is re-engineered towards native mosquito sequences to disrupt the genes essential for vectorial capacity and viability or introduce anti-pathogenic genes at selected loci. However, this has proven challenging, given that modifying sequences to confer binding specificity could compromise endonuclease efficiency (Chan et al., 2013; Garrood et al., 2022; Thyme et al., 2014). Thus, several gene editing technologies have replaced native HEGs with more easily programmable nucleases (Garrood et al., 2022). For example, programmable nucleases like Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), known for targeted genome editing are used (Carroll, 2014).

2.6.3.7 The CRISPR/Cas9 system

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated proteins (Cas) (CRISPR/Cas or CRISPR) are the most used today, have revolutionalised the process of genome engineering and work by altering regions of DNA (Chen et al., 2020; Dance, 2015; Robb, 2019; Zhang et al., 2014). Using a gene drive, it is possible to alter an individual mosquito gene and insert into its genome the CRISPR system that involves the gRNA and Cas protein, thus allowing the modified gene to self-replicate in subsequent generations (Hammond & Galizi, 2017; Mudziwapasi et al., 2021).

Discovering the CRISPR/Cas9 system has transformed the field of gene drive systems and has been adopted by the scientific community, replacing nearly all other genetic editing tools that were initially used (Garrood et al., 2022).

Also, the readily programmable guide RNA that binds Cas9 and directs DNA cleavage to desired sites (Jinek et al., 2012) has resulted in the rapid development of a variety of gene drive systems in insects (Gantz et al., 2015; Gantz & Bier, 2015; Hammond, et al., 2016; Kyrou et al., 2018; Li et al., 2020), mammals (Grunwald et al., 2019), yeast (DiCarlo et al., 2015) and bacteria (Valderrama et al., 2019). Besides, its simplicity and ease of engineering set it apart from earlier gene drive technologies (HEGs, SFN, or TALEN nucleases) (Garrood et al., 2022). The CRISPR technique allows gene editing with precision, speed, and economy, and has the potential to ensure that the alterations made in the wild-type mosquitoes persist in nature (Mudziwapasi et al., 2021).

Although CRISPR-based gene drives are central to developing effective drive systems aimed at reducing wild-type malaria mosquito populations, they are not entirely immune to the emergence of resistance (Champer et al., 2016; Garrood et al., 2022; Hammond et al., 2017; Unckless et al., 2017). In fact, while they are designed to minimize the formation of resistant alleles by targeting highly conserved genomic regions, resistance can still evolve through mechanisms such as non-homologous end joining or selection for naturally occurring resistant variants (Garrood et al., 2022). Ongoing research is therefore focused on optimizing target site selection and multiplexing guide RNAs to further suppress or delay the development of resistance (Garrood et al., 2022).

2.7. Population genetics of malaria vectors

Population genetics is generally defined as the study of processes that influence gene and genotype frequencies (Ewens, 2006). This field focuses on understanding how evolutionary

factors such as mutation, genetic drift, migration, and natural selection modify these frequencies over time (Buffalo & Coop, 2020; Corbett-Detig et al., 2015). These dynamics help explain patterns of genetic diversity and adaptation in natural populations.

The population genetics theory is related to the extent of genetic variation between individuals in nature, the factors responsible for this variation, and is concerned with retrospective behavior that uses the present state to answer questions about the evolution to this state (Charlesworth, 2015; Hunter & Slate, 2019; Wright, 1951). Studying the population genetics of malaria vectors is important in predicting how genes spread and reveals new insights into disease transmission (J. M. Donnelly et al., 2002).

Genetic diversity is said to be lower in islands compared to mainlands because, in mainlands, migration between related populations is more frequent and allows the exchange of inheritable information, thus enhancing genetic diversity (therefore their limited dispersal ability is responsible for low genetic diversity) (Frankham, 1997). Genetic variation is said to be the raw material for evolution, given that it allows populations to evolve in response to environmental change regardless of whether it is changed by diseases, parasites, predators, and competitors, among others (Frankham, 1997). Waiting times for new favorable mutations are high unless population sizes are very large (Frankham, 1997). Natural selection influences the loss of genetic variation, given that selection for a favorable allele increases the rate of loss, whereas heterozygote advantage may slow it down (Frankham, 1997). The four genetic factors that contribute to the higher mosquito extinction rates of islands compared to mainland populations are inbreeding depression, loss of genetic variation, accumulation of mildly deleterious mutations, and genetic adaptations to island environments (Frankham, 1997).

Proof-of-concept mosquito transformation studies done in laboratories show promising results and as a result, candidate gene drive systems have been identified (Galizi et al., 2014; Gantz & Bier, 2015; Hammond et al., 2016). However, before these drive systems are rolled out, they must undergo field trials (Burt et al., 2018; Lanzaro, et al., 2021; Nolan, 2021). Therefore, geographically isolated sites are under consideration for the initial field trials as a key standard for field site selection(Lanzaro, et al., 2021; Wiltshire et al., 2018).

Malaria elimination strategies and interventions greatly depend on vector control methods (WHO, 2023); however, modeling studies show that the conventional methods are insufficient (Griffin et al., 2010; Walker et al., 2016), thus calling for urgent reinforcement using new techniques (Gantz et al., 2015; Hammond et al., 2016; Macias et al., 2020). A promising approach is the use of gene drive systems based on natural or artificial homing endonucleases to suppress malaria vector populations or to alter their capacity to transmit malaria by rapidly spreading through populations despite their reduced individual fitness (Alphey, 2014; Burt, 2014; Champer et al., 2016; Godfray et al., 2017).

Before a particular drive system is justified and approved for field testing, modeling is paramount in determining the number of generations over which stability, efficiency, and safety characteristics must be monitored in the laboratory (James et al., 2018). Modeling thus guides the specification of required properties of the construct, such as the homing rate, effector strength, and frequency of development of resistance, among others (Eckhoff et al., 2017; Lambert et al., 2018; North et al., 2020). Mathematical modeling plays an important role at each step of the testing pathway for gene drive mosquitoes, and its validity is influenced by the dataset it uses and its relevance to the conditions at the field trial sites, thus the need to collect relevant baseline field data (North et al., 2019, 2020; Selvaraj et al., 2020).

Several modeling studies indicate that female fertility traits are an important target for gene drive systems that result in population suppression (Beaghton et al., 2019; Deredec et al., 2008; North et al., 2013; North et al., 2020). Spatial and non-models are essential for rapidly assessing the differences among gene drive scenarios and in designing deployment strategies (North et al., 2020; Sánchez et al., 2020; Winskill et al., 2014).

Mathematical modeling is also significant in predicting how the different gene drive constructs will function given realistic values for fitness costs of the effector transgene and target vector species' population structure (Sinkins & Gould, 2006). It is also essential for predicting the utility of different drive systems, given that realistic values for the fitness costs of the effector transgene and the vector population structure are used (Sinkins & Gould, 2006). For example, modeling of a self-sustaining gene drive that aimed at suppressing the target population showed a significant reduction in adult mosquitoes in the rainy season even though it did not always show total elimination, something that could result from seasonal effects that have an impact on the wild-type malaria vector populations or the inadequate success of the desired trait (Eckhoff et al., 2017).

A crucial but under-explored aspect in these models is the dry season survival of malaria mosquitoes, which involves aestivation, a dormancy strategy during dry periods, and long-distance migration, vital for sustaining mosquito genetic diversity (Adamou et al., 2011; Krajacich et al., 2020; Lehmann et al., 2017; Magombedze et al., 2018; North et al., 2018). Incorporating these survival mechanisms into models can refine estimates of effective population size by accounting for reduced yet persistent populations, and prevent overestimation of genetic drift and diversity loss, improving predictions of intervention efficacy by revealing how dry season survival can buffer population reductions in current vector tools, and enhancing gene drive strategies by addressing the challenges posed by

limited mating opportunities during dormancy (Ickowicz et al., 2021; Magombedze et al., 2018; Morris et al., 2021; A. R. North et al., 2019; Selvaraj et al., 2020). Integrating dry season dynamics into mathematical models provides a realistic framework for designing sustainable and contextually relevant vector control interventions, by aligning these efforts with ecological and evolutionary realities (Jones et al., 2021; Madgwick & Kanitz, 2022; Magombedze et al., 2018).

Therefore, to understand the dry season ecology of major malaria vectors in different environments, even if it is still unclear, it is important to know how these processes impact the widespread maintenance of populations in highly seasonal regions (North et al., 2020). So, there is need for further research on all aspects of *Anopheles* dry season ecology, which will improve our ability to understand and predict gene drive spread. Once implemented successfully, gene drive systems will be effective against all spatial (outdoor, indoor, urban, and rural) and temporal (day or night) malaria vector biting preferences, with access to the larval breeding sites that are hard to target, providing accessible protection to all without requiring human effort or behavior compliance (James & Santos, 2023; World Health Organisation, 2014).

Gene drive systems are specific, ecologically friendly compared to chemical insecticides (World Health Organization, 2014), highly cost-effective, and relatively easy to deliver and deploy, given that little or no change is required in people's behavior (Mudziwapasi et al., 2021). For example, for population modification gene drive strategies, only a few releases are required to achieve long-lasting effects, and the process does not disrupt ecological balance, given that the targeted mosquitoes are not eliminated (Dimopoulos, 2023; Powell, 2022).

2.7.1 Trends in mathematical modeling and simulations

Novel malaria vector control techniques can be analyzed through mathematical modeling and simulations to determine their feasibility, effectiveness, or risks (Korsah et al., 2024; Mandal et al., 2011). They capture the modes of transmission for malaria mosquitoes, including seasonal changes in mosquito population size, gene flow among populations, selection pressures due to environmental conditions and vector control measures (Adegbite et al., 2023; Adom-Konadu et al., 2022; Agusto, 2020; Korsah et al., 2024; Mandal et al., 2011; Ukawuba & Shaman, 2022). Mathematical models and simulations are essential for evaluating the effectiveness of new vector control strategies by simulating different scenarios to predict how gene drive systems will spread within a population and their effects on malaria transmission (Adegbite et al., 2023; Adom-Konadu et al., 2022; Agusto, 2020; Mandal et al., 2011; Ukawuba & Shaman, 2022; B. J. White et al., 2011).

Using these simulations, we can predict the rate at which gene drive mosquito influx will occur, the probability of resistance development, and the effects on mosquito population size and malaria transmission rates (Eckhoff et al., 2016; Hancock et al., 2024; Leung et al., 2022; Sánchez et al., 2020). Researchers can now investigate a wide range of scenarios involving ecological factors, genetic variation in populations, and intervention strategies for mosquito populations (Agboka et al., 2024; Mandal et al., 2011; Reiner et al., 2013).

Over the years, mathematical models have been utilized to simulate malaria transmission, considering vector population dynamics, human-host interactions, and environmental factors (Bakary et al., 2018; Beloconi et al., 2023; Mandal et al., 2011). Earlier models, however, focused on basic reproductive numbers (R0) and vectorial capacity to estimate transmission potential under different intervention scenarios and have been instrumental in showing how significantly reducing mosquito survival or biting rates can greatly reduce

malaria transmission (Kamba et al., 2023; Kamgang & Thron, 2018; Richard et al., 2020; Smith et al., 2007; Smith & McKenzie, 2004).

While prior models emphasized the estimation of transmission potential under different intervention conditions, relied on basic reproductive numbers and vectorial capacity, and have been instrumental in demonstrating how reducing mosquito survival or biting rates can significantly reduce malaria transmission (Smith et al., 2007; Smith & McKenzie, 2004), they have now evolved to incorporate more complex biological and ecological processes, such as mosquito behavior, insecticide resistance, and seasonal population fluctuations (Castro, 2017; Ibrahim et al., 2024; Pigeault et al., 2018; Whittaker et al., 2023). Stochastic models and agent-based simulations can now be used to capture heterogeneity in mosquito populations and human-vector interactions across diverse geographic areas (Amadi et al., 2021; Le et al., 2018; Modu et al., 2023; Smith et al., 2018). The traditional models are thus often inadequate in explaining genetic changes within mosquito populations, especially in the context of emerging genetic control technologies (Leung et al., 2022; North et al., 2019; Wang et al., 2021). Mathematical models are crucial in assessing the potential spread and effectiveness of gene drive systems, a promising genetic control technology for malaria vector control, designed to spread the desired genetic traits through wild mosquito populations at a rate faster than traditional Mendelian inheritance (Hancock et al., 2024; Metchanun et al., 2022; Naidoo & Oliver, 2024; Selvaraj et al., 2020).

Deterministic modeling of gene drive propagation within mosquito populations is now a commonly used technique, which assumes ideal conditions and provides insight into the basic dynamics of gene drive propagation (Dhole et al., 2024; Hammond, et al., 2021; Sánchez, et al., 2020). Since the practical applicability of these drive systems necessitates

complex models that consider ecological variability, fitness costs associated with genetic modifications, and possible resistance mechanisms within mosquito populations, stochastic models have thus been developed to capture the probabilistic nature of gene drive spread, especially in small or isolated populations highlighting the importance of initial release size, mating dynamics, and the genetic structure of target populations (Cui et al., 2023; D'amato et al., 2024; Dhole et al., 2024; Marshall & Hay, 2012; Sánchez et al., 2020; Verma et al., 2023).

For instance, while population suppression drives aim to introduce traits that reduce mosquito fertility or viability, population replacement drives instead introduce traits that make mosquitoes resistant to malaria parasites without decreasing mosquito numbers (Hammond, et al., 2021; Hoermann et al., 2021).

Simulations will assist in showing that both approaches have strengths and limitations depending on ecological and genetic contexts (Champer et al., 2022; Faber et al., 2024; James et al., 2018). Despite significant advancements in gene drive system modeling, a critical gap in current models is the lack of information on malaria vector persistence and population rebound, which is particularly relevant in regions with strong dry and rainy seasons (Eckhoff et al., 2016; North et al., 2018). Understanding these patterns is thus essential for accurately predicting the long-term impact of interventions, like gene drives (Eckhoff et al., 2016; North et al., 2018). This study investigated the malaria vector dry season persistence mechanisms to improve predictions of population recovery and sustained transmission risk after interventions are applied.

2.8 Progress and Perspectives on Gene Drive Development and Potential Deployment

Proof-of-concept in laboratories is promising, candidate-stratified mosquito population loci for modification have been identified , and molecular tools for vector transformation and

engineering for malaria gene drive mosquitoes have been developed (Hammond et al., 2016; Windbichler et al., 2011). However, to evaluate the effectiveness of these gene-driven products in the natural environment, there is a need to understand several malaria vector propagation dynamics, such as how mosquito populations re-establish at the beginning of the rainy season (Lehmann, Weetman, Diana L Huestis, et al., 2017).

Laboratory-developed mosquito colonies could have some fitness costs that if compared to wild-type populations, even with introgression to a local genetic background, could still limit their spread (Garrood et al., 2022). Therefore, there is need to have isolated and well-characterized study sites whose parameters, such as mosquito species composition, diversity, density, propagation, biting behavior, genetics, and parasite infection rates are known. Hence, when using mathematical models and simulations, these parameters will aid in making precise predictions about where and how to deploy the novel malaria vector control approaches (Eckhoff et al., 2016; Lambert et al., 2018).

The broad spectrum of biological research required for developing genetic control strategies is expected to contribute to the more efficient application of the already existing malaria vector control tools to develop new and effective approaches (Alphey et al., 2002; Hemingway et al., 2016; James & Santos, 2023). The developed candidate gene drive products will be assessed based on the criteria that affect their potential for success, such as the fact that the drive mechanisms must (1) be powerful enough to spread the effector genes to fixation or close to it in a time frame that is appropriate for a malaria vector control program (2) be resistant to the potential loss of linkage between the drive mechanisms and the effector gene(s) (3) have the ability to spread new or modified effector genes over time to counteract the loss of linkage, mutational inactivation of the effector or development of resistance by the pathogen (4) can spread to large multi-gene constructs and (5) be as safe

enough without risk of causing undesirable side effects in the target and non-target vector species (Boë & Koella, 2003; James, 2005; Letourneau & Burrows, 2001; Moreira et al., 2004; Sinkins & Gould, 2006).

This study investigated the dry season population genetics of *Anopheles gambiae* s.l. malaria mosquitoes and compared populations from mainland and island sites in Uganda to evaluate their potential for gene drive field trials. By analyzing genetic diversity, structure, and seasonal persistence mechanisms, we assess how these dynamics influence the effectiveness of gene drive as a novel malaria control strategy. The findings provide critical insights for site selection and deployment of genetically-based interventions, addressing key challenges in vector control and supporting malaria elimination efforts.

CHAPTER THREE: GENERAL MATERIALS AND METHODS

3.1 Study Setting

Each specific objective used different settings to address specific research questions related to the population genetics and seasonal dynamics of *Anopheles gambiae, An. arabiensis,* and *An. coluzzii*, effective vectors in transmitting malaria. Below is a summary of the study settings for each specific objective.

3.1.1 Specific Objective 1: The Potential Persistence Mechanisms of Major Malaria Vectors in Sub-Saharan Africa

The study focused on the persistence mechanisms of major malaria vectors (*An. gambiae, An. arabiensis* and *An. coluzzii*) across sub-Saharan Africa with particular emphasis on contrasting ecological regions, namely, the Sahelian and Equatorial regions. The Sahelian region is characterized by extreme aridity, long dry seasons that last up to 8 months with scarce surface water. The equatorial region on the other hand features milder dry seasons, shorter dry periods of less than 3 months and residual water availability. Key locations include Mali, Senegal, Burkina Faso (in the Sahelian region), Uganda and Kenya (in the Equatorial region) where field studies, genetic analyses, and semi-field experiments were conducted to investigate dry season survival hypotheses such as aestivation, local refugia, local migration, and long-distance migration.

3.1.2 Specific Objective 2: Simulation of Population Genetics of Partial Diapause with Application to *An. coluzzii*

The study investigated the population genetics of partial diapause (aestivation) in *Anopheles coluzzii*, a major malaria vector, with a focus on Thierola village in the Sahelian region of Mali. Mosquito samples were collected at seven time points spanning 2 years

(Lehmann, Weetman, Diana L Huestis, et al., 2017). Thierola village has no surface water for larval sites for 4-6 months a year during the dry season (Lehmann, Weetman, Diana L Huestis, et al., 2017).

3.1.3 Specific Objective 3: The Population Genetics of *Anopheles gambiae*; Investigating Potential Seasonal Rebound Mechanisms (Aestivation and Migration) in Eastern Uganda

The model developed in specific objective 2 was extended to include migration and applied to an *An. gambiae* dataset that was collected for a period of 2 years (2017-2018) from 14 districts in Eastern Uganda. The 14 districts are: Amuria, Bugweri, Busia, Iganga, Jinja, Kaliro, Kamuli, Luuka, Manafwa, Mayuge, Mbale, Namayingo, Ngora and Soroti (Figure 3.1).

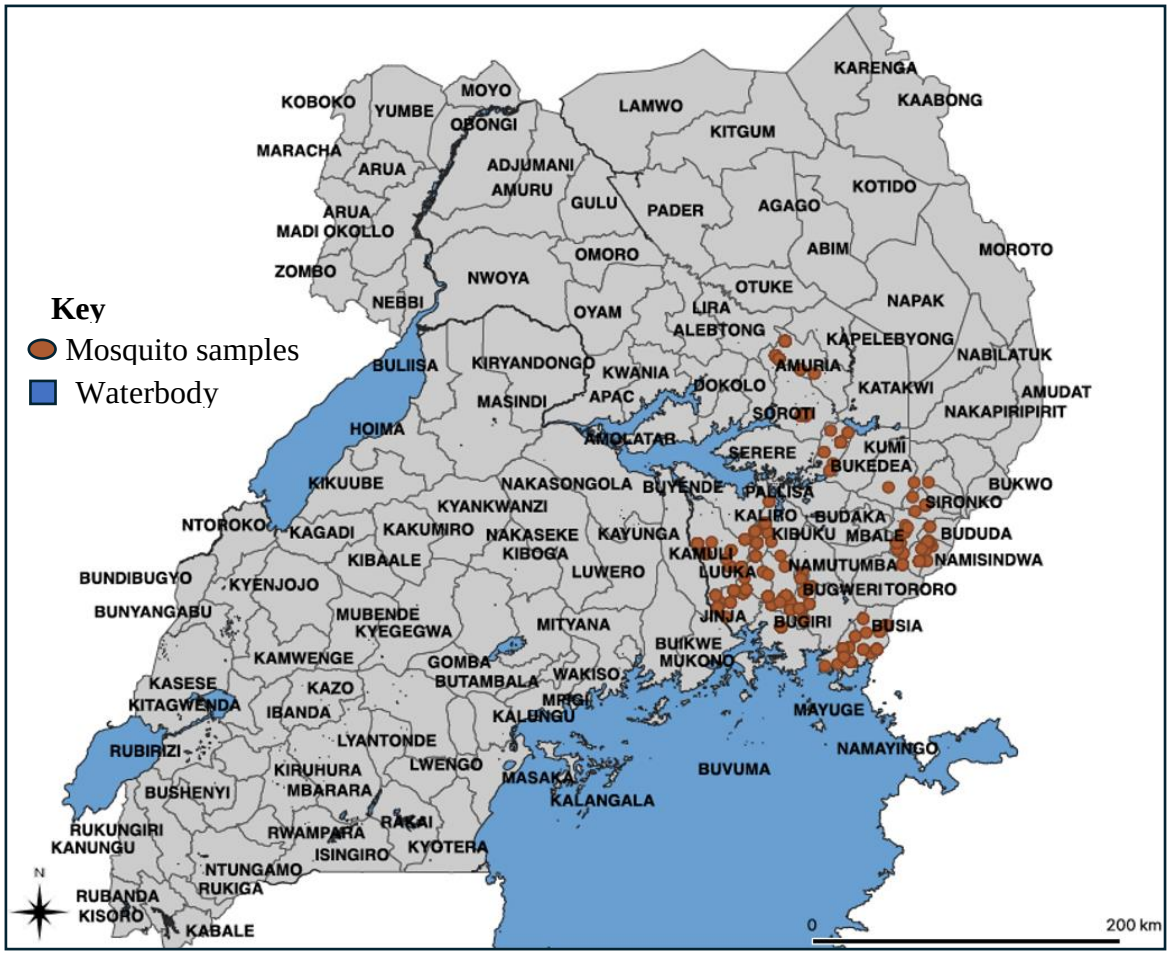

Figure 3.1 *Map of Uganda showing the districts in Eastern Uganda where malaria mosquito samples were collected*

3.1.4 Specific Objective 4: Assessing the Population Genetic Structure and Demographic History of *Anopheles gambiae* and *Anopheles arabiensis* at Island and Mainland Sites in Uganda: Implications for Testing Novel Malaria Vector Control Approaches

The study was conducted at six sites (Figure 3.2), three mainland (Kayonjo, Katuuso and Kibbuye) and three island sites (Bugiri, Kiimi and Kansambwe).

3.1.4.1 Mainland sites

Kayonjo is located in Kayunga district while Katuuso and Kibbuye villages are located in Mukono district (Fig. 3.2). These villages are located in central Uganda and normally experience two rainy and two dry seasons in a year. The climate is tropical and equatorial with two dry seasons, December to February and June to September (The Uganda Guide, 2020; Wikipedia, 2024). These three mainland sites are located in high malaria endemic areas and have a high malaria incidence of up to 150 malaria cases per 1000 people/year (Ugandan Ministry of Health, 2014).

Kibbuye and Katuuso villages are both located in the Seeta-namuganga subcounty in Mukono district. Kibbuye has a population of about 1500 inhabitants, while Katuuso has about 800 people. In both villages, the major economic activity is agriculture, and residents plant rice gardens in the swampy areas that border each village and coffee plants within the villages. Some residents are also involved in keeping livestock like cattle, pigs, chickens, and goats on a small scale.

Kayonjo village is located in Busaana subcounty in Kayunga district and has a population of about 1800 people. The major economic activity in Kayonjo is agriculture, and the areas bordering it are mostly large tracks of swampy grounds on which residents farm. Agriculture in Kayonjo is more diverse compared to Katuuso and Kibbuye, with a wide

range of food crops like maize, sweet potatoes, rice, and yams. Coffee is also widely grown in addition to evergreen trees, which are mostly fruit trees, especially mango trees. Livestock farming is at the subsistence level, and the swampy gardens contain most of the mosquito larval habitats.

3.1.4.2 Island sites

The island sites (Bugiri, Kiimi, and Kansambwe) are comprised of 600, 1500, and 1000 inhabitants, respectively. The main economic activity is fishing, and the housing structures are mainly temporary, constructed with grass or iron sheet roofs with walls made of mud or iron sheets. Information on human inhabitants and household characteristics at the island sites was collected during field surveys conducted between 2013 and 2016.

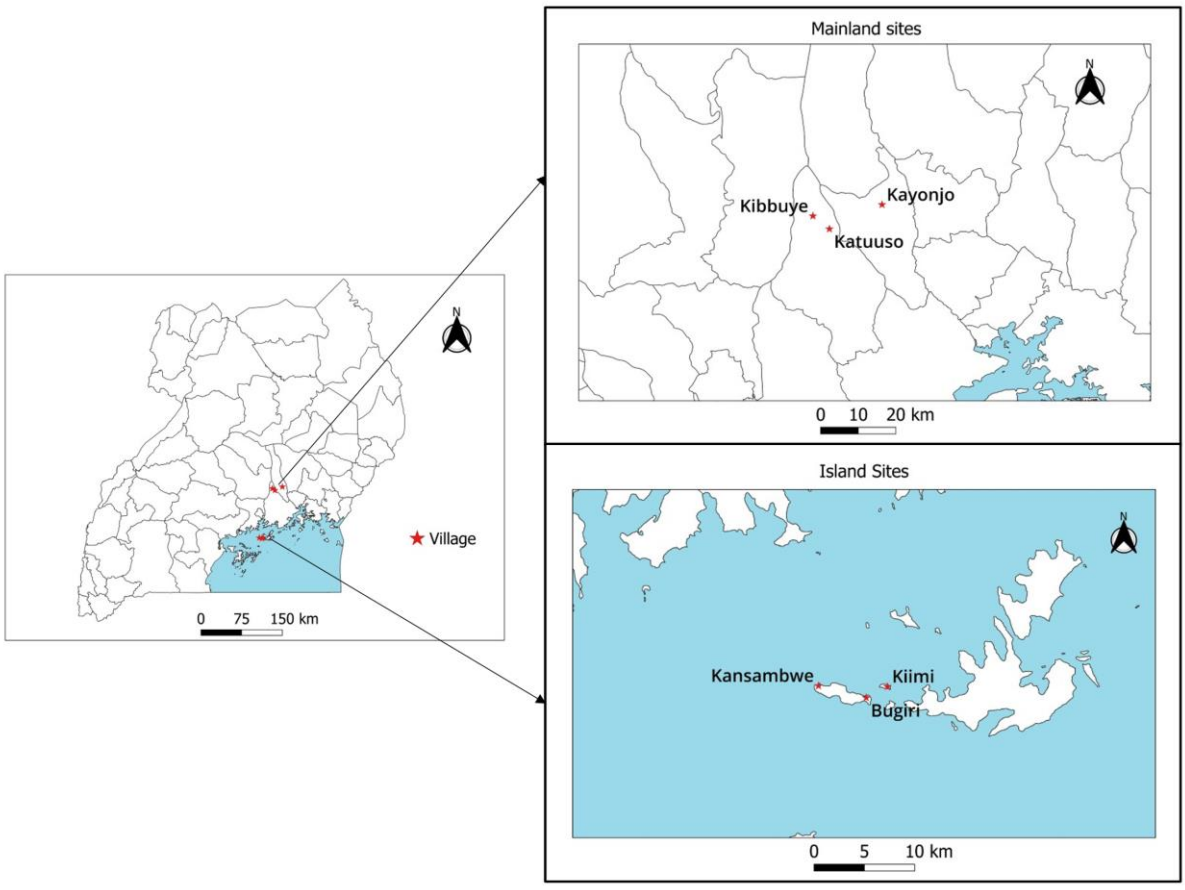

Figure 3.2 *Map of Uganda showing the location of the 3 mainland and 3 island study sites in Uganda.*

3.2 Study Design

3.2.1 Specific Objective 1: Potential Persistence Mechanisms of the Major *Anopheles gambiae* Species Complex Malaria Vectors in sub-Saharan Africa

This sub study used diverse methodologies such as the Mark-Release-Recapture, aerial sampling and genomic surveys to address gaps in understanding seasonal population rebounds of malaria mosquitoes. The study considered ecological parameters such as (a) dry season stressors like temperature fluctuations, low humidity and larval habitat desiccation, (b) behavioral adaptations like shifts in feeding, gonotrophic dissociation and microhabitat selections, and (c) genetic adaptations like chromosomal inversions linked to desiccation resistance, which setting generally underscored the interplay between environmental extremes and vector survival strategies which are critical for malaria control.

3.2.2 Specific Objective 2: The Population Genetics of Partial Diapause, with Application to the Aestivating Malaria Mosquito *Anopheles coluzzii*

Temporal *An. coluzzii* samples were collected from Thierola (2008-2010) in Mali, spanning three seasons, rainy, dry, and the next rainy season transitions (Lehmann, Weetman, Diana L Huestis, et al., 2017). Seven hundred thirty-eight (738) single-nucleotide polymorphisms (SNPs) from autosomal loci were considered. A novel population genetic framework that leverages temporal allele frequency dynamics was developed. This approach builds on the temporal method for estimating effective population size (N_e) and extends the classic Wright–Fisher (WF) model to incorporate a separate aestivating compartment during the dry season (DS). The theoretical predictions were verified using forward-time simulations, and the model was applied to empirical temporal genetic data from a well-characterized Sahelian mosquito population (Lehmann, Weetman, Diana L Huestis, et al., 2017).

Lehmann et al. (2017) studied the genetic signatures of aestivation, to find evidence that supports it using a mosquito dataset of about 738 SNPs. The main tool used was the

temporal F statistic which was compared to the null value (finite sampling but infinite N_e). If the observed F is larger than the null value, then drift is evident (Lehmann, Weetman, Diana L Huestis, et al., 2017).

3.2.2.1 Model Development and Theoretical Framework

This is a forward Wright-Fisher simulator, individual-based with recombination, simulating pairs of loci, to pick up signals of drift. This model is visualised in Figure 3.4, and the notations used are found in Table 3.1

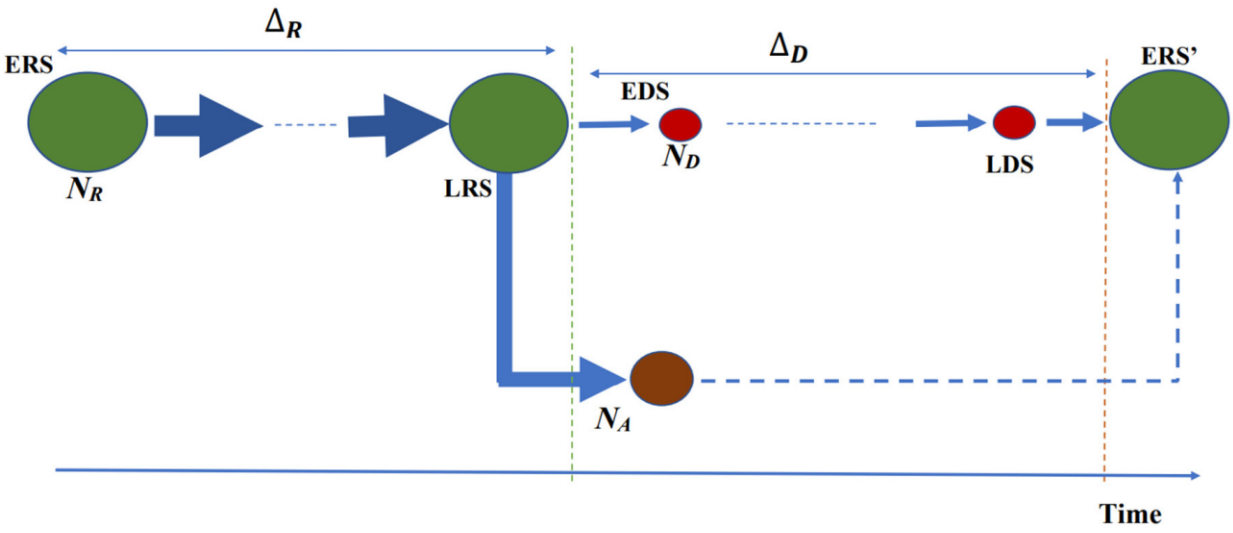

Figure 3.4 *Diagram for the aestivation model*

Table 3.1 *Notation used in this study*

RS	Rainy season
DS	Dry season (the unfavourable season for aestivation)
ERS (EDS)	The first generation of RS (DS)
LRS (LDS)	The final generation of RS (DS)
ERS'	ERS of the next cycle
N_R	RS breeding size
N_D	DS breeding size
N_A	Size of the aestivating (diapause) compartment NA⁄NR
Δ_R	Length of RS in generations
Δ_D	Length of DS in generations
V_t	Standardised variance of allele frequency, at generation t
F	Temporal F, standardised variance of allele frequency change (between two time points)

The model begins at ERS with breeding size N_R. Within the same season, the population reproduces (horizontal arrows) according to the WF model. Upon entering EDS, the population branches into two compartments: one continues to breed but with a smaller size N_D, another aestivates with N_A individuals. At the next ERS (denoted as ERS'), it is formed by previously aestivating individuals, and the descendants of the breeding compartment from LDS.

In this study, the Wright-Fisher (WF) model of genetic drift (Wright, 1931; Crow & Kimura, 1970) was modified to incorporate dual-compartment population dynamics with partial aestivation. The derived mathematical expressions for key population genetic statistics under this model, include:

1. The standardized variance of allele frequency over time (V_t)

Without other forces (migration, selection, mutation), allele frequency fluctuates randomly due to genetic drift. While the mean allele frequency remains unchanged over time, the variance increases. Under the WF model, the standardised V_t increases by approximately $1/_{2Ne}$ per generation.

$$V_{t+1} = V_t + \frac{1}{2N_e} \quad \text{[Equation 3.1]}$$

2. The temporal *F* statistic (standardized allele frequency change) can be derived from V_t (Waples, S., 1989). If two samples are taken Δ_t generations apart, then *F* is approximately

$$F \approx \frac{\Delta t}{2N_e} \quad \text{[Equation 3.2]}$$

3. and the impact of aestivation on estimates of N_e across seasons, which can be estimated from Equation 3.2 above.

These derivations enabled us to relate observed temporal changes in allele frequency to underlying demographic parameters, notably the breeding sizes during rainy season (N_R) and dry season (N_D), and the number of aestivators (N_A).

3.2.2.2 Simulation Framework

A custom forward-time simulator was implemented in R to validate the theoretical predictions. Simulations were conducted at multiple biallelic loci under varying aestivation proportions ($\alpha = N_A/ N_R$), using the following assumptions:

- Initial allele frequencies were sampled from a uniform distribution U(0.2, 0.8).

- Population sizes and season lengths were predefined: Δ_R and Δ_D (generations per RS and DS, respectively).
- Breeding occurred only within respective compartments (no migration between breeders and aestivators during the DS).
- Standard Wright-Fisher genetic drift operated during breeding generations.

Allele frequency variances and temporal *F* statistics across key timepoints were tracked from the start to end of RS and DS (ERS, EDS, and ERS') to verify that simulated patterns aligned with theoretical expectations. The model accurately predicted non-monotonic changes in allele frequency variance due to the reappearance of drift-unaffected aestivators at ERS'.

3.2.2.3 Allele Frequency Trajectories with Mosquito Aestivation

Using this model (Figure 3.1), the effect of aestivation on the allele frequency over time was visualised. Figure 3.5 is an illustrative example, with $N_R = 10000$, $N_D = 1000$, and $N_A = 7500$. $\Delta_R = \Delta_D = 6$ generations. The allele frequencies of 200 independent loci from the same population, with an initial allele frequency of approximately 0.5, were simulated over time. Due to random genetic drift, some alleles increase in frequency, while others decrease, however, the overall change in frequency is zero.

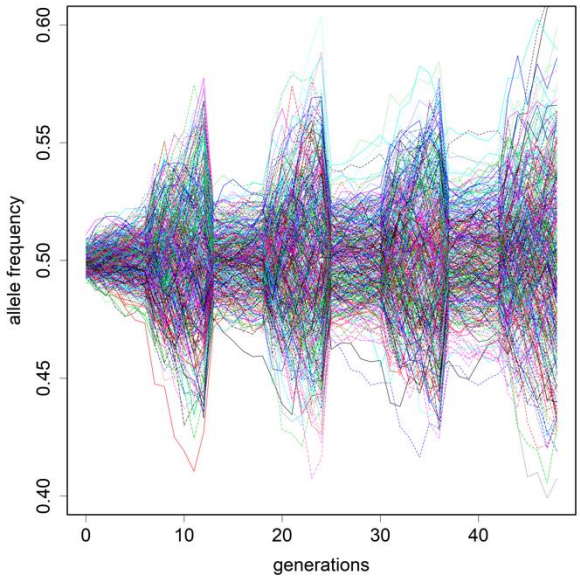

Figure 3.5 *Diagram illustrating allele frequency trajectories*

The sample variance of these allele frequency trajectories at each time point (i.e., along a vertical slice) was calculated.

3.2.2.8 Application of the model to Empirical Data

To demonstrate real-world applicability, the framework was applied to published *An. coluzzii* genotype data from Thierola village, in Mali (Lehmann, Weetman, Diana L Huestis, et al., 2017). This dataset comprised SNP genotype data from temporally sampled mosquitoes collected over two annual cycles (2008–2010), that spanned three critical seasonal points: RS, DS, and the following RS (ERS, EDS, ERS'). These temporal samples were used in the estimation of breeding and aestivating sizes.

The allele frequencies from each temporal sample, and effective sample sizes per locus (to account for missing genotypes) were extracted. These pooled allele frequencies were used to calculate the minor allele frequency cut-off so that only loci with maf >=0.05 were included in the estimation. For each annual cycle, three pairwise temporal *F* statistics were computed (ERS–LRS, LRS–EDS, EDS–ERS′). These were then used as summary statistics

in an Approximate Bayesian Computation (ABC) framework to infer the posterior distributions of:

- *NR* (RS breeding size),
- *ND* (DS breeding size),
- *NA* (number of aestivating individuals),
- and $\alpha = NA / NR$ (aestivation proportion).

3.2.2.9 Approximate Bayesian Computation (ABC) and Temporal F, corrected for finite sampling variance (F_c)

When analyzing the dataset of Lehmann et al. (2017) the F_c statistic was used to account for sampling errors. Let x_0 and x_t be the observed allele frequencies at two time points. Also let n_0 and n_t be the corresponding diploid sample sizes with complete information. The following quantalities were calculated for each locus:

$$F_c = \frac{(x_t - x_0)^2}{\frac{x_t + x_0}{2} - x_t x_o} - \frac{1}{2n_0} - \frac{1}{2n_t}$$

The overall F_c is the arithmetic average across all loci, similar to the procedure by Peel et al. (2013). If two samples are taken Δ_t generations apart, F will be:

$$F \approx \frac{\Delta_t}{2N_e}$$

From which N_e can be estimated (Waples, 1989). This method estimates the harmonic mean N_e of all the generations between the two samples (Waples, 2005)

Loci with pooled minor allele frequency >=5% and with complete information across all three temporal samples were included in the ABC. As a result, 435 loci were used to estimate the three sizes for the first year, and 496 loci for the second year. The maximum

accepted Euclidean distance was 0.0169 for the first year, and 0.00046 for the second year. These two numbers correspond to the tolerance δ in rejection-based ABC (Beaumont et al., 2002a).

3.2.2.10 Assumptions of the model

Key assumptions of the model include:

1. Individuals sampled during the DS are reproductively active (i.e., not aestivating).
2. The population is closed to gene flow during the study period (no significant immigration or emigration).
3. Generation time is constant (non-overlapping, discrete generations)
4. Random mating.
5. Sampling time points align closely with the first generation of each season.

3.2.3 Specific Objective 3: The Population Genetics of *Anopheles gambiae*: Investigating Potential Seasonal Rebound Mechanisms (Aestivation and Migration) in Eastern Uganda

This study utilised whole genome sequencing (WGS) data of *An. gambiae* mosquitoes collected from 14 districts in Eastern Uganda between 2017 and 2018. A total of 565 samples were obtained across 13 distinct time points (Table 3.2 A&B). The districts include Amuria, Bugweri, Busia, Iganga, Jinja, Kaliro, Kamuli, Luuka, Manafwa, Mayuge, Mbale, Namayingo, Ngora, and Soroti (Figure 3.1).

Sequencing data focused on biallelic SNPs located within intergenic regions of chromosomes 3L (15–41 Mega basepairs (Mbp)) and 3R (15–41 Mbp), selected to avoid polymorphic inversions, reduced recombination, and regions under strong selection. Intergenic SNPs from chromosome 3 (3L and 3R) were considered because they avoid regions of polymorphic inversions, reduced recombination and unequal divergence from

the reference genome. This allows for clearer detection of population-level processes like drift, migration, and bottlenecks, providing a more accurate representation of genetic diversity and evolutionary processes (Cáceres et al., 2012; Campos, Rona, et al., 2021; Miles et al., 2017; Wondji et al., 2007).

Table 3.2A *Samples collected per timepoint*

Year	**Month**	**Number of samples collected**
2017	Mar	71
2017	Apr	39
2017	May	100
2017	Jun	45
2017	Jul	26
2017	Aug	14
2017	Sep	35
2017	Oct	1
2017	Nov	11
2018	Mar	42
2018	May	116
2018	Sep	9
2018	Nov	56
Total		**565**

Table 3.2B *Number of samples per district*

District	**Number of mosquitoes**
Amuria	54
Namayingo	53
Soroti	33
Jinja	51
Kamuli	38
Busia	31
Iganga	35
Luuka	50
Ngora	33
Sironko	9
Kapchorwa	1
Kaliro	27
Bulamburi	1
Mbale	36
Bukedea	1
Namisindwa	9
Manafwa	34
Bududa	2
Bugweri	46
Mayuge	21
Total	**565**

3.2.3.1 Assessment of Spatial Structure

Before analyzing temporal dynamics, we evaluated spatial genetic structure to determine whether samples could be pooled as a single population. Principal Component Analysis (PCA) was used to visualize genetic clustering and differences between populations by district (Novembre & Stephens, 2008; Patterson et al., 2006a), and average pairwise Hudson's F_{ST} (using the ratio of averages) was computed between all district pairs, with standard errors computed using the block-jackknife method (Arnold et al., 2013; Bhatia et al., 2013). These analyses were performed using functions from MalariaGEN's population genetics API (MalariaGEN, 2023).

3.2.3.2 Temporal F and Effective Population Size (Ne) Analysis

The temporal F-statistics (F) and effective population size (N_e) for the Eastern Ugandan mosquito populations were computed by splitting the 565 mosquitoes according to the month/year of collection. This yielded 13 time points. For each chromosome, (1) allele frequencies were calculated across individuals for each time point, then (2) Temporal F statistics were calculated between all pairs of time points after a pooled minor allele frequency (MAF) filter of $\geq 5\%$ was imposed to remove low-frequency variants, and (3) Ne was derived by adjusting temporal F values based on the number of generations between time points (Waples, 1989). The resulting temporal F and Ne matrices enabled us to identify time points with sufficient temporal signal and finite drift, which were used in the subsequent parameter estimation. The temporal F's were used as summary statistics for parameter estimation (3.2.3.3).

3.2.3.3 Parameter Estimation Framework: Aestivation-Migration Model

This study estimated the dry-season persistence mechanisms (aestivation and migration) and seasonal breeding sizes by implementing an extended version of the genetic forward-

in-time simulation model that integrates both aestivation and LDM (Figure 3.6). This model adapted from (Mwima et al., 2024), includes four compartments:

- N_R: Rainy season breeding population size.
- N_D: Dry season breeding population size.
- N_A: Number of aestivating individuals that rejoin the population at the end of DS.
- N_M: Number of long-distance migrants entering at the start of the rainy season.

Sampling: Simulations accounted for finite population sizes using binomial sampling of allele frequencies across loci.

Simulations modeled allele frequency dynamics across seasons under Wright-Fisher assumptions, modified to include aestivation and migration processes. The model incorporated binomial sampling to simulate finite population sizes and tracked allele frequency changes over 5,000 loci. Pairwise temporal F-statistics were used as summary statistics for model fitting.

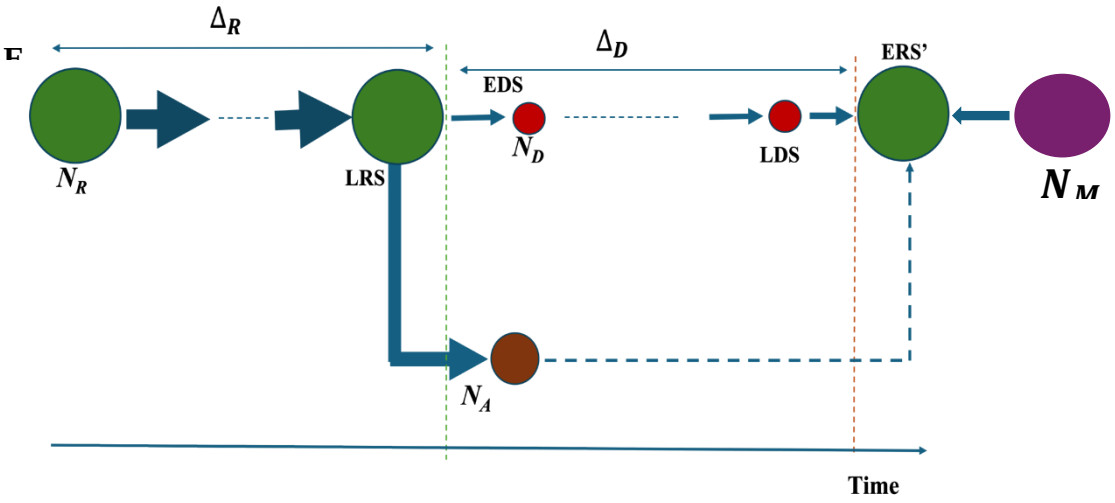

Figure 3.6 *Diagram for the aestivation-migration model*

The model begins at ERS with breeding size NR. Within the same season, the population reproduces (horizontal arrows) according to the WF model. Upon entering EDS, the population branches into two compartments: one continues to breed but with a smaller size ND, another aestivates with NA individuals. At the next ERS (denoted as ERS'), it is formed by previously aestivating individuals, the descendants of the breeding compartment from LDS and migrants from neighboring areas.

3.2.3.4 Parameter Estimation via Approximate Bayesian Computation (ABC)

Four time points were selected to capture key phases of the mosquito life cycle: early rainy season (ERS), peak rainy season (RS), dry season (DS), and next early rainy season (ERS'). These points showed finite N_e values and enabled estimation of pairwise temporal F-statistics. Uniform prior distributions were assigned to the parameters:

- N_D ~ U(500, 10,000)
- N_R ~ U(ND, 20,000)
- $\alpha = N_A/N_R$ ~ U(0, 0.5)
- $m = N_M/N_R$ ~ U(0, 0.5)

Simulations were run across the parameter space, and the posterior distributions were obtained by comparing observed temporal *F*-values with those generated from simulations. Approximate Bayesian Computation (ABC) was used to estimate posterior distributions, enabling probabilistic inference of N_R, N_D, N_A, and N_M.

The specific bounds reported (e.g. $N_D \sim \mathcal{U}(500,10,000)$) represent the ranges used for the preliminary ABC analyses presented here and are treated as tunable hyperparameters rather than intrinsic model constants. These bounds may be refined as part of further model calibration and sensitivity analyses.

3.2.3.5 Model Validation and Interpretation

To validate the model fit, posterior median parameter values were used to simulate expected *F*-statistics, which were then compared to the observed *F*-statistics. The close match between simulated and observed values confirmed the model's adequacy in capturing key features of seasonal allele frequency dynamics in the population. The posterior estimates were then interpreted to quantify the contributions of aestivation and migration to the early rainy season population. Model validation was conducted by partitioning the data into training and validation subsets, following procedures described in

the associated manuscripts. This ensured that model performance was assessed on independent data.

This analysis allowed us to partition the seasonal population rebound into contributions from dry-season survivors, aestivators, and long-distance migrants.

3.2.4 Specific Objective 4: Assessing the Population Genetic Structure and Demographic History of *Anopheles gambiae* and *An. arabiensis* at Island and Mainland Sites in Uganda: Implications for Testing Novel Malaria Vector Control Approaches

At the island sites mosquito collections were done from April 2013 to April 2017, and from January 2016 to December 2018 at the mainland sites. These collections were part of routine mosquito sampling done by the Department of Entomology at the Uganda Virus Research Institute. Adult mosquitoes were sampled indoors and outdoors using human landing catches (HLC), pyrethroid spray catches (PSC), indoor and outdoor aspirators, and catch basin traps (CBT). All mosquitoes were morphologically identified using the *Anopheline* morphological identification keys developed by Holstein (Gillies et al., 1987) and then stored in 80% ethanol in a -20 ^{0}C freezer for subsequent molecular analysis.

3.2.4.1 Mosquito Sampling Methods

3.2.4.1.1 Human Landing Catches (HLC)

Using HLC, trained human volunteers exposed parts of their body (usually legs or arms) to attract host-seeking mosquitoes (European Centre for Disease Prevention and Control; European Food Safety Authority;European Food Safety Authority, 2018; Service, 1993). Mosquitoes landing on exposed skin were captured using mouth aspirators prior to biting (European Centre for Disease Prevention and Control; European Food Safety

Authority;European Food Safety Authority, 2018; Service, 1993). This method estimates the biting rate of mosquitoes and helps identify which species prefer human hosts.

3.2.4.1.2 Pyrethroid Spray Catches (PSC)

Indoor spaces were sprayed with a pyrethroid insecticide and sealed for a brief period. Subsequently, knocked-down mosquitoes were collected from floors and surfaces to quantify indoor resting densities (European Centre for Disease Prevention and Control; European Food Safety Authority;European Food Safety Authority, 2018) . The PSC method is used for estimating the density of mosquitoes resting indoors and understanding their indoor behavior.

3.2.4.1.3 Indoor and Outdoor Aspirators

This method involves the use of aspirators, which are handheld or powered devices that suck up mosquitoes from the surfaces (European Centre for Disease Prevention and Control; European Food Safety Authority;European Food Safety Authority, 2018; Service, 1993). Indoor and outdoor resting mosquitoes were collected by systematically aspirating surfaces such as walls and ceilings either inside (indoor) or outside (outdoor) the house. Their purpose was to catch mosquitoes resting after blood meals or before seeking a host, helping to assess both indoor and outdoor resting mosquito populations.

3.2.4.1.4 Catch Basin Traps (CBT)

Traps were deployed in storm-water catch basins and similar habitats known for mosquito breeding and resting (European Centre for Disease Prevention and Control; European Food Safety Authority;European Food Safety Authority, 2018; Service, 1993). These traps captured adult mosquitoes utilizing these microhabitats.

Together, these complementary methods allowed for sampling across different mosquito behaviors and habitats, maximizing the chances of collecting a wide range of mosquito species for further analysis and disease risk assessment.

3.2.4.2 Deoxyribonucleic acid (DNA) extraction, library construction, and sequencing of mosquito samples

A total of 3515 whole mosquito samples, each stored in a well of a 96-well plate of 80% ethanol, were shipped to the Sanger Institute, UK, for sequencing. DNA extraction was done using a cost-effective, nondestructive method in which whole mosquitoes were incubated in 60 µl of custom-made lysis buffer (200 mM Tris-HLC pH 8.0, 25mM EDTA pH 8.0, 0.4 mg/ml Proteinase K, 0.05% Tween 20) at 56°C for up to 24 hours (Makunin et al., 2022). This protocol preserves specimen morphology for potential re-examination. No further DNA purification was required, crude lysates were diluted 10-fold with ultrapure water prior to PCR amplification, providing ample template quality and quantity for downstream applications (Makunin et al., 2022).

Amplicon libraries were generated through a two-step, highly multiplexed PCR protocol. The first PCR simultaneously amplified 62 selected *Anopheles* nuclear loci and two *Plasmodium* mitochondrial loci using a mixture of locus-specific primers tailed with Illumina adapter sequences (Makunin et al., 2022). The reaction conditions were carefully optimised for even amplification across all targets, and the second PCR introduced dual-index barcodes and complete Illumina flow cell adaptor sequences, enabling high levels of multiplexing (Makunin et al., 2022). This two-stage PCR setup eliminated the need for DNA purification or size-selection steps between reactions (Makunin et al., 2022).

Indexed libraries were pooled in equimolar volumes and purified using AMPure XP beads (Makunin et al., 2022). The final library pool was assessed for size distribution and

quantified, followed by denaturation and dilution to the appropriate concentration for Illumina MiSeq sequencing (2 × 150 bp paired-end reads, using 300-cycle v2 kits) (Makunin et al., 2022). This workflow enabled simultaneous sequencing of over a thousand individual mosquitoes per MiSeq run, facilitating efficient high-throughput screening for population and species identification as well as *Plasmodium* detection (Makunin et al., 2022). This streamlined method supports robust, accurate, and cost-effective genetic surveillance across large sample sets, and is particularly well-suited for studies requiring high-throughput processing of mosquito populations for population genetics, species assignment, and malaria parasite screening. For all other details regarding the sequencing techniques, and the functions of the 62 amplicon regions please refer to the original publication (Makunin, et al. 2022).

3.2.4.3 Bioinformatics Analysis

To analyze the amplicon data, an open-source computational pipeline, AmpSeeker (Nagi et al., 2025), was used. This pipeline is written in Snakemake (Mölder et al., 2021) facilitates automated, end-to-end analysis and can be applied to any Illumina amplicon sequencing data (Nagi et al., 2025). The raw reads were imported into AmpSeeker, which performed demultiplexing followed by alignment to the *Anopheles* reference genome (AgamP4 PEST) with bwa mem (H. Li & Durbin, 2009), and variant calling with bcftools mpileup (Danecek et al., 2021). Following variant calling, sample quality control was performed, removing samples with low depth. For all other details regarding the bioinformatics processing including quality checking, read alignment and variant calling, please refer to the original publication (Nagi et al., 2025). The output here was a Variant Call Format (VCF) file used for further analysis.

The resulting raw variant call format (VCF) file consisted of variant calls across all samples and comprised a total of 12,412 sites (inclusive of insertions and deletions (indels), primer regions, fixed and polymorphic sites). Subsequent subsetting was performed on the VCF file based on the metadata information, which provided details about the mosquito samples, including individual sample IDs, species, collection location, collection period, and season among others. In the filtering process, indels were removed, resulting in 9,890 remaining sites. These sites were distributed across the genome as follows: X (n = 952), 2L (n = 1881), 2R (n = 2815), 3L (n = 1615), 3R (n = 2627). Further downstream analyses were based on these 9,890 sites with potential further filtering, such as according to minor allele frequency and missing values.

3.2.4.4 Population genomic analyses

3.2.4.4.1 Principal Component Analysis (PCA)

The PCA was conducted in the R statistical environment (Version 4.3.1) (Posit team, 2025) using the prcomp() function (Version 4.3.1) (Harvey & Hanson, 2024). The PCA was performed on the genetic covariance matrix for all biallelic SNPs, first across all chromosome arms, then focusing on the 3L arm to assess fine-scale population structure. The PC axes were examined to reveal genetic clustering among populations (island and mainland sites) and between species (*An. gambiae* and *An. arabiensis*).

3.2.4.4.2 Genetic differentiation using Hudson's pairwise F_{ST} estimation

Calculation of Pairwise F_{ST}

Genetic differentiation was estimated between populations using Hudson's F_{ST} (Bhatia et al., 2013), a robust estimator that avoids biases associated with unequal sample sizes and rare variants. The analysis was performed on biallelic SNPs from the 3L chromosome to maintain consistency in genomic coverage and filtered for $\leq 10\%$ missing data and a minor allele frequency (MAF) $\geq 1\%$ to ensure reliability.

F_{ST} was computed using the fst_hudson_pairwise function from the Popkinsuppl R package (Ochola, 2022). This function implements Hudson's estimator (Bhatia et al., 2013), defined as:

$$F_{ST}^{Hudson} = \frac{\left(\tilde{P}_1 - \tilde{P}_2\right)^2 - \frac{\tilde{P}_1\left(1-\tilde{P}_1\right)}{n_1 - 1} - \frac{\tilde{P}_2\left(1-\tilde{P}_2\right)}{n_2 - 1}}{\tilde{P}_1\left(1-\tilde{P}_2\right) + \tilde{P}_2\left(1-\tilde{P}_1\right)}$$

[Equation 3.3]

where:

- $\tilde{P}_1, \tilde{P}_2$ are allele frequencies in Population 1 and Population 2, respectively.
- n_1, n_2 are the respective sample sizes for Populations 1 and 2.

1. Standard Error Estimation

Uncertainty in F_{ST} was assessed using a leave-one-out jackknife resampling method (Bhatia et al., 2013; Weir & Hill, 2002) and involved;

- Recalculating F_{ST} iteratively, each time excluding one SNP
- Estimating the standard error (SE) from the variance of these pseudo-values.

2. Statistical Significance Testing

- Z-scores were attained by dividing each F_{ST} estimate by its SE
- P-values were obtained by comparing Z-scores against the standard normal distribution, testing the null hypothesis of no genetic differentiation ($F_{ST} = 0$)

3. Population Definitions

- All *An. gambiae* samples from the same geographic location were treated as a single population
- All *An. arabiensis* samples were grouped as a seventh population for cross-species comparisons.

3.2.4.5 Mantel Test Analysis of Isolation-by-Distance (IBD)

To investigate the role of geographic distance in shaping genetic differentiation, a Mantel test was done to test for isolation by distance (IBD) between populations by combining Euclidean geographic distances (calculated from geographic coordinates) and genetic distances based on 9999 permutations using GenAlEx 6.51b2 (Diniz-Filho et al., 2013a; Peakall & Smouse, 2012). For *An. arabiensis* populations, the same F_{ST} and Mantel tests were also conducted using the 3L chromosome arm biallelic SNPs but only for the three mainland populations. The islands samples were not analyzed because of their small sample sizes.

3.2.4.6 Doubleton Analysis

Doubletons are loci where the minor allele is present exactly twice in the dataset, across all sampled individuals (Mathieson & McVean, 2014; Reynolds et al., 2025; The Anopheles gambiae 1000 Genomes consortium, 2017a). Numbers of doubleton heterozygote pairs were tabulated within and between population groups (*An. arabiensis*, mainland *An. gambiae*, island *An. gambiae*). Chi-square tests compared the observed to the expected counts (calculated based on group sample size proportions), revealing patterns of gene flow or isolation.

3.2.4.7 Bayesian Clustering (STRUCTURE Analysis)

To infer population genetic structure, Bayesian clustering was conducted using the software STRUCTURE version 2.3.4 (Pritchard et al., 2000). This method probabilistically assigns individuals to genetic clusters based on multilocus genotype data, while accounting for potential admixture and allele frequency correlations among populations (Pritchard et al., 2000).

3.2.4.8 Parameter Settings and Run Conditions

STRUCTURE was run with 20 independent runs for each hypothesized number of clusters (K), from 2 to 8. Each run consisted of:

- A burn-in period of 100,000 iterations to allow the Markov chain to reach convergence
- A subsequent 100,000 Markov chain Monte Carlo (MCMC) iterations to sample from the posterior distribution of parameters (Evanno et al., 2005a).

With a burn-in period of 100,000 iterations followed by 100,000 MCMC iterations. Convergence was verified by the high consistency of results across 20 independent replicate runs for each *K*, as assessed using the CLUMPAK pipeline

3.2.4.9 Post Processing and Visualization

To account for stochastic variation among runs and align cluster labels across replicates, we used CLUMPAK software that employs a Markov clustering algorithm to; (1) identify distinct clustering modes among replicate runs for each K, (2) generates concensus ancestry proportions for each mode and (3) aligns clusters across different K values for consistent interpretation (Kopelman et al., 2015a). The final ancestry proportions and cluster assignments were visualised using distruct, which provides a graphical representation of individual admixture patterns (Rosenberg, 2004).

3.2.4.10 Genetic Diversity and Neutrality

To assess the genetic diversity within each population, the nucleotide diversity (π) which represents the average number of nucleotide differences per site between two randomly sampled sequences was calculated (Nei & Miller, 1990a). Nucleotide diversity was computed for each population and chromosome arm to evaluate the extent of genetic variation.

To test for deviations from the standard neutral model of molecular evolution, Tajima's D was computed for each population and chromosome arm. Tajima's D compares two estimates of genetic diversity:

- number of segregating sites (S) and
- average pairwise nucleotide differences (π), which two estimates are expected to be equal under neutrality (Tajima, 1989b).

The significance of Tajima's D was assessed by comparing the observed values with the expected distribution under neutrality (Tajima, 1989a). Both computations (nucleotide diversity and Tajima's D) were done using the Pegas package in R (Paradis, 2010a) which aligns the methodologies described in Tajima (1989) for neutrality testing and Nei and Miller (1990) for estimating nucleotide substitutions, ensuring robust and comparable results.

3.2.4.11 Effective Population Size Estimation

Contemporary effective population size (N_e) was estimated using linkage disequilibrium (LD) method (Waples & Do, 2008), as implemented by NeEstimator program (Do et al., 2014a). NeEstimator calculates Ne based on non-random associations of alleles at different loci, with estimates generated for each population using SNP markers and provided insights into the demographic stability of island versus mainland populations (R. S. Waples & Do, 2008).

Data preparation and estimation procedure

Genotype data was changed into a format compatible with the NeEstimator, which accepts standard file formats like GENEPOP or FSTAT, and only SNP markers with MAF $\geq$ 1% were included in the analysis to minimise bias from rare alleles (Do et al., 2014a). The LD method was used to calculate pairwise r^2 as a measure of LD for all combinations of

unlinked loci, adjusted for finite sample size and the overall mean r^2 was computed as a weighted average across locus pairs (Waples & Do, 2008). Ne was then derived from the bias-corrected relationship between r^2 and effective size (Waples & Do, 2008). To ensure robust estimates, NeEstimator employs a weighted harmonic mean approach to adjust for missing genotypes (Peel et al., 2013a), and confidence intervals of Ne estimates quantified using jackknife resampling across locus pairs (Waples & Do, 2008).

3.3 Target Malaria

This research was conducted within the framework of Target Malaria, a not-for-profit research consortium whose focus is on developing and sharing new, cost-effective and sustainable genetic technologies to modify mosquitoes and reduce malaria transmission (Target Malaria, 2025). It is developing innovative genetic technologies in a stepwise approach, for malaria vector control that will be sustainable and cost-effective to implement, given that the mosquitoes themselves will help to control malaria transmission (Target Malaria, 2025). Target Malaria consists of scientists, stakeholder engagement teams, regulatory affairs experts, project management teams, risk assessment specialists and communications professionals from Africa, North America and Europe (Target Malaria, 2025).

Target Malaria specifically focuses on developing genetic technologies for suppressing numbers of malaria-transmitting mosquitoes in sub-Saharan Africa through a stepwise development pathway (Target Malaria, 2025). These technologies are designed to be sustainable and cost-effective, a key component of which is the gene drive system which is built to bias inheritance patterns and spread desired traits rapidly through wild mosquito populations (Burt, 2014; Burt and Crisanti, 2017). Target Malaria's preparatory activities include entomological surveillance, genomic characterization of malaria vector

populations, stakeholder engagement and ecological risk assessments (Target Malaria, 2025). These efforts are essential to generate baseline data for field trials to ensure the safe and context-appropriate implementation of genetic technologies.

This thesis directly contributes to the Target Malaria objectives, specifically;

- Objective 1 presented a comprehensive narrative review (Mwima et al., 2023) of the potential mechanisms by which malaria vectors persist through the dry season including aestivation, long-distance migration, local migration, and refugia, highlighting methodological limitations in previous studies and laying the groundwork for the genetic inference work that followed.

- Objective 2 (Mwima et al., 2024) introduced a novel population genetic framework to estimate the proportion of mosquitoes undergoing aestivation based on temporal allele frequency dynamics, using data from *Anopheles coluzzii* in Mali. This work demonstrated the feasibility of detecting aestivating proportions using genomic data and probabilistic modeling given the challenge of sampling aestivators .

- Objective 3 was an extension of the novel population genetic framework to estimate both aestivating and LDM proportions that contribute to the rebounds at the start of a rainy season. This work revealed striking differences in aestivation rates between climatic regions: Sahelian (Mali) and equatorial (Eastern Uganda) populations and underscores how seasonality gradients shape mosquito survival tactics. These insights are critical for Target Malaria's gene drive strategies and help inform where and when releases could maximize impact by disrupting rebound dynamics.

- Objective 4 was designed to investigate the genetic structure and connectivity between island and mainland populations of *An. gambiae* and *An. arabiensis* at

Ugandan island and mainland sites using amplicon sequencing. The study focused on assessing levels of genetic diversity, population differentiation and patterns of gene flow across these sites. This work addresses one of the foundational needs of Target Malaria, by identifying and characterizing populations that may serve as suitable sites for potential gene drive releases. This work also contributes to parameterizing models that predict gene drive spread and persistence and informs the design of post-release surveillance and resistance management plans.

This thesis thus builds the scientific and operational foundation for evidence-based decision-making regarding the implementation of novel malaria vector control interventions, such as gene drive in malaria endemic settings. These findings also align with Uganda's National Malaria Strategic Plan, which emphasizes integrated vector control management and evidence-based evaluation of emerging tools as the country transitions toward malaria elimination.

CHAPTER FOUR: PERSISTENCE MECHANISMS OF THE MAJOR ANOPHELES GAMBIAE SPECIES COMPLEX MALARIA VECTORS IN SUB-SAHARAN AFRICA

Preamble

Even with enormous progress over the years, malaria remains a burden globally, especially in the African region, because of the exceptionally robust vector dynamics of *Anopheles* mosquitoes (Bhatt, Weiss, Cameron, Bisanzio, Mappin, Dalrymple, K. E. Battle, et al., 2015; Lehmann et al., 2010). Sub-Saharan Africa carries the greatest burden(WHO, 2024) with *Anopheles gambiae s.s*, *Anopheles coluzzii*, *Anopheles arabiensis* and *Anopheles funestus* as the key malaria vectors (Magombedze et al., 2018). Malaria transmission is highly seasonal and associated with vector populations that exhibit strong seasonal fluctuations in abundance, being present in large numbers during the rainy season (Mabaso et al., 2005; Yamana & Eltahir, 2013), but drop to deficient numbers during the dry season when the breeding sites dry up (Charlwood et al., 1995; Hidalgo et al., 2016). These species survive in areas that experience relatively long dry periods (Adamou et al., 2011; Lehmann et al., 2010).

Despite efforts to reduce malaria transmission, malaria mosquito population rebounds that are associated with their persistence remain a crucial hindrance to local malaria elimination, and this is due to the poorly understood issue of how the malaria vector survives long dry spells to re-emerge after the rains start (Magombedze et al., 2018). Understanding dry season survival attributes, which may be the driving force behind population persistence, is important given that it could have implications for vector control strategies and, in turn, help to specifically target the malaria vectors that survive during the dry season. A critical

and unresolved question is how these mechanisms of persistence, dry-season survival and post-dry-season population rebound differ between geographically distinct regions with contrasting ecologies. This comparative question leads to the central hypothesis that geographic variation in environmental drivers fundamentally shapes the survival strategies of mosquito populations, governing their recovery and the subsequent risk of malaria transmission

In this study, we differentiate between the persistence mechanisms used by malaria vector species in the equatorial regions, such as Uganda, Kenya, and Rwanda, among others, and Sahelian regions, which include, among others, countries like South Sudan, Mali, and Burkina Faso. In the Equatorial region, there could always be surface water available nearly all year round, or the dry season could be short relative to the mosquito life cycle, while in the Sahelian region, there is no surface water in vast areas spanning the long dry season that usually lasts between 3 and 8 months.

In the basin region of Lake Victoria in Western Kenya, a mosquito field survey showed that malaria mosquitoes survived in two ways: (1) they continued reproducing throughout the year and (2) embryos survived in moist soils for several days (Minakawa et al., 2001). In another study carried out in Thierola village in Mali, one mosquito was recaptured about 7 months after several mosquitoes were captured, marked and released, being proof of malaria mosquito persistence by aestivation (Lehmann et al., 2010).

On this note, we propose that malaria mosquito persistence mechanisms are investigated for different regions to inform the effectiveness of the already existing and new malaria vector control tools such as gene drive systems which constitute a new set of tools that either replace or suppress malaria mosquito populations. This work has been published.

The word version of this article published in 2023 by the *Malaria Journal* is attached (https://malariajournal.biomedcentral.com/articles/10.1186/s12936-023-04775-0) (Mwima et al., 2023).

Potential persistence mechanisms of the major *Anopheles gambiae* complex malaria vectors in sub-Saharan Africa: a narrative review

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Conflict of Interest Statement

The authors declare that there is no conflict of interest

Abstract

The source of malaria vector populations that re-establish at the beginning of the rainy season is still unclear, yet knowledge of mosquito behaviour is required to effectively institute control measures. Alternative hypotheses like aestivation, local refugia, migration between neighbouring sites, and long-distance migration (LDM) are stipulated to support mosquito persistence. This work assessed the malaria vector persistence dynamics and examined various studies done on vector survival by aestivation, local refugia, local or long-distance migration across sub-Saharan Africa, exploring a range of methods used, ecological parameters and highlighting the knowledge trends and gaps. The results about a particular persistence mechanism that supports the re-establishment of *Anopheles gambiae*, *Anopheles coluzzii* or *Anopheles arabiensis* in sub-Saharan Africa were not conclusive given that each method used had its limitations. For example, the Mark-Release-Recapture (MRR) method whose challenge is a low recapture rate that affects its accuracy, and the use of time series analysis through field collections whose challenge is the uncertainty about whether not finding mosquitoes during the dry season is a weakness of the conventional sampling methods used or because of hidden shelters. This, therefore, calls for further investigations emphasizing the use of ecological experiments under controlled conditions in the laboratory or semi-field, and genetic approaches, as they are known to complement each other. This review, therefore, unveils and assesses the uncertainties that influence the different malaria vector persistence mechanisms and provides recommendations for future studies.

Keywords *Anopheles*, persistence mechanisms, dry season survival, malaria

Background

Malaria vector populations exhibit strong seasonal fluctuations in abundance and are present in large numbers during the rainy season but drop to extremely low levels when the larval habitats dry up (Lemasson et al., 1997; Taylor et al., 1993; Touré et al., 1994). This has been observed within members of the *Anopheles gambiae* species complex (or *Anopheles gambiae sensu lato*) (Diptera: Culicidae) and beyond and across diverse ecological or geographical set-ups, including the West-African Sahel and East Africa Savanna. Prevailing hypotheses suggest that the possible ways that could explain the seasonal malaria mosquito population dynamics are: (1) local mosquito populations experience dry season bottlenecks and are sustained by a few survivors (aestivation) (Lehmann et al., 2010); (2) local populations become extinct and few migrants from neighbouring areas, where permanent breeding occurs, recolonize the area at the beginning of the rainy season (local migration) (Atieli et al., 2023; Service, 1997); (3) the local population gets extinct during the dry season and is recolonized by long-distance migrants from stable areas (Long-Distance Migration, LDM) (A. S. Dao et al., 2014); and (4) large populations survive locally but are hidden with respect to sampling methods (also known as hidden or local refugia) (Charlwood, et al., 2000; Connell & Slatyer, 1977)

Despite the findings at hand from different studies, the source of malaria mosquito populations that re-establish at the start of a rainy season remains a mystery mostly because getting direct evidence of adults in their hidden shelters or even recapturing marked mosquitoes around the release sites is difficult to attain (Faiman et al., 2022a; Lehmann et al., 2010). Genetic studies have been conducted to test whether populations undergo annual dry season bottlenecks (Lehmann et al., 1998, 2017) but have not yielded conclusive results. This could be because of the type of loci that are targeted, using an insufficient number of loci that negatively impacts the statistical power, unavailability of mosquito

samples with longer alternating time series, using limited sample collection methods (which are not representative of both endophilic and exophilic fractions of a particular population to account for behavioral heterogeneity and aid in estimating total effective population size (Ne)), and no knowledge of how selection affects allele frequency changes and consequently Ne estimates (Lehmann et al., 2017; Lehmann, , et al., 1998; Simard et al., 2000; Taylor et al., 1993).

It is essential to distinguish between the persistence mechanisms used by malaria vector species in either the Equatorial or Sahelian regions. It is important to note that in the Equatorial region, there could always be surface water available nearby all year round or the dry season could be short relative to their life cycle (that is, less than 2 months). Therefore, mosquito persistence mechanisms might not be required or could be by local migration or local refugia. In the Sahelian region on the other hand, there is never surface water in vast areas spanning the long dry season that usually lasts between 3 to 8 months.

The actual persistence mechanisms used by malaria vector species in sub-Saharan Africa (Fig. 4.1) is a conundrum, given that the four hypotheses explain the rapid mosquito rebounds at the beginning of each wet season (Adamou et al., 2011; Lehmann et al., 2010, 2017). Various studies concerned with which populations contribute to the early rainy season malaria mosquito rebounds have been carried out, and in this review, their strengths and weaknesses were accessed based on the study design, the methods used and whether the conclusions support the results and thereafter highlight the gaps that remain therein (Table 4.1). This review, therefore, focuses on the uncertainties of the persistence mechanisms utilized by malaria vectors across sub-Saharan Africa.

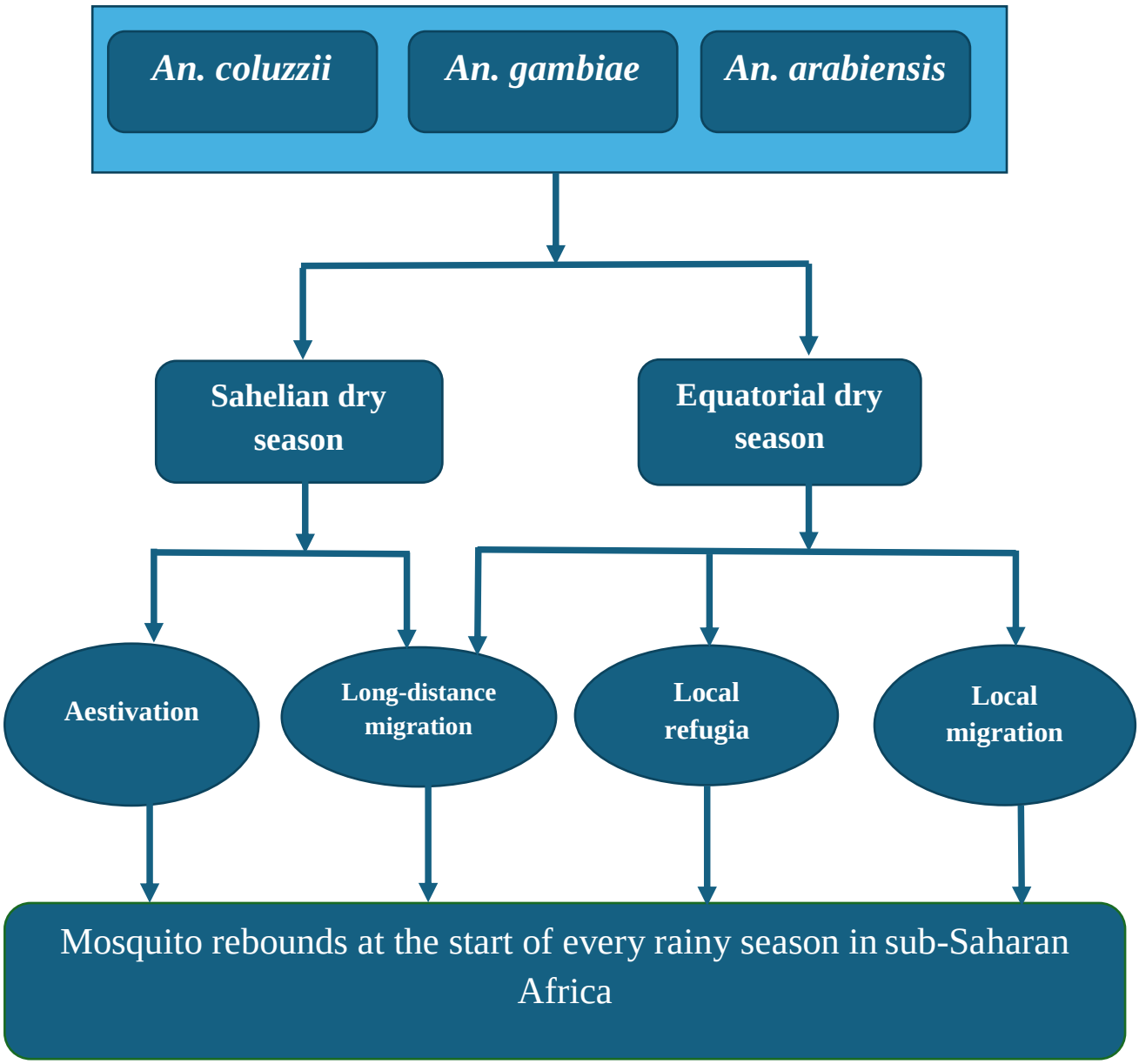

Figure 4.1 Schematic diagram for persistence mechanisms

Schematic diagram showing the different persistence mechanisms responsible for the early rainy season malaria mosquito rebounds across sub-Saharan Africa. The four hypotheses could be responsible for population rebounds of An. gambiae species complex at the start of each rainy season

Anopheline Mosquitoes in sub-Saharan Africa

In sub-Saharan Africa, the main groups of malaria vectors are *An. gambiae*, *An. coluzzii*, *An. arabiensis*, and *Anopheles funestus* (Sinka et al., 2012; Wiebe et al., 2017b), which are genetically distinct (Harbach, 2004b). *Anopheles gambiae* and *An. coluzzii* were once

considered as one species until recently. They remain as part of the *An. gambiae* species complex alongside *An. arabiensis*, hence are morphologically inseparable. It is worth mentioning *An. funestus*, which belongs to its own group of species (A. Y. Afrane et al., 2012; Coetzee et al., 2000, 2013). The four are amongst the most efficient, broadly distributed, and dominant malaria vectors in sub-Saharan Africa. These species inhabit diverse environments that include areas where the water that is required for larval development is absent for more than four months (Lehmann et al., 2010). Their bionomics vary according to species and in several aspects, such as biting rates, duration of their gonotrophic cycles, fecundity, survival, and development of immature and adult stages. *Anopheles arabiensis* lives in dry savannah environments but occupies similar larval habitats to *An. gambiae* (Tandina et al., 2018a), thus, occurs in sympatry with their relative abundance dependent on local ecological conditions (Hay et al., 2000b). It is known that "*An. gambiae* is predominantly anthropophagic and endophilic, and together with its longevity, has a higher vectorial capacity than other species of the *An. gambiae* complex" (Hay et al., 2000b). The *An. gambiae* species complex is the major malaria vector characterized by endophagy (preferences for obtaining blood meals indoors), anthropophily (blood meals from humans), and endophily (indoor resting following blood meals) (Touré, 1994).

Its distribution spans most of sub-Saharan Africa and can survive under a wide range of ecological, geographical and seasonal conditions (Hay et al., 2000b). *Anopheles coluzzii* has high ecological plasticity; thus, it can exploit different habitats (Kamdem et al., 2012; Lehmann & Diabate, 2008) and has an opportunistic host-seeking behaviour (Lefèvre et al., 2009b). However, *An. arabiensis* is known for its ecophenotypic plasticity and is predominantly exophagic (feeds outdoors) and exophilic (rests outdoors) (Hay et al., 2000b). Because of its ability to develop in residual pools of water in dry riverbeds, it can

survive arid conditions and in turn rapidly become abundant at the onset of rains (Hay et al., 2000b).

The Biology of Malaria Mosquito Persistence

The *Anopheline* mosquito populations withstand dry conditions which could last three to 8 months (Arcaz et al., 2016), equivalent to several generations of their lifetime (Magombedze et al., 2018). The hypotheses that explain malaria mosquito persistence mechanisms are aestivation (Lehmann et al., 2010), persistence at local refugia (Charlwood, et al., 2000) local migration (Lehmann et al., 2014) and LDM (Huestis et al., 2019). Aestivation is a repeated state of summer dormancy that constitutes suppressed reproduction and growth to ensure extended mosquito survival during the harsh conditions of the dry season (Lehmann et al., 2010). Local refugia populations are those that can survive under adverse conditions but remain hidden with respect to conventional sampling methods and can only be found by actively searching for them (Charlwood, et al., 2000). Local migration involves mosquito movement from adjacent areas, while LDM is the movement of mosquitoes to favourable areas from further fields, potentially hundreds of kilometres (km) away and is predominantly wind-aided (Huestis et al., 2019).

The mechanisms by which *An. gambiae* species complex persist throughout the dry season vary from the Equatorial to Sahelian region across sub-Saharan Africa (Ramsdale et al., 1970). Unlike the Sahelian region, the Equatorial region experiences a milder dry season during which some larval sites remain available within a 5 to 10 km radius (Charlwood, , 2000; Minakawa et al., 2001). These few but constantly available larval sites during the dry season are known to act as a strong selection force against aestivation as the persistence mechanism used (Huestis & Lehmann, 2014). Instead, refugia populations are said to occupy these distinct hidden habitats during the dry season, which sites could be difficult to detect using conventional sampling methods (Charlwood, et al., 2000)

Distinguishing between whether the lack of direct evidence for aestivating females during the dry season could be because of the conventional sampling methods or total absence is very difficult (Charlwood, et al., 2000). However, the difference between aestivating mosquitoes and those maintained as refugia is that aestivating females become gonotrophically discordant, and could either fail to develop eggs after taking a blood meal (Omer et al., 1970), or because they lack suitable oviposition sites, do not lay eggs, but instead, dissolve them and use that as their source of energy (Chisulumi et al., 2022), while refugia populations continue to breed. It is thought that they can still be found by actively searching for them (Charlwood, et al., 2000).

It is believed that aestivation is predominantly activated by the absence of water at all the stages of malaria mosquito growth (Depinay et al., 2004). The eggs of *Anopheles* cannot survive more than 15 days on dry soil (Koenraadt et al., 2003), therefore, with several months without rain or surface water, the adult stage provides the most possible route for survival (Depinay et al., 2004). During the dry season, malaria vectors generally become susceptible to water loss caused by increased evaporation rates through their spiracles and cuticles (Holmes & Benoit, 2019). This water loss is linked to reduced survival and oviposition (Canyon et al., 1999), reduced nutritional reserves and egg production (Benoit & Denlinger, 2007) and changes in macrogeographic and microgeographic distributions (Reidenbach et al., 2014).

Dehydration stress has over time resulted in genetic alterations and behavioural adaptations that interact with mosquito physiology, survival and distribution (Reidenbach et al., 2014). This could imply that these species experience fitness trade-offs deduced from the fact that, the 2La inversion is associated with higher desiccation resistance and are high in frequency (higher fitness traits) among *An. gambiae* and *An. coluzzii* populations found in arid areas;

however, this is rare or even absent in areas where water is readily available (Reidenbach et al., 2014). The 2La chromosomal inversions are reported to drive the cuticle thickness and cuticular hydrocarbon (CHC) composition that are responsible for the desiccation-resistant phenotype (Reidenbach et al., 2014). Within the *An. gambiae* species complex, dry season metabolic characteristics are evidently similar but show that suppression in metabolic and reproductive processes support the adaptive potential to survive by changing their cuticular, metabolic and behavioural traits (Hidalgo et al., 2014).

In a genome-wide laboratory-based survey of *An. gambiae* species complex populations, 33 *An. gambiae* desiccation-responsive genes that exhibited reduced transcript accumulation when mosquitoes were exposed to the desiccation treatment and 50 desiccation-responsive genes with known metabolism-related functions altered in response to dehydration were identified (Wang et al., 2011). The results from this survey also showed that the number of genes expressed is dependent on the duration of desiccation stress (Wang et al., 2011). *Anopheles gambiae* and *An. coluzzii* are known to have the 2La and 2Rb chromosomal inversions (Reidenbach et al., 2014), which could be associated with aestivation, body size (Fouet et al., 2012) and dry season survival mechanisms (Cheng et al., 2018).

In addition to 2La and 2Rb chromosomal inversions, *An. gambiae* species complex has other inversions and combinations (2Rc, 2Rd and 2Ru) that are said to be non-randomly correlated with adaptations to arid conditions (White et al., 2009). These inversions are controlled by the environment and could contribute to local adaptation, habitat range, and desiccation tolerance (Gray et al., 2009; Reidenbach et al., 2014; B. J. White et al., 2011), and may also influence some of the variations in competence for *Plasmodium* (B. J. White et al., 2011). Inversion polymorphisms among local populations could temporally change

depending on the seasonal dynamics (Touré et al., 1999), which explains how various molecular forms of *An. gambiae* species complex develop acclimatization to dry season and increased survival (Hidalgo et al., 2014).

However, apart from the genetic characteristics, because of the high rates of evaporation through their respiratory spiracles and cuticle, mosquitoes are predisposed to water loss which they could deal with by employing several behavioural adaptations, and altering their body size, metabolism and cuticular hydrocarbon composition (Benoit & Denlinger, 2007; Holmes & Benoit, 2019; Rinehart et al., 2006; Yang et al., 2017). Phenotypic differences such as adult body size, reproductive output and longevity could indicate that malaria mosquito molecular forms are adapted to specific niches (Lehmann & Diabate, 2008).

The adult *Anopheles* mosquito has a lifespan of less than a month however, some studies indicate that they could survive for over three months during the dry season (Dao et al., 2014; Depinay et al., 2004; Lehmann et al., 2010; Yaro et al., 2012). Results from the studies that have been carried out in the *An. gambiae* species complex on how they survive more than 4 months of harsh dry season conditions have showed that compared to the wet season; there was a dramatic extension of lifespan (Lehmann et al., 2010; Omer et al., 1968), they were reproductively suppressed in a state of gonotrophic dissociation (Omer et al., 1970); had a 70% reduction in reproduction (between the wet and dry season, the oviposition rate dropped from 70 to 20%, the mean number of eggs per female reduced from 173 to 101 and gonotrophic dissociation increased from 5 to 45%) (Yaro et al., 2012), an 80% reduction in flight activity and the metabolic rate was highest during the dry season (Huestis et al., 2012).

A key feature of aestivation is that it involves a pre-programmed suite of physiological changes that occur in response to one or more external cues such as changes in photoperiods

and high temperatures that predict future environmental changes and trigger certain changes in the mosquito to enable it to survive (Huestis & Lehmann, 2014). For mosquitoes in the Sahelian region, the primary forces known to drive aestivation are (1) the absence of surface waters for larval site development (2) temperature fluctuations (3) changes in relative humidity which could confine flight to certain times of the night (Huestis & Lehmann, 2014). This means that mosquito behavioral changes in selecting suitable microhabitats, suitable times of activity and rest may contribute to physiological changes and not necessarily rely on them (Huestis & Lehmann, 2014). Other behavioral changes that are noted to occur during the dry season include modification of their feeding habits by switching from human blood to other sources, such as flower nectar and woody-plant juices (Müller, Beier, Traore, Mahamoudou B. Toure, et al., 2010), which are low in protein and could in part be the reason for gonotrophic dissociation that is observed in aestivating adults (Huestis et al., 2012; Huestis & Lehmann, 2014; Yaro et al., 2012).

In addition to that, when anticipating the coming dry season, *An. coluzzii* have been observed to nearly disappear from villages approximately one month before the larval sites dry up (Adamou et al., 2011; Huestis et al., 2012; Lehmann et al., 2010; Yaro et al., 2012). The work by Huestis and Lehmann (Huestis & Lehmann, 2014) hypothesized that behavioural changes in selecting suitable microhabitats in shelters and suitable periods of activity and rest, play a large role in complementing physiological changes, rather than relying on them completely, as is the case for winter diapause. This can also be supported by the results from the Magombedze *et al*. (Magombedze et al., 2018) study in which two selection bottlenecks that drive phenotypic plasticity occurred: at the beginning of a dry season and selected for mosquitoes able to survive the long dry season, and at the start of the new wet season. These results were comparable to other studies that suggest that malaria mosquitoes in the Sahel region do not use inherited traits (mosquito adaptation) to survive

ever-changing environmental conditions but instead employ a phenotypic switch (Bell & Collins, 2008; Chevin et al., 2010; Denlinger & Armbruster, 2014).

When reproductive depression was assessed in *An. coluzzii* populations from the Sahel region, the results showed marked seasonality in the reproductive physiology, a drop in response to oviposition, and increased gonotrophic dissociation, which are signs that support survival throughout the dry season by aestivation (Yaro et al., 2012). Depressed reproduction is, therefore, the most fundamental feature of diapause in adult insects (Yaro et al., 2012), which generally means that for aestivating mosquitoes, during the long dry spell, resources are diverted from reproduction to survival (Yaro et al., 2012). The key changes noted to happen during the dry season are (1) reduced reproduction (Yaro et al., 2012), (2) reduced flight activity (Huestis et al., 2012), (3) increased tolerance to desiccation attributed to changes in cuticular hydrocarbons (Arcaz et al., 2016), and (4) metabolic and protein changes (Mamai et al., 2014).

The major *An. gambiae* species complex is noted to undergo these changes only in response to certain external stimuli or cues such as changes in photoperiod, temperature, and moisture availability, among others that predict the beginning of an environmental change (Huestis & Lehmann, 2014). The cues that have predictors are better suited to initiate aestivation while those without may instead reinforce or maintain it (Huestis & Lehmann, 2014). For example, changes in moisture content (disappearance of larval sites) are a result rather than a predictor of a dry season, while changes in photoperiod are a predictor that a change in day lengths has occurred and, therefore, initiate aestivation (Huestis & Lehmann, 2014). Case in point was when the responses of *An. coluzzii* and *An. arabiensis* to changes in photoperiod and temperature were compared under dry season conditions, results showed that longevity, body size and total lipids of *An. coluzzii* increased, while those for

An. arabiensis decreased, a signal that *An. coluzzii* entered the diapause initiation phase (Huestis et al., 2017).

Thus, given that *An. gambiae* species complex is highly sensitive to temporary oviposition-site deprivation, even dry spells that last just a few days during the wet season can reduce reproductive success (Faiman et al., 2017). This means that their physiology modifies the effect of oviposition-site deprivation on their reproductive output (Faiman et al., 2017), and because oviposition is largely controlled by water availability, with contributions from humidity and rainfall (Wagoner et al., 2014), not finding suitable larval sites may be an indication used by mosquitoes to switch from their reproductive state to reproductive depression during the dry season (Charlwood, et al., 2000; Huestis et al., 2017; Lehmann, et al., 2014; Yaro et al., 2012). The wind-aided LDM is the other mechanism by which *An. gambiae* species complex persists through the dry season. So far, studies show that LDM takes place in both the Equatorial and Sahelian regions as a means of survival for members of this species (Atieli et al., 2023; Huestis et al., 2019).

However, from earlier studies carried out in the Sahel, there was scepticism on whether the surge in population was an indication of migrants from the neighbouring areas or whether they were hidden in the same locality (Lehmann et al., 2010). This was because the neighbouring villages could not serve as a source of migrants, and given that there were low densities of adults throughout the whole area, the Sahelian villages were isolated (Adamou et al., 2011; Lehmann et al., 2010) with studies at that time pointing to the fact that mosquito dispersal over a distance of 2-3 kilometres was unusual (Constantini et al., 1996; Touré et al., 1998). However, an extensive aerial sampling experiment of mosquitoes at 40-290 metres above ground level confirmed the occurrence of windborne migrations among malaria vectors and was estimated to span tens to hundreds of kilometres in a single

night (Huestis et al., 2019). The same study collected 23 *An. coluzzii*, but only 1 *An. gambiae*, among the 235 *Anopheline* mosquito migrants, something that contradicted the initial predictions that *An. coluzzii* solely survive the long dry spell by aestivating locally and not through migration in the Sahelian region (A. S. Dao et al., 2014; Lehmann et al., 2010). *Anopheles coluzzii* could, therefore, survive the long dry spells in the Sahel region by aestivation accompanied by long-distance migration that is said to take place in the late rainy season, otherwise, without migration, the small Sahelian population that survives the dry season through aestivation would become locally extinct (Lehmann et al., 2017) because of the unpredictable dry spells that occur during the rainy season (Hastenrath & Polzin, 2011, 2014; Salack et al., 2014). This attests to the complexity of species, presenting the two strategies (aestivation and LDM) as mutually exclusive.

Following wind-borne migration, the ability of each migrant to arrive at a favourable habitat is influenced by changing windspeeds and direction together with the distribution of habitat patches (Gatehouse, 1994). Migrants could be displaced over hundreds of kilometres in one night, and this may happen for several days (Drake & Farrow, 1988), depending on the flight capacity and the flight period (Gatehouse, 1994). The key predictors of long-distance migration include; (1) extinction of the local population during the dry season followed by an abundant rise in population by migrants from areas with favourable climatic conditions that maintain larval sites, (2) the genetic make-up of migrants that arrive from other areas at the start of the rainy season will be distinct from that of the previous dry season, and (3) when populations are sampled at different time points, large genetic drift is expected, a sign that continuous reproduction has been taking place (Lehmann et al., 2017). In genetic studies, these predictors enable the evaluation and differentiation of various explanations for dry-season survival.

Approaches to Studying Malaria Mosquito Dry Season Survival and Population Rebounds

Two approaches, direct (ecological) and indirect (genetic) are used to study the seasonal dynamics of malaria vectors (Simard et al., 2000). The direct approach mainly utilizes the mark-release-recapture (MRR) experiments (Simard et al., 2000), while the indirect approach relies on the genetic information from the samples collected. These include genetic diversity, population differentiation parameters, and temporal variation in allele frequencies, as a measure of genetic drift and N_e (Lehmann et al., 2017; Taylor et al., 1993). Results from indirect and direct approaches complement each other but are also usually different because the population size varies greatly throughout the year with estimates from the direct approach made when the population is near its maximum while that of the indirect approach is the effective population size (Ne) estimate which represents some sort of yearly average (harmonic mean) (Touré et al., 1998). Several studies using direct or indirect approaches to investigate the different mosquito persistence mechanisms across sub-Saharan Africa have been carried out and are summarized in Table 4.1.

Table 4.1: *Summary of studies on persistence mechanisms of malaria mosquitoes in sub-Saharan Africa*

Method, persistence mechanism being tested & reference	**Strengths of the method used**	**Weaknesses of the method used**	**Key assumptions of the method**	**Comments**
Mark Release Recapture (MRR) to determine whether malaria mosquitoes survive the dry season by aestivation (Epopa et al., 2017; Faiman et al., 2022; Lehmann et al., 2010; Touré et al., 1998)	Informs population size, survival rate and movement Maybe the only method that can provide unequivocal proof for aestivation and migration.	Affected by the dry season with few or no mosquitoes Without sequencing of recaptured mosquitoes, results are not confirmatory (aestivation/local refugia) Low recapture rate, thus affecting the accuracy of the method Does not reveal where mosquito shelters are and how they cope with the DS	The marked mosquitoes become re-integrated into the rest of the population Marking mosquitoes does not adversely affect them Mortality of marked mosquitoes caused specifically by their recapture is ignored Mortality rate is constant throughout	**Aestivation** The process is difficult to reproduce Some females break their aestivation more readily than others
Lab studies to determine whether malaria mosquitoes survive the dry season by aestivation (Krajacich et al., 2020a)	In this study, the maximum lifespan of *Anopheles* mosquitoes was over 100 days representing maximum longevity compared to standard insectary conditions by 2.2-3.5-fold	Laboratory colonies lose genetic diversity in a few generations Laboratory conditions do not recapitulate all the possible cues present in the field. The lack of unambiguous markers of aestivation in *Anophelines* made it difficult to clearly confirm whether it really happened Demonstrating aestivation in its entirety in the lab is still a challenge (Krajacich et al., 2020a)	This study used somewhat exaggerated climatic conditions to induce longevity with reduced temperature and photoperiod	Using exaggerated photoperiods beyond what happens in Mali is likely to have pushed *An. gambiae* to have similar longevity to that of *An. coluzzii*, something studies carried out to date have not reported
Field collections to confirm whether mosquitoes survive by	No specific functional approach (Krajacich et al., 2018a)	Vector density too low during dry season	Changes occur in mosquito physiology and behaviour in dry season.	In dry season, females occupy hidden habitats

aestivation or as local refugia (Adamou et al., 2011b; Charlwood, Vij, Billingsley, et al., 2000a; Lehmann, Dao, Yaro, Diallo, et al., 2014; Minakawa et al., 2001; Omer et al., 1968)	Areas with determinants of high mosquito density are established to show sources of dry-season populations.	Distinguishing between absences that are a result of poor sampling and those which are legitimate is a challenge	Ovaries undergo one gonotrophic cycle in dry season and develop slowly	Low temperatures and relative humidity induce a state of arrested development.
Time series analysis (Field collections) to confirm whether mosquitoes survive by aestivation or migration (A. S. Dao et al., 2014)	More reliable results as mosquitoes are collected over a relatively long period of time	Not clear whether not collecting mosquitoes during dry season is a weakness of the sample collection method or because of hidden shelters.	When the mosquito recapture rate was less than 3%, the effect of removing them from the subsequent density instead of releasing them was negligible	Climate is one of the Selective pressures responsible for the ecological divergence between *An. coluzzi*i and *An. gambiae* species The *An. arabiensis* collected during the dry season Could be representative of backcrossed hybrids between *An. coluzzii* and *An. arabiensis*
Aerial sampling of mosquitoes at 40-290m above ground level to confirm whether mosquitoes undergo long distance migration (et al. Huestis et al., 2019)	Results disprove previous studies that malaria mosquito dispersal doesn't exceed 5km (Constantini et al., 1996; Touré et al., 1998)	There is need to separate the role of Odyssean malaria from windborne migrants Protocol optimisation is time consuming, takes close to 12 months	Mosquitoes ascend by their own flight but are also passively carried by wind altitude. Mosquitoes fly in a layer between 50 and 250m above ground level (and probably higher).	The likelihood of capturing *Anopheles* species increased with altitude. Malaria mosquitoes migrated over tens to hundreds of kilometres in a single night. Females outnumbered the males collected (4:1)

			Mosquito flights started at or after 18:00 and ended by 06:00 the following morning LDM-based migrants remain viable /reproductively fit	
Semi-field study (SFS) to test whether malaria mosquitoes survive the dry season by aestivation or migration (Mamai et al., 2016)	SFS bridge the conceptual and methodological gaps between laboratory and field experiments. Lifecycle completion is feasible inside the SFS	Laboratory colonies do not represent the wild type as they lose genetic diversity in a few generations A few larvae are sampled to avoid population depletion The hidden mosquito shelters used give a biased representation the natural environment	Aestivation and migration are the main mechanisms that explain variation in population dynamics	Study results showed that *An. coluzzii* and *An. arabiensis* aestivate while *An gambiae* could adopt a different dry season survival strategy such as LDM. Host feeding preferences could be involved in causing species variation of the SFS
The indirect approach: Using genetic data (Atieli et al., 2023; Lehmann et al., 2017; Müller et al., 2010; Touré et al., 1994)	The method used is sensitive to bottlenecks of population size (robust) Ne depends on both population density and patterns of movement Additional inference based on inter-annual and inter-seasonal changes in private alleles and other measures of pop genetic constitution may be key to identify continuation of breeding vs. migration.	Reliable estimates of Ne are difficult to obtain for natural populations. Violation of the assumptions considered could result in larger Ne values. More information is required to assess the effect of constraints on Ne estimates. Ne is not meaningful if we don't know the geographical area it represents and the population structure model these species follow.	Random mating between individuals, discrete generations, a sex ratio of one, negligible selection, migration and mutation.	Large populations are maintained throughout the dry season. Large populations could be maintained by individuals hidden with respect to sampling. Large populations could be maintained by extensive movement of adults.

Computer Simulations and Dynamic Models in Population Genetics to Study Mosquito Persistence Mechanisms

Malaria mosquito population genetic studies provide information about gene exchange between populations which is beneficial in making conclusions about the dispersal patterns of malaria vectors and in answering other ecological questions (F. H. Collins et al., 2000). These patterns make it possible to predict vector competence, whose knowledge is critical in vector control, especially in understanding malaria vector genetic population structure and barriers to gene flow (F. H. Collins et al., 2000).

Computer simulations assist to assess the potential validity of the different hypotheses, determine which areas to consider for experimental studies, establish expected genetic signatures under different hypotheses and guide experimental work (North et al., 2018. The use of dynamic models (used to simulate trajectories of change under different scenarios) is still in its infancy and is very important in highlighting several parameters such as changing temperature, mosquito dispersal, humidity, and mosquito size among others that contribute to vector dynamics observed in laboratory settings, semi-field conditions and the field (Lunde et al., 2013). The use of forward-time simulations (known to start from an initial population and follows its evolution from generation to generation) in population genetics to determine the origin of early wet season rebounds is promising and could be the most effective way to test between hypotheses (Hamad et al., 2002). Forward-time population genetic simulations play an important role in generating and testing evolutionary hypotheses that would be difficult to attain in laboratory settings because of the complexity of the process often known to be burdensome or even expensive (Ruths & Nakhleh, 2013).

The increase in population genomic data over the years has resulted in the use of more complex analyses using advanced simulation models (Adrion et al., 2020). These simulations are important for gaining an understanding of specific datasets used and in assessing and validating biological models (Escalona et al., 2016), while evaluating the sampling properties of any statistics used on genome-wide association studies to compare the performance of different methods used (Carvajal-Rodriguez, 2008). Simulations usually allow for the inclusion of stochasticity in a natural way to investigate the entomological parameters relating to dry season ecology and movement behaviour which are still unclear in malaria vector species (A. R. North et al., 2018b).

Discussion

Over the years, several studies on the dry season persistence of *An. gambiae* species complex in sub-Saharan Africa have been carried out in the field, laboratory, and in-silico and have generated vast information and insights. How malaria vectors survive the long dry season remains unclear but could be associated with locality and niche-specific influences. Results from a study done on *An. coluzzii* populations in the Sahel and Riparian areas showed a difference in the aestivation phenotypes within and between the two environments, which signifies that there is a possibility that various populations of the same species have specific dry season survival strategies that depend on the strength and duration of the dry season in that locality (Yaro et al., 2012). That could be the reason why *An. coluzzii* populations of similar geographic origins undergo persistent local adaptations, which are also anticipated to be influenced by specific microhabitats (Arcaz et al., 2016; A. S. Dao et al., 2014). These adaptations may also be responsible for the fact that *An. gambiae*, a highly anthropophilic species has become both anthropophilic and/or endophilic (Holmes & Benoit, 2019).

Whereas some studies provide evidence for aestivation, local refugia, local or long-distance migration, repeating similar studies usually does not replicate the results (Lehmann et al., 2010), thus, the need to handle each geographical area independently because different populations may present different dry-season survival strategies depending on the strength and duration of the dry season. Aboud *et al*., (2014) study in which *An. arabiensis* populations in South Sudan exhibited two phenotypic forms, one which was large and heavily melanized, while the other had the usual characteristics as found in other African settings (normal colour and size), results showed that the melanic form survived throughout the long dry season by partial aestivation, and was similar to populations found in *An. arabiensis* populations in Senegal (Besansky et al., 1997). The normal form, however, was inferred to persist by LDM (Adamou et al., 2011), which was further confirmed by Atieli et al., (2023). Therefore, more studies that are geared towards comparing *An. gambiae* species complex populations from various environments, especially where they occur in sympatry, are important.

Using a combination of approaches, both direct and indirect in tandem because they complement each other, could be a more credible way to not only understand dry season persistence mechanisms in *An. gambiae* species complex determine but also provides more insights into malaria vector population dynamics and how they affect vector control implementation. The marked mosquito recaptured at the start of the new rainy season (*An. coluzzii*) (Lehmann et al., 2010), and the *An. arabiensis* mosquitoes found at the end of the dry season (Omer et al., 1970) could either have survived by aestivation or as local refugia. Therefore, using both direct and indirect approaches in these studies could have resulted in more concrete and informed conclusions. Also, studies in genetic evolution and phenotypic plasticity combined with demography will assist in making predictions about population persistence in a changing environment. Population genetics using malaria mosquito genetic

data will create a better understanding of the extent to which mosquitoes at the start of a rainy season are genetically distant from the previous season populations (Lehmann et al., 2017).

Further studies could consider sequencing the whole *Anopheles* genome of mosquito populations from various areas in sub-Saharan Africa collected over several seasons to further elucidate the balance between longevity, reproduction, and migration of the three *Anopheles* species. Developing a modelling framework that could be extended into a spatial meta-population could also allow an assessment of the relative roles of different mosquito persistence mechanisms, together with their environmental triggers. This will assist in predicting which genetic signatures are responsible for the different persistence mechanisms since the possible views that could explain each of them, as mentioned earlier, if tested using population genetic structure and temporal stability of genetic composition within populations, have different expected outcomes (Simard et al., 2000). Key parameters such as within-sample genetic diversity, between-sample genetic distance, and temporal variance in allele frequency (Lehmann et al., 2017) could assist in making predictions based on each of the persistence mechanisms considered.

Using forward-time simulations in population genetics to determine the origin of early rainy season rebounds is promising and could be an effective way to test which persistence mechanism is more readily used by the three *Anopheles* species. Forward-time population genetic simulations track complete ancestral information and are significant for deriving and testing evolutionary hypotheses that could be burdensome or expensive (Ruths & Nakhleh, 2013).

Conclusion

Following studies to date, it remains still unclear which persistence mechanism(s) are responsible for the survival of each of the three *Anopheles* species known to contribute most to the malaria burden in sub-Saharan Africa. Using combined approaches (both ecological and genetic) is promising and has the added advantage of providing results that complement each other and provide more insights. This should reinforce the inexplicit theories that surround malaria vector population rebounds at the start of every rainy season. The clarity in this subject matter should also inform the effectiveness of the already existing and new malaria vector control tools which may include the use of genetically modified mosquitoes, which constitute a new set of tools noted to either replace malaria vector populations with introduced genes for refractoriness to limit malaria transmission or disrupt fertility genes and thus lower mosquito numbers to achieve vector population suppression.

These findings highlight dry-season survival as a critical ecological bottleneck that may determine the success of gene drive interventions targeting mosquito populations during periods of low abundance, and as a result provide a biological rationale for the modeling approaches developed in subsequent chapters.

CHAPTER FIVE: THE POPULATION GENETICS OF PARTIAL DIAPAUSE, WITH APPLICATIONS TO THE AESTIVATING MALARIA MOSQUITO *ANOPHELES COLUZZII*

Preamble

One of the key biological mechanisms that facilitates seasonal mosquito survival is aestivation, characterized by halted development and reproduction (Jiang et al., 2023; Krajacich et al., 2020b; Lehmann et al., 2010). Aestivating mosquitoes suspend reproductive activity and reduce metabolic rates, effectively surviving through the unfavorable conditions (Jiang et al., 2023; Krajacich et al., 2020b; Lehmann et al., 2010). Even though direct detection and sampling of aestivating individuals have proven difficult due to the cryptic nature of dormant mosquitoes which evade traditional sampling methods, understanding the extent and genetic implications of partial diapause is critical for characterizing population dynamics and informing vector control interventions (Dao et al., 2014; Denlinger & Armbruster, 2014; Yaro et al., 2012). This study addresses this knowledge gap by developing a novel genetic approach to infer the aestivating proportion without directly sampling dormant individuals and understanding how genetic drift and effective population size are impacted as a result. The study leverages temporal allele frequency dynamics to estimate key parameters, including the proportion of the population undergoing aestivation (α) and the effective breeding sizes during the RS and DS. By extending the classical Wright-Fisher model to incorporate partial reproductive arrest and applying the model using Approximate Bayesian Computation to temporal genetic data from *An. coluzzii* in Mali, the work provides quantitative insights into seasonal population structure and demographic fluctuations without requiring direct sampling of aestivating individuals. This approach deepens our understanding of malaria mosquito persistence

mechanisms during the dry season, with broad applicability to other species that undergo diapause.

This work highlights how a combination of applied entomology, the population genetics theory, and mechanistic models address ecological and evolutionary questions in vector biology, which insights are essential for designing effective vector management strategies that are tailored to seasonal dynamics and for predicting the evolutionary trajectories of vector populations in response to control pressures.

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The population genetics of partial diapause, with applications to the aestivating malaria mosquito *Anopheles coluzzii*

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Conflict of Interest Statement

The authors declare that there is no conflict of interest

Abstract

Diapause, a form of dormancy to delay or halt the reproductive development during unfavorable seasons, has evolved in many insect species. One example is aestivation, an adult-stage diapause enhancing malaria vectors' survival during the dry season (DS) and their re-establishment in the next rainy season (RS). This work develops a novel genetic approach to estimate the number or proportion of individuals undergoing diapause, as well as the breeding sizes of the two seasons, using signals from temporal allele frequency dynamics.

Our modelling shows that the magnitude of drift is dampened at early RS when previously aestivating individuals reappear. Aestivation severely biases the temporal effective population size (N_e), leading to an overestimation of the DS breeding size by $1/(1-\alpha)^2$ across one year, where α is the aestivating proportion. We find that sampling breeding individuals in three consecutive seasons starting from a RS is sufficient for parameter estimation and perform extensive simulations to verify our derivations. This method does not require sampling individuals in the dormant state, the biggest challenge in most studies. We illustrate the method by applying it to a published dataset for *Anopheles coluzzii* mosquitoes from Thierola, Mali.

Our method and the expected evolutionary implications apply to any species in which a fraction of the population diapauses for more than one generation and are difficult or impossible to sample during that stage.

Keywords: diapause, aestivation, persistence, genetic drift, temporal allele frequency

Background

Many insect species have evolved the mechanism of diapause, a form of dormancy, which halts development and reproduction under unfavorable environmental conditions (Ojima et al., 2018; Cloutier et al., 2021). Some forms of diapause may also include reduced activity such as flying and feeding. Metabolism is suppressed, and most energy resources are diverted toward survival. Diapause is pre-programmed and genetically coded, then activated by changing of environments (Ojima et al., 2018). Depending on the species, known stimuli include day length, temperature, and access to water. Examples of species that undergo diapause include *Drosophila suzukii*, *Phormia regina* (Queen blow flies), *Danaus plexippus* (Monarch butterfly), multiple moth species, tropical walking-stick insects, and many more (Herman and Tatar, 2001; Tatar and Yin, 2001; Toxopeus et al. 2016; Salmon et al., 2019; Yoder and Delinger, 1992). While the definition of what being "unfavourable" varies across species, as does the life stage (embryonic, larval, or adult) in which diapause occurs, the common aim is to increase organismal survival, and ultimately enable them to re-establish when the next favourable season arrives (Denlinger and Armbruster, 2014). If the duration of the unfavourable season lasts for multiple generations, then diapause has a direct impact on the rate of evolution and the genetic constitution of the population.

In many locations, malaria vector populations exhibit strong seasonal fluctuations in abundance, being present in large numbers during the rainy season (RS) and then dropping to low levels or even becoming undetectable when the breeding sites disappear in the dry season (DS) (Charlwood et al., 1995; Hidalgo et al., 2016; Kabbale et al., 2013). Various hypotheses have been suggested to account for their persistence in the DS and the source of their re-establishment soon after the first rains. For some vector species, like *Anopheles coluzzii*, aestivation, a DS adult-state diapause, is thought to be the primary route (Wang et

al., 2011). Aestivation is an interesting biological process that is predominantly activated by the absence of water (Depinay et al., 2004). However, using conventional mosquito trapping methods, it is almost impossible to detect aestivators; thus, they are thought to occupy distinct hidden habitats during this state (Charlwood et al., 2000; Lehmann et al., 2017). There are other hypotheses, such as long-distance migration, which assumes (near) extinction of the local DS population, then recolonisation by migrants from further afield where breeding sites exist year-round. Another is local refugia, where populations survive locally but are hidden from sampling, and maintain themselves by low-level breeding during the DS, but this implies the constant availability of larval sites (Omer & Cloudsley-Thompson, 1970).

Many studies have focused on finding direct evidence of aestivating mosquitoes, notably via the mark-release-recapture (MRR) experiment. For example, Lehmann et al. (2010) recaptured one marked female *An. coluzzii* after it had endured a 7-month-long DS in the Sahelian region. More recently, a modified MRR using hydrogen isotopes as markers estimated that aestivation contributed at least 20% in census size to the persistence of *An. coluzzii* in the Sahelian villages of Mali until the following RS (Faiman et al., 2022b). One obvious challenge of the direct methods is the sampling of aestivating individuals, as these laborious MRR provided a disproportionate amount of evidence with low recapture rates (Epopa et al., 2017). There have been fewer indirect (i.e. genetic) studies. Lehmann et al. (2017) explored the population genetics of several Malian populations of *An. coluzzii* through successive seasons and attempted to interpret the results in the context of possible aestivation, noting that drift should be negligible in aestivating populations due to reproductive arrest, but the qualitative nature of the work did not present a formal model or statistical testing. There have been other studies to test whether populations undergo annual bottlenecks (Lehmann, Hawley, Grebert, & Collins, 1998; Luikart & Cornuet,

1998), but these drift-based analyses are limited to the DS only. They did not directly infer aestivation nor provide any means for parameter estimation.

In this work, we first study from a theoretical perspective the magnitude of genetic drift under diapause. If we define temporal effective population size (N_e) as that estimated from the change in allele frequencies between two time points under the idealised Wright-Fisher (WF) model (Waples, 1989), we can address several key questions: How does diapause affect temporal N_e estimates, if samples are collected across seasons? What are these N_e estimates referring to? What happens if only a fraction of individuals undergoes diapause, while the others continue to breed? Can we estimate the number (or proportion) of individuals entering the diapause stage during the unfavourable season from allele frequency dynamics? Below we give answers by mathematical derivations and computer simulations.

We use aestivation to represent diapause and the DS as the unfavorable season in the following paragraphs. The tested example of the genotypic dataset primarily concerns *An. coluzzii*, but the theory is also applicable to other diapausing species.

Theory

1. Standardized variance of allele frequency

Without other forces (migration, selection, mutation), allele frequency fluctuates randomly due to genetic drift. While the mean allele frequency remains unchanged over time, the variance increases. Under the WF model, the standardized variance of allele frequency V_t (Waples, 1989) increases by approximately $1/2N_e$ per generation:

$$V_{t+1} = V_t + \frac{1}{2N_e}$$

[Equation 5.1]

(The full derivations of this and other equations in this section can be found in the supplementary information, SI). A relevant statistic that can be derived from V_t is the temporal F, which is the standardised variance of allele frequency change (Waples, 1989). If two samples are taken Δ_t generations apart, then F is approximately:

$$F \approx \frac{\Delta_t}{2N_e}$$

[Equation 5.2]

from which N_e can be solved or estimated (Waples, 1989). If N_e is not a constant, then this method estimates the harmonic mean N_e of all the generations between the two samples (Waples, 2005).

To extend the model to incorporate aestivation, we assume two seasons per cycle (or year, for most organisms), the RS and DS, lasting Δ_R and Δ_D generations, and with breeding population sizes of N_R and N_D respectively. While the population still reproduces under the WF manner between most generations, the transitions between seasons are of particular interest. These include the generations of ERS (E for early, the first generation of RS), LRS (L for late, the last generation of RS), and similarly EDS and LDS. Upon entering EDS, N_D offspring enter the breeding compartment, and N_A to another compartment to aestivate (N_A is the number that survive aestivation to re-emerge the next ERS; individuals that die during aestivation do not affect allele frequencies and are ignored). The breeding compartment continues to reproduce during DS but with a smaller size, while reproduction halts for the aestivators. The two compartments remain isolated throughout the DS. At the next ERS, those N_A aestivators reappear and unite with the descendants of the breeding individuals from the LDS. This completes the population dynamics of one cycle. The model can be visualized in Figure 5.1, and the notations used in the paper are found in Table 5.1.

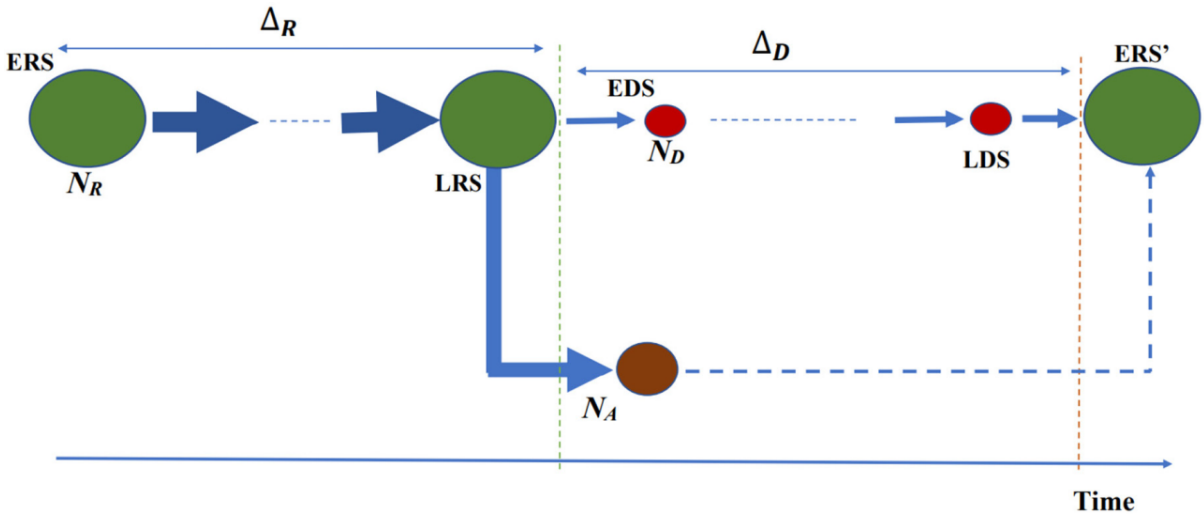


Figure 5.1 *Diagram for the aestivation model*

The model begins at ERS with breeding size N_R. Within the same season the population reproduces (horizontal arrows) according to the WF model. Upon entering EDS, the population branches into two compartments: one continues to breed but with a smaller size N_D, another aestivates with N_A individuals. At the next ERS (denoted as ERS'), it is formed by previously aestivating individuals, and the descendants of the breeding compartment from LDS.

Table 5.1: *Notation used in this study*

RS	Rainy season
DS	Dry season (the unfavourable season for aestivation)
ERS (EDS)	The first generation of RS (DS)
LRS (LDS)	The final generation of RS (DS)
ERS'	ERS of the next cycle
N_R	RS breeding size
N_D	DS breeding size
N_A	Size of the aestivating (diapause) compartment NA⁄NR
Δ_R	Length of RS in generations
Δ_D	Length of DS in generations
V_t	Standardised variance of allele frequency, at generation t
F	Temporal F, standardised variance of allele frequency change (between two time points)

With this model in mind, we then quantify the effect of aestivation on the temporal allele frequency dynamics, such as how V_t changes over time, the expected temporal F, and the interpretations of N_e estimates. Given that the model begins with the ERS, V_t follows the

WF for all generations up to the LDS, but under two different breeding sizes. For instance, we have

$$V_{EDS} = V_{ERS} + \frac{\Delta_R - 1}{2N_R} + \frac{1}{2N_D}$$

[Equation 3]

where Δ_R is the duration of the RS (similarly for Δ_D). The next ERS (denoted as ERS') can be viewed as a mixture of the two compartments, one experiencing no drift (other than that associated with the establishment of the aestivating class) and another with Δ_D generations of drift. Then $V_{ERS'}$ is calculated from their standardised variances and covariance (see SI):

$$\begin{aligned} V_{ERS'} &= V_{LRS} + \left(\frac{N_R - N_A}{N_R}\right)^2 \left(\frac{\Delta_D}{2N_D}\right) + \frac{1}{2N_R} \\ &= V_{LRS} + (1-\alpha)^2 \left(\frac{\Delta_D}{2N_D}\right) + \frac{1}{2N_R} \end{aligned}$$

[Equation 4]

We further introduce $\alpha = \frac{N_A}{N_R}$ as the fraction of aestivators contributing to ERS'. One can quickly examine the limiting cases: For complete reproductive arrest, $\alpha = 1$, then ERS' is in effect the next generation of LRS hence with only one generation of drift with breeding size N_R. If $\alpha = 0$ then it reduces to the pure WF process with Δ_D generations of drift in the DS, and one generation when the population enters the next RS.

Note that the variance at the beginning of a RS ($V_{ERS'}$) can be lower than that from the previous generation (V_{LDS}):

$$V_{ERS'} - V_{LDS} = -\alpha(2-\alpha)\left(\frac{\Delta_D}{2N_D}\right) + \frac{1}{2N_R}$$

[Equation 5]

The difference is negative when

$$\alpha > 1 - \sqrt{1 - \frac{N_D}{N_R \Delta_D}}$$

2. Temporal F and N_e estimates

With V_t no longer monotonically increasing we need to examine its impact on temporal N_e estimates. Here our derivations focus on the following three time points: ERS, EDS, and ERS' (Figure 1), from which three temporal N_e estimates can be calculated. Without much ambiguity, the expected temporal F between ERS and EDS is

$$F_{ERS,EDS} = \frac{\Delta_R - 1}{2N_R} + \frac{1}{2N_D}$$

[Equation 6]

As there are Δ_R generations between them, the corresponding temporal N_e estimate refers to the harmonic mean of $(\Delta_R - 1)$ generations of size N_R and one generation of size N_D. A more complex scenario is when the two samples are from exactly one cycle apart from successive ERS's (SI):

$$F_{ERS,ERS'} = \frac{\Delta_R}{2N_R} + (1-\alpha)^2 (\frac{\Delta_D}{2N_D})$$

[Equation 7]

The temporal N_e estimate between two consecutive ERS is equivalent to that of the WF process with Δ_R generations of size N_R, and Δ_D of $\frac{N_D}{(1-\alpha)^2}$.

Finally, between EDS and ERS' (SI):

$$F_{EDS,ERS'} = \frac{2\alpha - 1}{2N_D} + (1-\alpha)^2 \left(\frac{\Delta_D}{2N_D}\right) + \frac{1}{2N_R}$$

[Equation 8]

With Δ_D generations between them, we are unable to express it analytically as any kind of harmonic mean within the sampling horizon. Note that Equations 6-8 are functions of the three parameters, hinting at the potential use of the three temporal F's for parameter estimation. Another key observation is that when there is aestivation ($N_A > 0$), the sum of the two shorter F's exceeds the overall F, with the difference determined by the three sizes:

$$F_{ERS,EDS} + F_{EDS,ERS'} = F_{ERS,ERS'} + \frac{N_A}{N_R N_D}$$

[Equation 9]

Methods

We built a forward WF simulator with breeding and aestivating compartments to verify the above derivations. Biallelic loci were simulated, and the allele frequencies of the breeding compartment over time were recorded. The model further assumed non-overlapping, discrete generations, with random mating. The three sizes are non-negative with $N_R \geq N_A + N_D$, and Δ_R and Δ_D are known. The first simulation aimed at visualizing the V_t dynamics under different aestivation proportions and comparing them to our theoretical expectations. Then, we aimed to validate the temporal F's we derived, focusing only on ERS, EDS, and ERS'.

Results

Simulation results can be found in Figures 5.2-5.3, and the SI. Figure 5.1 plots V_t over time for several values of α. Within a cycle starting from the first ERS, V_t increases with two different slopes because of the two different breeding sizes. The shape of V_t within one cycle was the same for all α, as the breeding sizes were the same. The only difference was between LDS and the next ERS when aestivating individuals reappear. Larger α gave a bigger drop in V_t between the two generations, concurring with our theory (Equation 4).

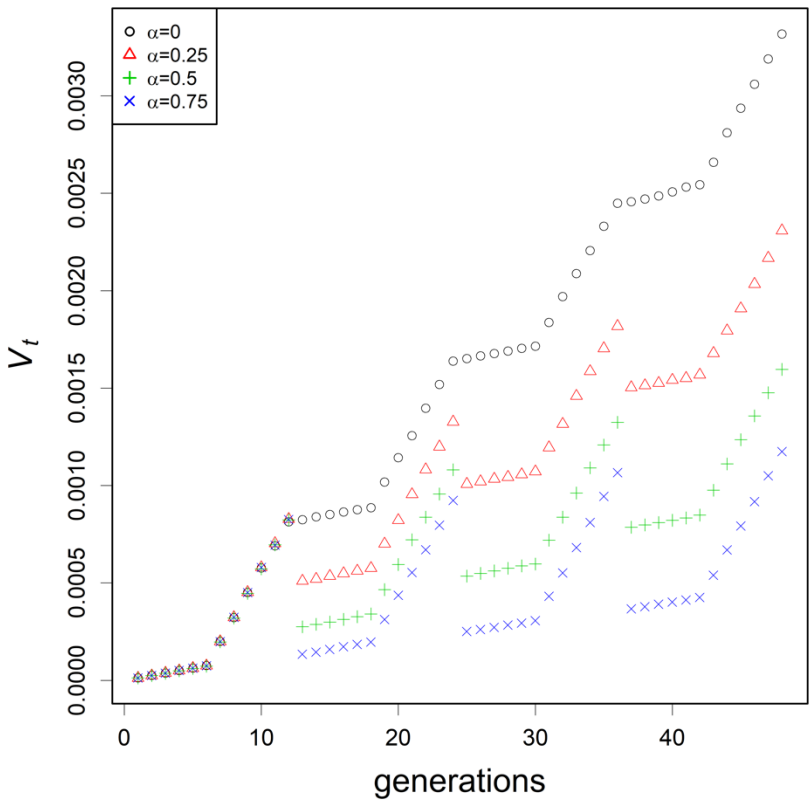


Figure 5.2. *Simulated V_t over time under different aestivating proportion α*

Four values of α were examined. Note that $\alpha = 0$ is equivalent to WF. Simulation parameters are as follows: $N_R = 10000$, $N_D = 1000$, $N_A = \alpha N_R$, $\Delta_R = \Delta_D = 6$. V_t were calculated from 10000 independent loci, with an initial frequency of 0.5. Four complete cycles (48 generations) were simulated, starting from a RS.

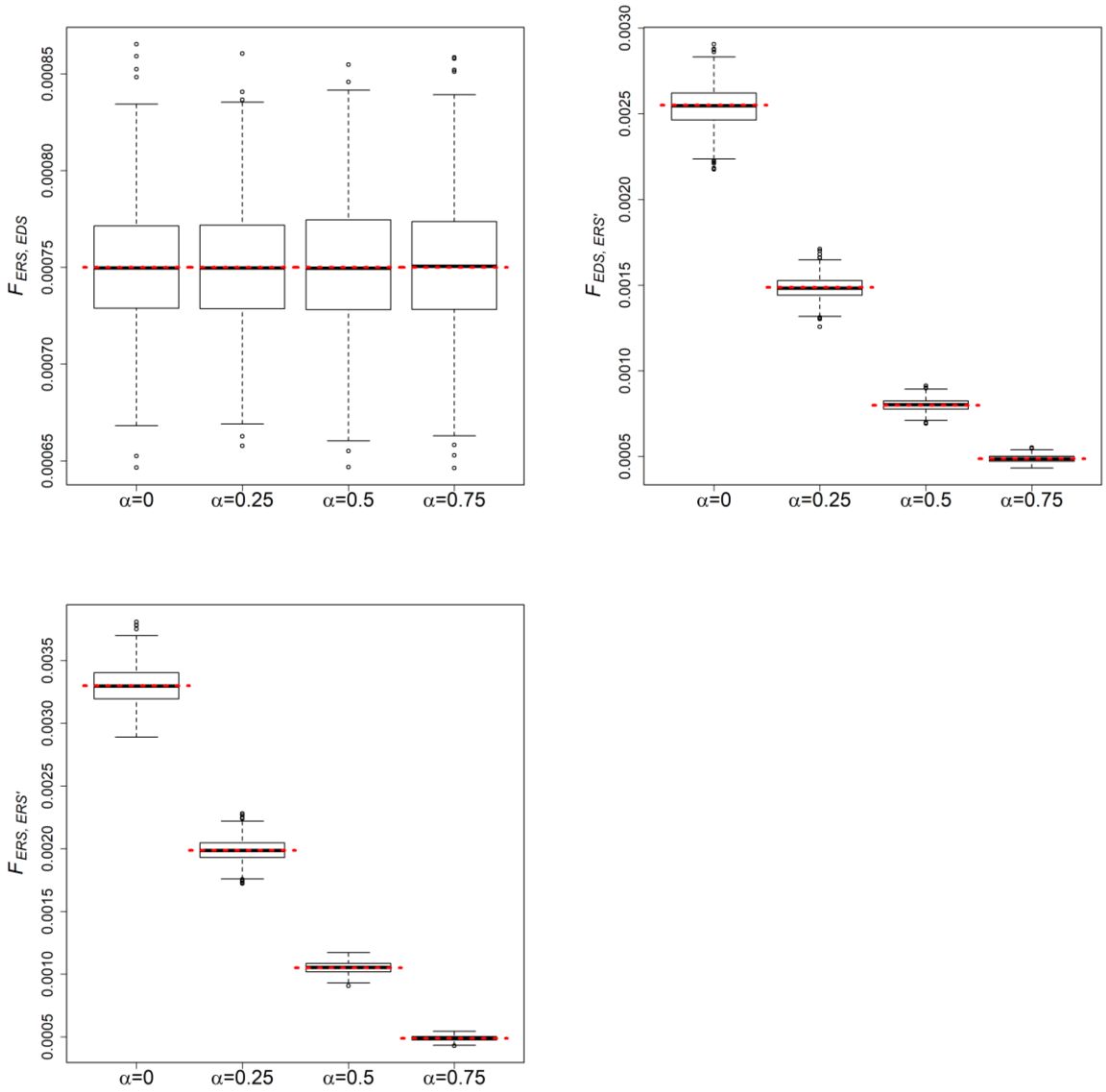


Figure 5.3. *Simulated temporal F statistics against α*

The parameters were identical to those stated in Figure 1. Each box plot was generated from 1000 repeated simulations. The red dotted lines show the theoretical expectations of *F*'s (Equations 7-9). Exhaustive sampling was assumed in this simulation.

In the second simulation, we focused on the three pairs of temporal F's among ERS, EDS, and ERS'. Each box plot and error bar in Figure 5.3 came from 1,000 repeated simulations. Between the first two time points, different values of α produced the same mean F as no aestivation was involved. The widths of the error bars appeared to be similar as well. Both $F_{EDS,ERS'}$ and the overall $F_{ERS,ERS'}$ decreased with α. Because of their inversely proportional relation, larger α gave larger N_e estimates, despite having the same breeding sizes. In short, the results all agreed with our theoretical expectations (Equations 7-9).

Parameter estimation on a real dataset

To illustrate how the theory can be used to estimate parameters, we searched for datasets in which the same population had been genotyped at multiple loci in 3 successive seasons (RS, DS, RS'). The most appropriate we found was the study of Lehmann, et al. (2017), though it is not clear whether it meets all the assumptions of the model. We particularly focused on the temporal *An. coluzzii* samples collected between 2008 and 2010 from the Sahelian village of Thierola in Mali. Sample sizes per time point varied from about 30 to 60 and were scored for 738 SNPs (Lehmann et al., 2017).

To estimate parameters, we used Approximate Bayesian Computation (ABC; (Beaumont et al., 2002b). As the dataset spanned two years, we analysed the two dry seasons (DS) separately, with non-overlapping intervals. For the first DS, the three temporal samples were collected in August `08, May `09, and October `09, and for the second DS, they were collected in October `09, April `10, and June `10. As the generation time was assumed to be one month (The Anopheles gambiae 1000 Genomes consortium, 2017b), the sampling horizons for the two years were 14 and 8 generations, respectively. According to the original authors, the DS runs from December to May (Lehmann et al., 2017). For summary

statistics we used the three temporal F's, which were computed via F_c to account for finite sampling and missing data (Peel et al., 2013; Pollak, 1983; Waples, 1989).

In each ABC simulation, a burn-in period of 24 generations (2 cycles) was run. The initial allele frequency was sampled from $U(0.2,0.8)$, where $U(a,b)$ denotes a uniform distribution with bounds a and b. For the priors, N_D was drawn from $U(10,1000)$ based on preliminary studies on it being not too large; $N_R|N_D \sim U(N_D+2,100{,}000)$; and $N_A|N_R,N_D \sim U(1,N_R-N_D)$ conditionally. Finally, samples were taken binomially from the population, with sample sizes equal to those of the real dataset. The simple Euclidean distance between the observed and simulated summary statistics was calculated. For each year, 2 million ABC simulations were run, and the 1,000 (0.05%) with the smallest distances were accepted as realizations of the posteriors. We report medians from the posterior distributions and 90% confidence limits (CI) calculated by excluding the 5% on either extreme.

The inferred posterior distributions from the ABC are shown in Figures 5.4 and 5.5. For the first year, the posterior medians for N_R, N_D and N_A were 265, 45, and 210 respectively. In addition to the three sizes, the posterior of α was also computed from the pairs of accepted N_A and N_R. The posterior median for α was 0.79. For the second year, the posterior median for N_D and N_A were 578 and 15,972. Note that the posterior N_A was highly skewed. The result for N_R was inconclusive because the posterior was flat (see discussion below). The posterior of α had a sharp peak with a median of 0.39. Confidence intervals (C.I.) are reported in the figure legends.

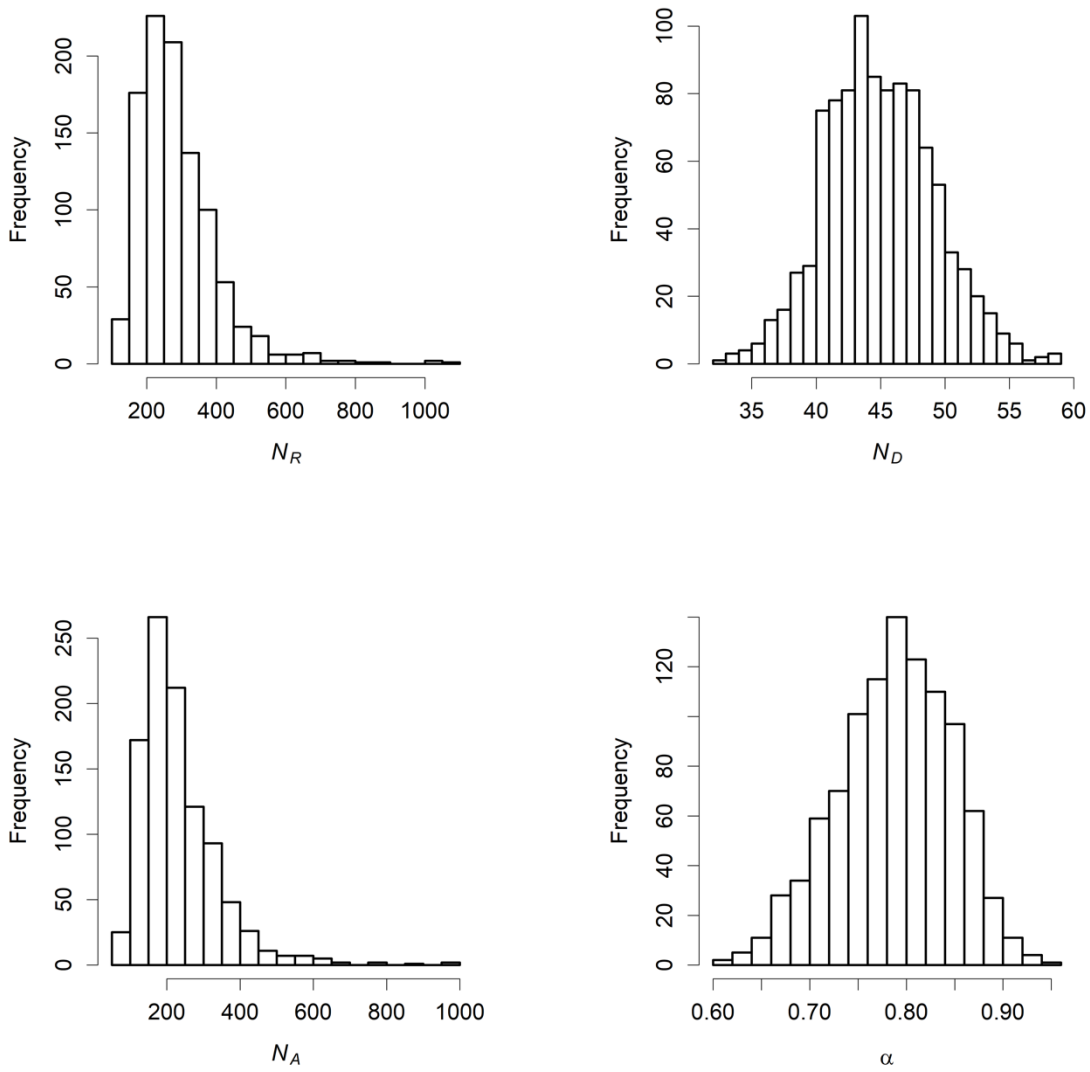


Figure 5.4. *Posterior distributions for the first DS (2008-2009)*

The 90% C.I. were estimated as follows: N_R: [163, 486], N_D: [39, 53], N_A: [113, 420], α: [0.68, 0.88]. The corresponding temporal F estimates were 0.0714, 0.0669 (shorter-term), and 0.0290 (overall).

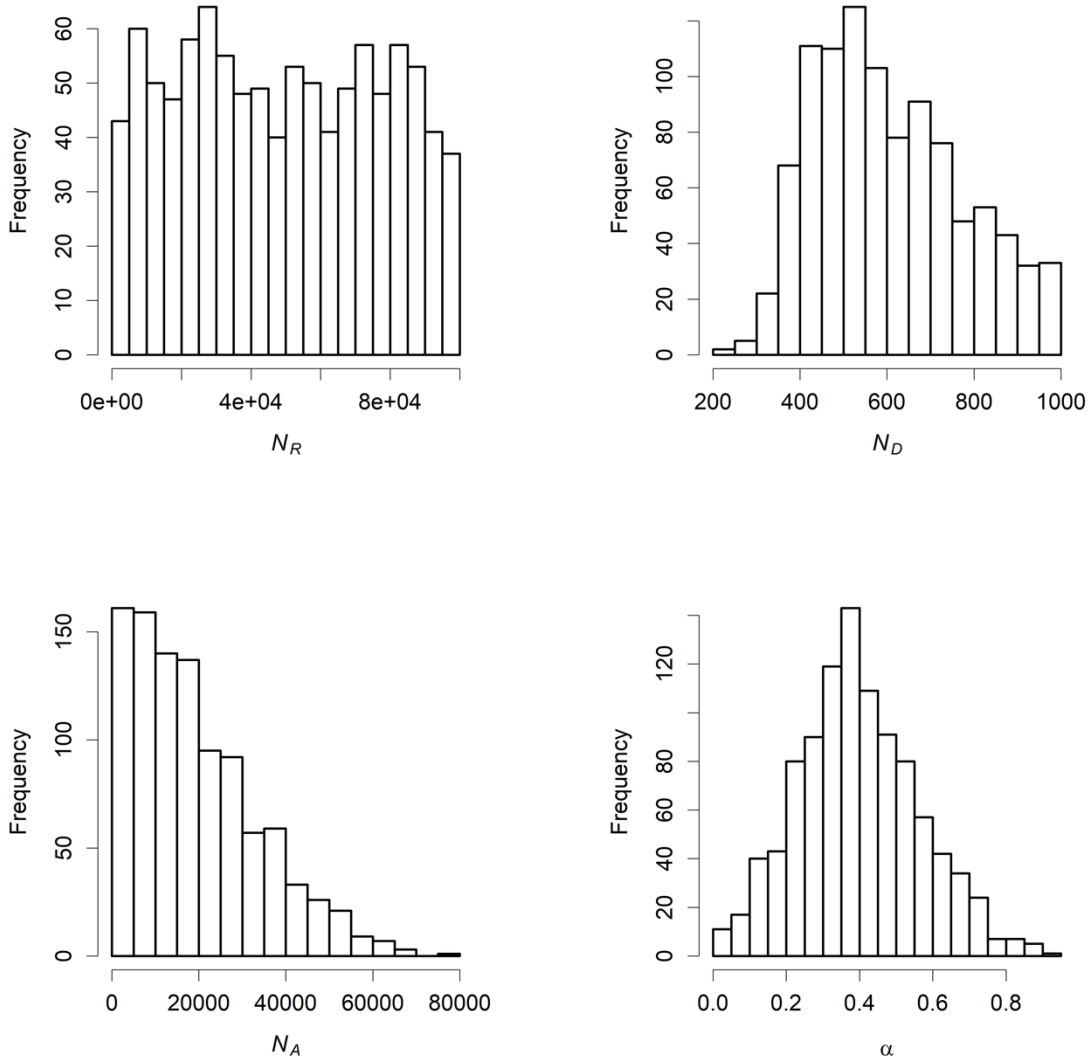


Figure 5.5. *Posterior distributions for the second DS (2009-2010)*

The 90% C.I. were estimated as follows: N_D: [365, 918], N_A: [1430, 47385], α: [0.13, 0.68]. The estimates for N_R was not reported as its posterior was flat. The corresponding temporal F estimates were 0.0071, 0.0024 (shorter-term), and 0.0036 (overall).

Discussion

Diapause allows many insect species to survive through their unfavourable seasons. It is interesting mechanisms have been studied for model organisms such as *Drosophila melanogaster* (Piper and Partridge, 2016; Ojima et al., 2018). Many genera of mosquitoes

are also known to undergo diapause and at different life stages: egg and larva diapause occurs in *Aedes*, while adult diapause is usually found in *Culex* and *Anopheles* (Denliger and Armbruster, 2014). The mechanisms by which reproductive arrest is achieved can be different: some take blood meals but do not develop eggs, some do neither (Yaro et al., 2012). Even within the same species, diapause may or may not be activated depending on the climate the populations live in (Lee and Duvall, 2022). For most species, mosquitoes and beyond, the unfavorable season is usually the harsh winter, but for *An. coluzzii* this becomes the tropical DS in the Sahel region. Aestivation, a special case of DS diapause at the adult stage, provides a route for the populations to persist then rebound when the rain returns. Aestivation, by its very nature, is difficult to study because the individuals in this state are difficult or impossible to collect (Lehmann et al., 2017). In this work, we have explored how an indirect approach, using allele frequency changes across seasons, can be used to estimate the proportion of aestivation. Our model extends the classical WF model by incorporating two separate compartments in the DS for partial aestivation. Here, the meaning of partial is twofold: First, our model still allows breeding during the DS under a smaller size, as opposed to the more restrictive complete reproductive arrest (Lehmann et al., 2017). Second, $(N_A + N_D)$ can be smaller than N_R such that the total population size can be smaller in the DS. The formulae we derived for V_t and temporal F assumed that only the breeding compartment is observed. One advantage of this method is that it does not require sampling of aestivating individuals, which, thus far, have been difficult or almost impossible to find, while sampling breeding DS individuals is more feasible (Lehmann et al., 2017; The Anopheles gambiae 1000 Genomes consortium, 2020).

Cross-seasonal dynamics are crucial to understanding the population genetic consequences of aestivation. One characteristic is the drop in V_t at every ERS'. Previously aestivating individuals, who have experienced no drift through the DS, reappear and admix with the

rest of the population, lowering the combined V_t. If the proportion of the RS population that aestivates (α) is sufficiently large, then V_t is severely dampened or even reset to a level similar to the LRS. Populations with huge seasonal variation in population density, such as *An. coluzzii*, whose N_D/N_R can be as low as 0.01 (Bomblies & Eltahir, 2009; Khatri & Burt, 2019; Mabaso et al., 2007; Minakawa et al., 2002), require only a tiny α to give such an effect (Equation 5).

Signals from allele frequency over time have been utilised for N_e estimation, often via the well-studied temporal F statistic (Hui et al., 2021; Waples, 1989). Before this work, little was known about the interpretation of these estimates for aestivating populations, especially when the two samples are taken across different seasons, or when they sandwich an ERS. The temporal F across two consecutive ERS overestimates N_D by a factor of $1/(1-\alpha)^2$ (Equation 7). It may therefore give the false impression of having a very large DS size, contradictory to within-season estimates or the ecology of the species of interest. Note that the temporal N_e estimates between a DS and the subsequent RS (Equation 8) has more terms than the number of generations between them, presumably because there are two routes linking them, one via the breeding compartment of DS, and another via LRS (backward in time) then through the aestivating compartment.

Equations 7-9 show that sampling from three consecutive seasons starting with a RS is sufficient to estimate the three different population sizes (N_R, N_D, N_A), which serves as the basis for our ABC. With aestivation the sum of the two shorter-term F's is greater than the overall F, and the difference increases with α (Equation 9). As a result, the overall N_e estimate will be larger than either of the two shorter-term estimates. While the theory was developed assuming samples were taken at the first generation of each season, it could be modified to consider samples taken in most other generations from their respective seasons,

with slightly different expectations. The theory could also be extended to consider populations with overlapping generations (Waples & Yokota, 2007).

The inequalities between the shorter- and longer-term temporal F's from the Thierola samples (Equation 9, and the legends of Figures 5.4, 5.5) are indicators of aestivation. The first-year DS effective breeding size was estimated to be about 50, several times smaller than that of the RS. For the second year, N_D was also small compared to its corresponding RS but was larger than the previous year. This result concurred with the original publication that the first year was genetically less stable (Lehmann et al., 2017). In both years, the C.I. of N_D and α excluded 0, suggesting the coexistence of aestivation and reproduction in the DS (partial aestivation). The N_R estimate for the second year was inconclusive as the posterior was flat and almost identical to the uniform prior. We believe this was caused by two factors: First, the sample size might be inadequate, that sampling noise overwhelmed the relatively weak drift signals (Waples, 1989). This was further supported by the small temporal F estimates (Figure 5.5). The second possibility was the relatively short sampling interval. It began rather late in RS (October) and ended at the next ERS (June); hence it may not have covered enough RS generations. The original authors also found very little differentiation among the samples collected from October 09 and beyond, which also explained the challenges we faced when estimating the parameters for the second year. While the N_R estimate was inconclusive, we could still estimate α with precision. This is not uncommon in population genetics, that only composite or ratio parameters can be resolved when information is limited.

The proportion of the population that aestivates is unlikely to be a fixed characteristic of the species, but rather to vary geographically, depending on the severity of the DS. Indeed, there is likely to be an adaptive cline in aestivation phenotypes, given their heterogeneity

between climate regions (Yaro et al., 2012). In areas such as the Equatorial where surface waters are found year-round, α is expected to be 0 (see Supplementary Information). In contrast, Sahelian *An. coluzzii* requires specific mechanism like aestivation to persist through the DS (Mwima et al., 2023). We believe partial aestivation occurs along this gradient, but this hypothesis requires further investigation. There are other potential factors that may affect the α estimate, such as the switching of individuals between the two compartments in the DS, or if aestivators begin their dormancy at different DS generations.

As in other temporal N_e studies, perfect knowledge of generation time is usually assumed. Additionally, we require information on seasonality and its transition. The beginning of RS is usually associated with the first rain and thus is not always on the exact same date or week of the year. Sampling too close to the beginning of each season may risk obtaining individuals that are from the previous one. The proposed sampling regime of RS-DS-RS is only an example, and further investigations are encouraged. As mentioned, our model and derivations are applicable to any diapause scenario that lasts for more than one generation, which is exactly the case for most short-lived insect species.

Key assumptions for our model and method of analysis are (1) that individuals collected during the DS are reproductive, and (2) that the population under study is closed, without significant genetic impacts due to immigration over the course of the study. It is not clear how well the population of *An. coluzzii* from Thierola fits these assumptions. For example, no breeding sites were found during the DS, and it is possible that individuals collected then were aestivators that briefly became active to feed before returning to aestivate. It will be worthwhile to expand the model to allow for this possibility, and for possible large-scale immigration at the beginning of the RS, which has also been proposed for these populations (Dao et al. 2014; Lehmann et al., 2017). Each of these processes should leave a

distinguishable genetic signature which will allow appropriate statistical inference. We hope our work will stimulate and accelerate relevant studies; to shed light on questions such as why aestivating proportions vary between populations and years, and how they interact with vector control programmes. All else being equal, aestivation is expected to slow down the rate of evolution in calendar time, including insecticide resistance. Considering genetic control strategies, a large aestivating population will slow down the spread of a gene drive (North et al., 2020) but can also serve as a reservoir for the gene drive mosquitoes so that they do not locally go extinct after a DS, potentially having an effect similar to repeated releases (Eckhoff et al., 2017). The consequences hence vary from case to case, requiring further analyses. More generally, understanding the persistence mechanisms is essential for all vector control programmes to identify and target the susceptible phases of the vector populations (Lehmann et al., 2017). It is foreseeable that long-distance migration and aestivation, the two main hypotheses of their persistence and re-establishment in highly seasonal environments, require different vector control strategies. Future genetic investigations will be necessary to help differentiate these processes with quantitative testing, but they will almost certainly require intensive sampling and a combination of genetic parameters, such as linkage disequilibrium, clustering, and kinship

Supplementary Information (SI)

1. **Allele frequency trajectories with aestivation**

With our model (Figure 5.1, main text) in mind, we can visualise the effect of aestivation on the allele frequency over time. Figure 5.6 is an illustrative example, with $N_R = 10000$, $N_D = 1000$, and $N_A = 7500$. $\Delta_R = \Delta_D = 6$ generations. We simulate the allele frequencies of 200 independent loci from the same population over time, with the same initial allele frequency of about 0.5. Because of random genetic drift, some alleles increase in frequency, some go down, but the mean change is 0.

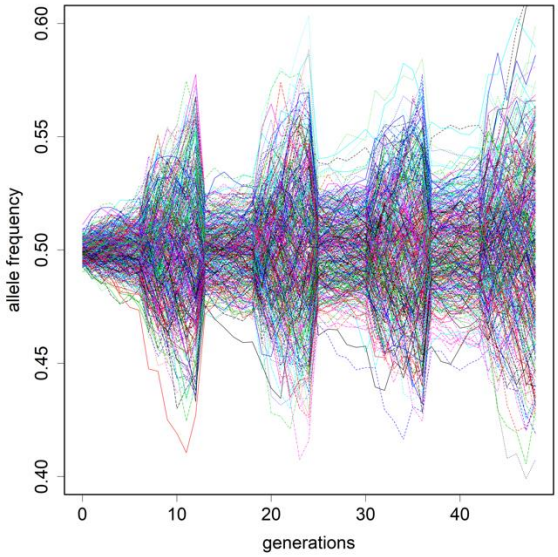


Figure 5.6 *Illustrative example of the effect of aestivation on the allele frequency over time*

We can calculate the sample variance of these allele frequency trajectories at each time point (i.e. along a vertical slice), and this is the V_t plot (Figure 2, main text). Specifically, in this case, we have $\alpha = 0.75$, corresponding to the blue crosses in Figure 2. Of course, in the making of the V_t plot we used the sample variance from 10000 loci for accuracy.

2. Standardised variance of allele frequency and temporal *F*

Let p_t be the allele frequency at generation t. Assume the initial condition p_0 is known. Under the discrete WF process, drift increases the variance of allele frequency over time. From Waples (1989) we have

$$Var(p_t) = p_0(1-p_0)[1-\left(1-\frac{1}{2N_e}\right)^t]$$

where N_e is the constant effective population size. If we define $V_t = Var(p_t)/(p_0(1-p_0))$ as the standardised variance

$$V_t = 1-\left(1-\frac{1}{2N_e}\right)^t$$

The difference between V_{t+1} and V_t is

$$\begin{aligned} V_{t+1} - V_t &= 1 - \left(1 - \frac{1}{2N_e}\right)^{t+1} - 1 + \left(1 - \frac{1}{2N_e}\right)^{t} \\ &= \left(1 - \frac{1}{2N_e}\right)^{t} - \left(1 - \frac{1}{2N_e}\right)^{t+1} \\ &= \left(1 - \frac{1}{2N_e}\right)^{t} \left(1 - 1 + \frac{1}{2N_e}\right) \\ &= \left(1 - \frac{1}{2N_e}\right)^{t} \left(\frac{1}{2N_e}\right) \end{aligned}$$

If we assume N_e is reasonably large and t is small, then $\left(1 - \frac{1}{2N_e}\right)^{t} \to 1$ and we have

$$V_{t+1} = V_t + \frac{1}{2N_e}$$

In short, drift increases the variance of allele frequency almost linearly over a short period of time, with slope inversely proportional to N_e. In the main text we also introduce temporal F as a measure of drift (Waples, 1989). The formula for F may have given the impression that it equals the difference between the two $V's$, but it is only a coincidence. The correct interpretation should be $F = V(p_{t+\Delta t} - p_t)$ between generation t and $(t + \Delta t)$.

3. Deriving $V_{ERS'}$

In DS the population is split into an aestivating and breeding compartment. Let p_A and V_A be the allele frequency and the corresponding standardised variance of the (hidden) aestivating compartment. The model assumes that N_A offspring enter this compartment. With LRS being the reference point,

$$V_A = V_{LRS} + \frac{1}{2N_A}$$

Note that V_A is constant throughout a DS. Next, we consider the standardized variance for the breeding compartment during the same period. At LDS we have

$$V_{LDS} = V_{LRS} + \frac{\Delta t_D}{2N_D}$$

But when entering the next generation (ERS') the standardised variance is further increased by $\frac{1}{2(N_W - N_A)}$, as $(N_W - N_A)$ offspring are sampled to join up with the aestivating individuals. If we call the allele frequency of such a breeding compartment as p_B (and that we also have V_B):

$$V_B = V_{LRS} + \frac{\Delta t_D}{2N_D} + \frac{1}{2(N_W - N_A)}$$

The ERS' population is a mixture of the aestivating and breeding compartments, with a proportion α

$$p_{ERS'} = \alpha p_A + (1 - \alpha) p_B$$

Applying the standardised variance on both sides gives

$$V_{ERS'} = \alpha^2 V_A + (1 - \alpha)^2 V_B + 2\alpha(1 - \alpha) C(p_A, p_B)$$

The remaining challenge is to find $C(p_A, p_B)$, the standardised covariance between p_A and p_B. We apply the total law of covariance, conditioning on p_{LRS}:

$$C(p_A, p_B) = E[C(p_A, p_B | p_{LRS})] + C[E(p_A | p_{LRS}), E(p_B | p_{LRS})]$$

The conditional variance is 0 because p_A and p_B are offspring of LRS and their WF samplings are independent. For the latter term, neither aestivation nor drift changes the mean allele frequency, therefore $C[E(p_A | p_{LRS}), E(p_B | p_{LRS})] = C(p_{LRS}, p_{LRS}) = V_{LRS}$. Putting everything together we have

$$V_{ERS'} = \alpha^2 \left(V_{LRS} + \frac{1}{2N_A} \right) + (1 - \alpha)^2 \left(V_{LRS} + \frac{\Delta_D}{2N_D} + \frac{1}{2(N_R - N_A)} \right) + 2\alpha(1 - \alpha) V_{LRS}$$

$$= V_{LRS} + (1 - \alpha)^2 \left(\frac{\Delta_D}{2N_D} \right) - \frac{\alpha^2}{2N_A} + \frac{(1 - \alpha)^2}{2(N_R - N_A)}$$

$$= V_{LRS} + (1-\alpha)^2\left(\frac{\Delta_D}{2N_D}\right) + \frac{1}{2N_R}$$

4. Deriving $F_{ERS,ERS'}$

By definition the temporal F is the standardised variance of the change in allele frequency between the two time points. Here our focus is on between two consecutive ERS (one cycle apart). We drop the subscript here for simplicity.

$$\begin{aligned} F &= V(p_{ERS'} - p_{ERS}) \\ &= V_{ERS'} + V_{ERS} - 2C(p_{ERS'}, p_{ERS}) \end{aligned}$$

The first two terms are known. For the last term, we can apply the total law of covariance again, conditioning on p_{ERS}. It is then found that the covariance is in fact V_{ERS}.

$$\begin{aligned} F &= V_{ERS'} + V_{ERS} - 2V_{ERS} \\ &= \frac{\Delta_R}{2N_R} + (1-\alpha)^2\frac{\Delta_D}{2N_D} \end{aligned}$$

Note that all V_{ERS} are cancelled out.

5. Deriving $F_{EDS,ERS'}$

Similarly, the expected temporal F between EDS and ERS' is

$$\begin{aligned} F &= V(p_{ERS'} - p_{EDS}) \\ &= V_{PES'} + V_{EDS} - 2C(p_{ERS'}, p_{EDS}) \end{aligned}$$

We then apply the same trick that $p_{ERS'}$ is a mixture of the two compartments, and that covariance is linear

$$\begin{aligned} F &= V_{ERS'} + V_{EDS} - 2C(\alpha p_A + (1-\alpha)p_B, p_{EDS}) \\ &= V_{ERS'} + V_{EDS} - 2\alpha C(p_A, p_{EDS}) - 2(1-\alpha)C(p_B, p_{EDS}) \end{aligned}$$

Using almost the same argument as above, we have $C(p_A, p_{EDS}) = V_{LRS}$ and $C(p_B, p_{EDS}) = V_{EDS}$

$$
\begin{aligned}
F &= V_{ERS'} + V_{EDS} - 2\alpha V_{LRS} - 2(1-\alpha)V_{EDS} \\
&= (2\alpha - 1)(V_{EDS} - V_{LRS}) + (1-\alpha)^2\left(\frac{\Delta_D}{2N_D}\right) + \frac{1}{2N_R} \\
&= \frac{2\alpha - 1}{2N_D} + (1-\alpha)^2\left(\frac{\Delta_D}{2N_D}\right) + \frac{1}{2N_R} \\
&= \frac{\alpha^2}{2N_D} + (1-\alpha)^2\frac{\Delta_D - 1}{2N_D} + \frac{1}{2N_R}
\end{aligned}
$$

Note that there are Δ_D generations between EDS and ERS' but there are more terms in the formula (you may consider it as the sum of $\Delta_D + 1$ terms), making it difficult to interpret the corresponding N_e estimate.

6. ABC and F_c

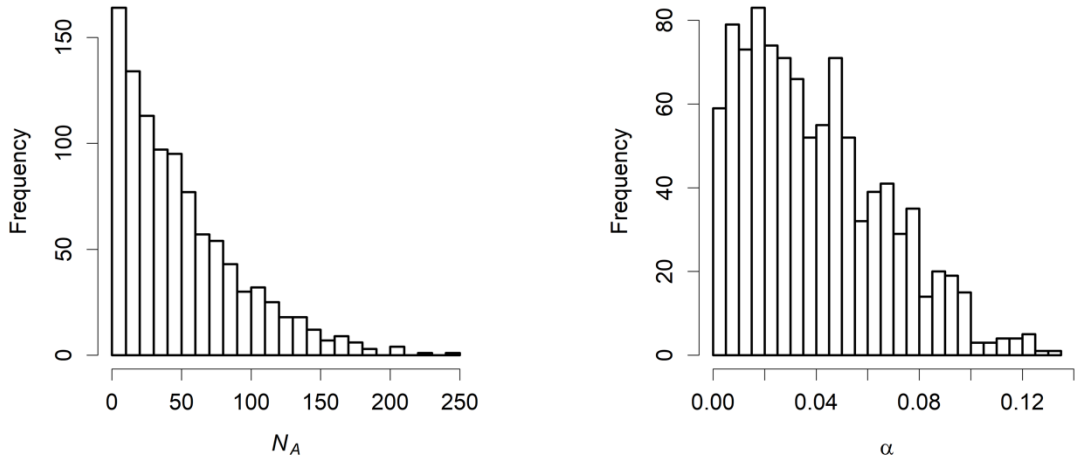
When analysing the dataset of Lehmann et al. (2017) we used F_c statistic to account for sampling errors. Let x_0 and x_t be the observed allele frequencies at two time points. Also let n_0 and n_t be the corresponding diploid sample sizes with complete information. The following quantality was calculated for each locus:

$$
F_c = \frac{(x_t - x_0)^2}{\frac{x_t + x_0}{2} - x_t x_o} - \frac{1}{2n_0} - \frac{1}{2n_t}
$$

And the overall F_c is the arithmetic average across all loci, similar to the procedure by Peel et al. (2013). N_e can be estimated via Equation 2 of the main text. In the ABC (Figures 3-4, main text), we included loci with pooled minor allele frequency >=5% and with complete information across all three temporal samples. As a result, 435 loci were used to estimate the three sizes for the first year, and 496 loci for the second year. The maximum accepted Euclidean distance was 0.0169 for the first year, and 0.00046 for the second year. These two numbers correspond to the tolerance δ in rejection-based ABC (Beaumont et al., 2002). The dataset is publicly available, please consult the original paper Lehmann et al. (2017).

7. Additional ABC simulation with no aestivation

In areas such as the Equatorial or Sudan where surface water and breeding sites are available year-round, no DS persistence mechanism is required. An additional ABC was run to examine the method's behaviour under no aestivation. An artificial observed dataset was generated by fixing $N_A = 0$. Note that it is identical to a WF population with alternating RS and DS breeding sizes. We further assumed sample sizes were plentiful to illustrate the effect. The posteriors of N_A and α were as follows:



Without aestivation the typical posteriors for N_A and α should have lie close to 0 with only one peak and tail.

CHAPTER SIX: THE POPULATION GENETICS OF *ANOPHELES GAMBIAE*: INVESTIGATING POTENTIAL SEASONAL REBOUND MECHANISMS (AESTIVATION AND MIGRATION) IN EASTERN UGANDA

6.1 Background

Malaria remains a significant public health challenge in sub-Saharan Africa, with Uganda being one of the most affected countries. The primary malaria vectors in Uganda are *Anopheles gambiae*, *Anopheles arabiensis*, and *Anopheles funestus* (E. M., Sinka et al., 2012; Ugandan Ministry of Health, 2014; WHO, 2023), and exhibit population dynamics that correlate strongly with environmental conditions. While climate-vector relationships are well established in malaria vector ecology (Y. A. Afrane et al., 2006; Bomblies, 2012; Koenraadt et al., 2004; Lindblade et al., 2000; Patz & Olson, 2006; Stresman, 2010), studies in Uganda have documented climate-associated dynamics (Iga et al., 2023; Kigozi et al., 2016; Okello et al., 2006). Therefore, climatic factors together with human interventions contribute to seasonal variations in both malaria vector populations and transmission intensity (Beck-Johnson et al., 2017; Kigozi et al., 2016), even though local-scale drivers, specifically in Eastern Uganda, require further empirical characterization.

Understanding the genetic structure of mosquito populations across seasonal fluctuations is critical for optimizing malaria vector control strategies, including the emerging gene drive technologies in the high-burden regions like Eastern Uganda. These seasonal population dynamics may significantly impact vector genetics through three key mechanisms: (1) altering the frequency of resistance alleles through observed seasonal shifts in resistance genotypes (Abdalla et al., 2014; Jeon et al., 2025) (2) revealing

migration patterns through genetic differentiation between seasonal populations (Kaddumukasa et al., 2020a); and (3) identifying evolutionarily vulnerable periods in which targeted interventions could maximize impact (Donnelly et al., 2002).

One potential mechanism that contributes to seasonal fluctuations in mosquito populations is aestivation, a form of dormancy during dry seasons (DS), where adults may enter a state of reduced metabolic activity to survive dry conditions (Diniz et al., 2017; Lehmann et al., 2010; Mwima et al., 2023). Aestivation enables mosquito populations to persist through unfavorable dry conditions and rebound quickly with the return of the rainy season (RS) (Faiman et al., 2022b; Mwima et al., 2024). But while this phenomenon has been demonstrated in *An. coluzzii* populations in arid and highly seasonal environments like the Sahelian Africa (Dao et al., 2014; Lehmann et al., 2010), its role an Equatorial region such as, Eastern Uganda remains hypothetical.

In contrast, while some populations persist locally through aestivation, others, particularly those in areas with ephemeral water sources and no year-round breeding habitats are likely to go extinct during the DS and rely on re-establishment by migrants from distant, hydrologically stable regions (Lehmann, Weetman, Diana L Huestis, et al., 2017). These vulnerable populations are characterized by: (1) complete absence of larval sites during the DS, (2) dramatic mosquito density declines, and (3) genetic signatures of bottlenecks that are followed by recovery that includes reduced genetic diversity following the dry season (Lehmann, Weetman, Diana L Huestis, et al., 2017; Luikart et al., 1998). Understanding the interplay between these mechanisms is essential for predicting patterns of genetic variation and adaptation in mosquito populations across different seasons (Caldwell et al., 2021; Williams et al., 2017).

Genetic analysis of malaria mosquito populations during seasonal rebounds is critical for understanding how vector populations recover and adapt following a dry season (Dao et al., 2014; Huestis et al., 2019; Lehmann et al., 2010; Yaro et al., 2012). Characterizing gene flow, population bottlenecks, and the distribution of genetic variation, reveal the key factors that impact the effectiveness of existing and novel mosquito control interventions, such as gene drive (Odero et al., 2023; Powell, 2022; The Anopheles gambiae 1000 Genomes consortium, 2017). These insights directly inform their design, including the selection of target genes, prediction of spatial spread, and the development of field trial frameworks to assess efficacy and ecological impact (Carballar-Lejarazú et al., 2020; James et al., 2020; Marshall & Akbari, 2018; North et al., 2019).

In Eastern Uganda, the seasonal dynamics of malaria mosquito populations could present challenges for the deployment of gene drives, for instance, if migration between populations is high, it could facilitate the spread of a gene drive across a broader geographic range into non-target regions, on the other hand, if populations are genetically isolated due to aestivation or other factors, gene drives may be contained or experience slower spread (Connolly et al., 2024; Garrood et al., 2022). Also, seasonal bottlenecks could affect the genetic diversity of mosquito populations, potentially influencing the persistence of gene drive alleles over time (North et al., 2019; Weaver et al., 2021). Understanding these dynamics is therefore crucial for tailoring gene drive approaches to local ecological conditions and for assessing potential unintended consequences, such as the evolution of resistance to the gene drive (Legros et al., 2021; Price et al., 2020b; Thorogood et al., 2023).

Previously, we proposed a method to estimate the aestivation fraction from temporal allele frequency changes (chapter 6). We now extend the model to jointly estimate aestivation and migration rates, as well as the RS and DS breeding sizes. This allows us to examine

the relative contributions of the two main DS persistence mechanisms. These are critical for mapping how malaria mosquito populations change over time and space in response to environmental factors (Luikart et al., 2018; Stange et al., 2021). Ultimately, this knowledge will improve strategies for implementing gene drives in integrated vector control programs, potentially accelerating efforts to reduce malaria transmission in Uganda and beyond (Eckhoff et al., 2017; Garrood et al., 2022).

6.2 Methods

6.2.1 Mosquito Data

This chapter utilised an *An. gambiae* dataset from the MalariaGEN database (last accessed on 24th January 2024) collected from 14 districts in Eastern Uganda between 2017 and 2018: Amuria, Bugweri, Busia, Iganga, Jinja, Kaliro, Kamuli, Luuka, Manafwa, Mayuge, Mbale, Namayingo, Ngora and Soroti were considered in this study (Figure 6.1). This yielded 565 *An. gambiae* samples in total across 13 different time points (Table 6.1A, figure 6.2). Table 6.1B shows the number of mosquitoes for each district. For details on mosquito sampling please consult the Ag1000G user guide (https://www.malariagen.net/partner_study/1288-vo-ug-donnelly/, https://www.malariagen.net/partner_study/1178-vo-ug-lawniczak/).

This whole genome sequenced (WGS) data mapped millions of biallelic SNPs from the intergenic regions of the 3L (15-41Mbp) and 3R (15-41Mbp) chromosome arms. These regions were selected to ensure that we avoid regions of polymorphic inversions, reduced recombination, and unequal divergence from the reference genome. Intergenic regions often show higher levels of genetic variation compared to coding regions because the mutations that happen in these regions are less likely to be deleterious (Ellegren & Galtier, 2016a; Koonin & Wolf, 2010; Neafsey & Waterhouse, 2015; Ranson & Lissenden, 2016).

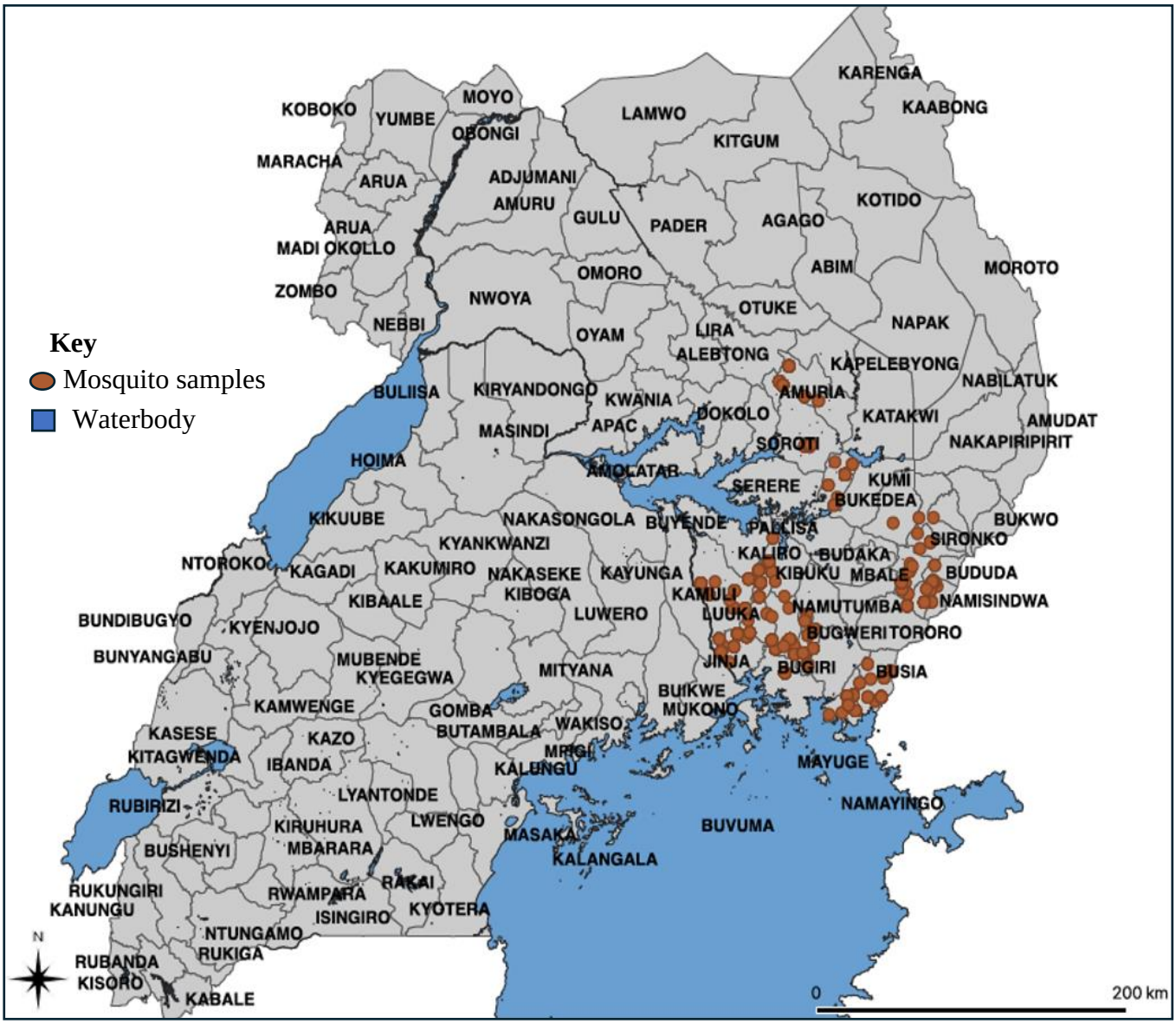


Figure 6.1: *Map of Uganda showing the districts in Eastern Uganda where malaria mosquito samples were collected*

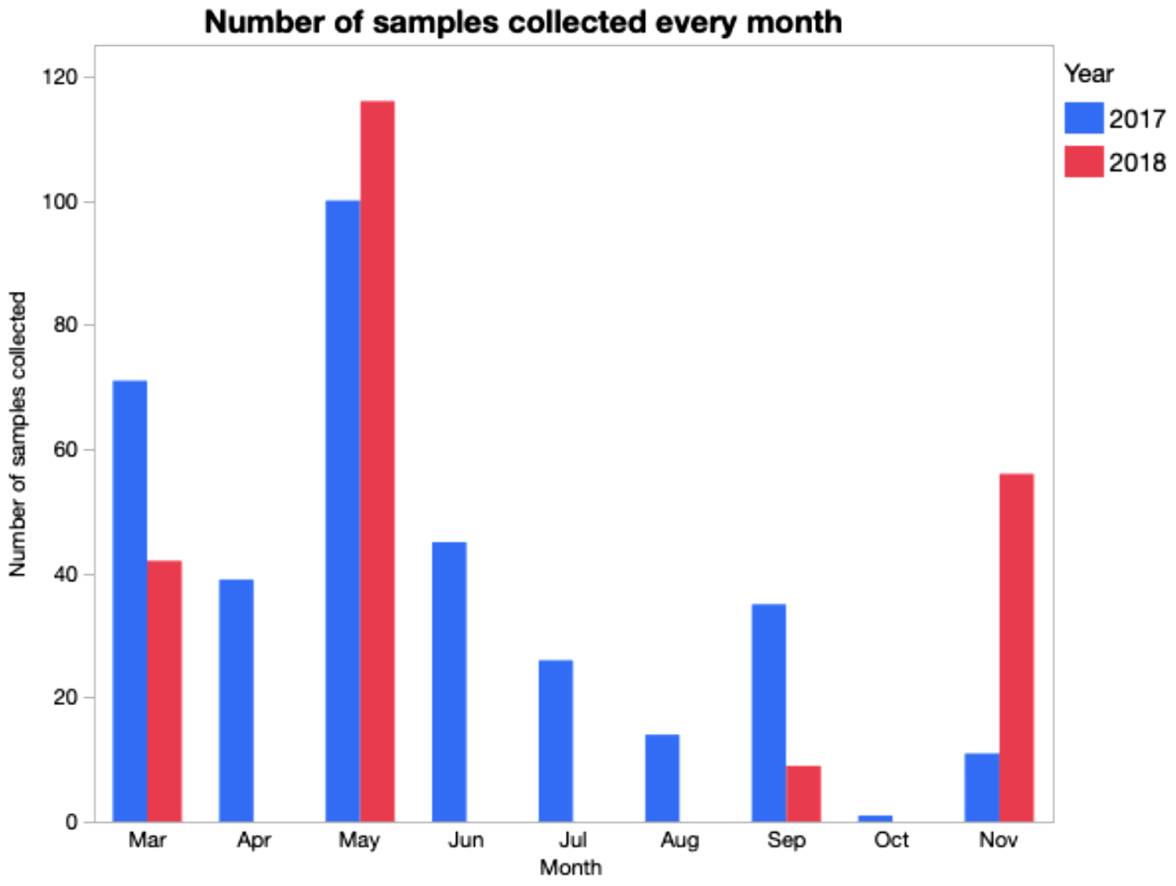


Figure 6.2: *Graph showing the distribution of the 13 time-points regarding actual seasons in the two years*

Table 6.1A: *Mosquito collections per time-point*

Year	Month	Number of samples collected
2017	Mar	71
2017	Apr	39
2017	May	100
2017	Jun	45
2017	Jul	26
2017	Aug	14
2017	Sep	35
2017	Oct	1
2017	Nov	11
2018	Mar	42
2018	May	116
2018	Sep	9
2018	Nov	56
Total		**565**

Table 6.1B: *Collections per district*

District	Number of mosquitoes
Amuria	54
Namayingo	53
Soroti	33
Jinja	51
Kamuli	38
Busia	31
Iganga	35
Luuka	50
Ngora	33
Sironko	9
Kapchorwa	1
Kaliro	27
Bulamburi	1
Mbale	36
Bukedea	1
Namisindwa	9
Manafwa	34
Bududa	2
Bugweri	46
Mayuge	21
Total	**565**

6.2.2 Seasonality per year in Eastern Uganda

The Eastern region has two cycles of RS and DS every year. The dry season months extend from December to February and from June to August while the wet season months are March to May and September to November (Climate Change Knowledge Portal, 2021; Jury, 2018; The Uganda Guide, 2020).

Although some rainfall occurs during the dry season (DS), supporting limited breeding habitats, aestivation remains an important survival strategy for certain species. The reduced precipitation and higher temperatures of the DS can still impose significant physiological stress, particularly in areas farther from Lake Victoria or at lower elevations where

conditions are hotter and drier. While proximity to the lake sustains higher humidity and rainfall, allowing some organisms to remain active species in more arid microclimates, or those with energy-conserving life-history strategies may rely on aestivation to endure harsh intervals. Additionally, even where breeding persists, aestivation can optimize survival during suboptimal conditions, ensuring readiness for the more favorable wet season (RS). Thus, the need for aestivation is context-dependent, shaped by local climate variability and species-specific adaptations (MetMatters, 2021; Williams et al., 2014).

Eastern Uganda has diverse vegetation influenced by climate, altitude, and human activities. The main types of vegetation are; (1) savanna grasslands which are predominantly in lowland areas (2) tropical rainforests mainly located in the higher altitudes and around mountain Elgon (3) wetlands located along rivers, lakes, and floodplains, and (4) agricultural landscapes which are widespread due to extensive farming activities, with crops such as maize, beans, bananas, ground-nuts and rice (especially in the swampy areas) (Chapman & Lambert, 2000; A. C. Hamilton, 1981; Ministry of Water and Environment, 2009; Ssemmanda et al., 2005).

6.2.3 Population Genomics Analysis

6.2.3.1 Spatial Population Structure

We first assessed the dataset to determine whether there was a genetic relationship between mosquito samples collected from the individual districts as a precaution. Using principal components analysis (PCA), an exploratory data analysis method that reveals genetic similarities and differences between individuals or populations (Novembre & Stephens, 2008; Patterson et al., 2006b) and average pairwise Hudson's F_{ST} used to calculate pairwise F_{ST} values between each pair of sampling populations, considering the ratio of averages, and standard errors computed using the block-jackknife method (Arnold et al., 2013; Bhatia

et al., 2013), we were able to detect how much spatial structure occurred between the populations. These were calculated using functions from the MalariaGEN API (MalariaGEN, 2023). We considered intergenic SNPs from the 3L (15-41Mbp) and 3R (1-37Mbp) chromosome arms which avoid regions of polymorphic inversions, reduced recombination, and unequal divergence from the reference genome. This allows for a clearer analysis of genetic variation and evolutionary patterns without the confounding effects of structural variations and recombination suppression, thus providing a more accurate representation of genetic diversity and evolutionary processes (Cáceres et al., 2012; Campos, Rona, et al., 2021; Miles et al., 2017; Wondji et al., 2007). Results from these statistics showed very low diversity between the populations and thus justify our pooling of 565 mosquitoes as one population and focusing on temporal structure.

6.2.3.2 Temporal F and Effective Population Size (Ne) Analysis

Like the spatial F_{ST}, we estimated the temporal F-statistics (F) and effective population size (Ne) for the Eastern Ugandan mosquito population by splitting the 565 mosquitoes according to the month/year of collection. This yielded 13 time points. For each chromosome, (1) allele frequencies were calculated across individuals for each time point, then (2) Temporal F statistics were calculated between all pairs of time points after a pooled minor allele frequency (MAF) filter of >=5% was imposed to remove low-frequency variants, and (3) Ne was derived by adjusting temporal F values based on the number of generations between time points (Waples, 1989). The temporal F's were used as summary statistics for parameter estimation

6.2.3.3 Parameter estimation using the Eastern Uganda *Anopheles gambiae* mosquito dataset

This study utilized a novel genetic approach to estimate the number or proportion of individuals undergoing aestivation and migration, as well as the breeding sizes of the two

seasons, using signals from temporal allele frequency dynamics (Mwima et al., 2024). Here, we extended the model in Mwima et al (2024) by incorporating long-distance migration.

Building on the same model, we introduce an additional mainland compartment that is genetically very stable (no drift). Upon entering ERS, N_M individuals from the mainland immigrate to the focal population and are mixed with the descendants (Figure 6.3). The previously aestivating individuals (N_A) reemerge as well. Hence the model has four parameters (N_A, N_M, and the breeding sizes in RS and DS respectively).

The model we used was designed to simulate genetic processes such as allele frequency changes due to sampling, aestivation, and long-distance migration across several loci. The key compartments of this model include (1) an aestivation simulation module that models allele frequency dynamics in populations undergoing aestivation. The aestivating individuals are said to enter aestivation during the dry season and rejoin the rest of the population later.

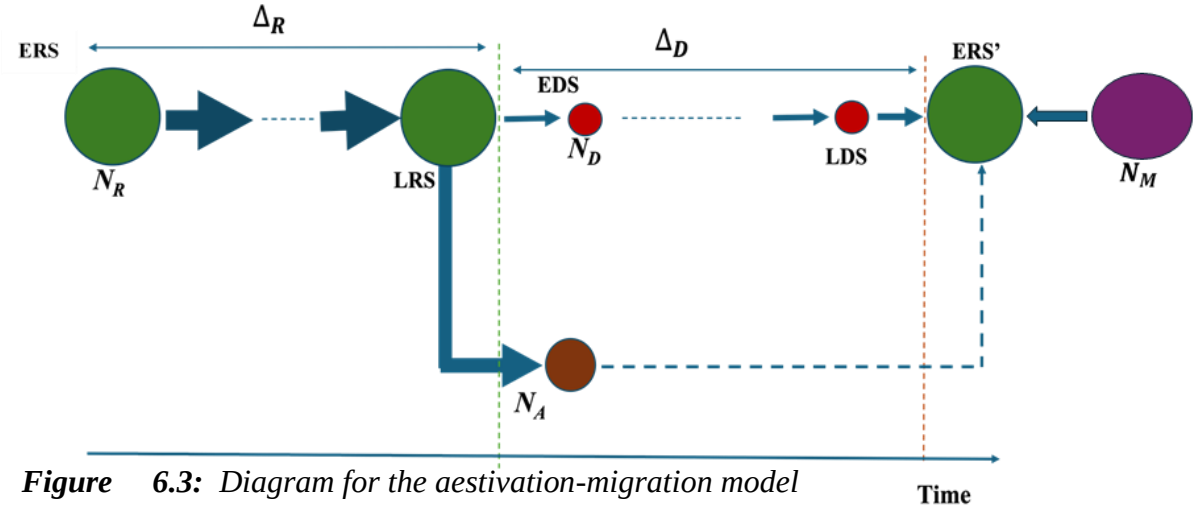


Figure 6.3: *Diagram for the aestivation-migration model*

The model begins at ERS with breeding size NR. Within the same season, the population reproduces (horizontal arrows) according to the WF model. Upon entering EDS, the population branches into two compartments: one continues to breed but with a smaller size N_D, another aestivates with N_A individuals. At the next ERS (denoted as ERS'), it is formed by previously aestivating individuals, the descendants of the breeding compartment from LDS and migrants from neighboring areas.

The simulation updates allele frequencies based on reproduction and aestivation cycles while tracking samples at defined intervals, (2) the long-distance migration simulation module which introduces migration between populations by adding long-distance migrants to the reproducing population, altering allele frequencies, including a burn-in period to ensure quasi-equilibrium before sampling begins, and finally, (3) a combined migration and aestivation simulation module which allows for the simultaneous modeling of both processes by incorporating aestivating individuals and long-distance migrants into the reproduction cycle, while updating allele frequencies accordingly and (4) the sampling module that handles finite population sizes and allele frequencies updated using binomial sampling. With this model, we can quantify the effect of aestivation and long-distance migration on the temporal allele frequency dynamics over a given time, and how N_e estimates can be interpreted. Simulations were run over multiple loci, and the output is a matrix of sample allele frequencies.

We assumed 3 generations per season (i.e. two complete cycles per year): June to August and December to February belong to DS, and March to May and September to November belong to RS. It takes four temporal samples to estimate four parameters. Observed temporal F statistics were considered for specific time points (March 2017 (ERS), April 2017 (RS), July 2017 (DS), and September 2017 (ERS')) that corresponded to various phases in the mosquito lifecycle. These F-statistics were then used to assess the changes in genetic variation across the mentioned time points. They were considered as the key summary statistics for parameter estimation and quantified the genetic drift between populations sampled at different time intervals.

Then using the aestivation-migration model, an improved version of the first simulator (Mwima et al., 2024), genetic drift across seasons was simulated with varying Ne estimates

for the rainy (N_R) and dry (N_D) seasons, as well as aestivation (α) and migration (m) rates. The priors for N_R, and N_D, were drawn from uniform distributions, N_D~U (500, 10,000), and N_R~ $[N_D + 1,\ 20{,}000]$, while α and m were ratios based on N_R. The posterior distributions of N_R, N_D, N_A, and N_M were obtained by comparing the observed with simulated *F* statistics.

Parameters were estimated using Approximate Bayesian Computation (ABC) (Beaumont et al., 2002a). We analyzed the seasonality with non-overlapping intervals, and for each time point, we calculated the temporal *F*, using the ratio of sums, with sample size correction imposed for us to have finite drift and temporal N_e if the F is positive.

6.3 Results

6.3.1 Spatial Population Structure

The PCA visualises how the individuals were genetically clustered within and between collection sites. The output PCA plots from both 3R and 3L chromosome arms showed that all individuals but two clustered together, generally suggesting low genetic variation within the whole dataset (Figure 6.4).

The average Hudson's F_{ST} between pairs of *An. gambiae* populations (districts) are shown in Table 2. All pairwise comparisons produced low spatial F_{ST} values (ranging from 0 to 0.00229), and most of these low F_{ST} values were associated with statistically non-significant p-values ($Z < 1.96$, $p > 0.05$, Table 6.2) though a small number of comparisons reached significance. Given their rarity and absence of a consistent pattern, these few significant results likely reflect minor divergence rather than robust population structure.

The uniformly low pairwise F_{ST} values across all sampled districts indicate minimal genetic differentiation. This pattern is consistent with high, ongoing gene flow, demonstrating that *Anopheles gambiae* populations in this region of Eastern Uganda function as a single, well-

connected metapopulation rather than as isolated subpopulations. Therefore, populations in different districts in Eastern Uganda are not genetically isolated but instead exchanging genes frequently enough to act as a single, large, interbreeding population (a metapopulation). This prevents genetic divergence between districts.

To summarise, PCA and F_{ST} both suggested low spatial population differentiation among the 565 *An. gambiae* from Eastern Uganda, thus it is valid for us to group them as one spatial population and focus on temporal variations.

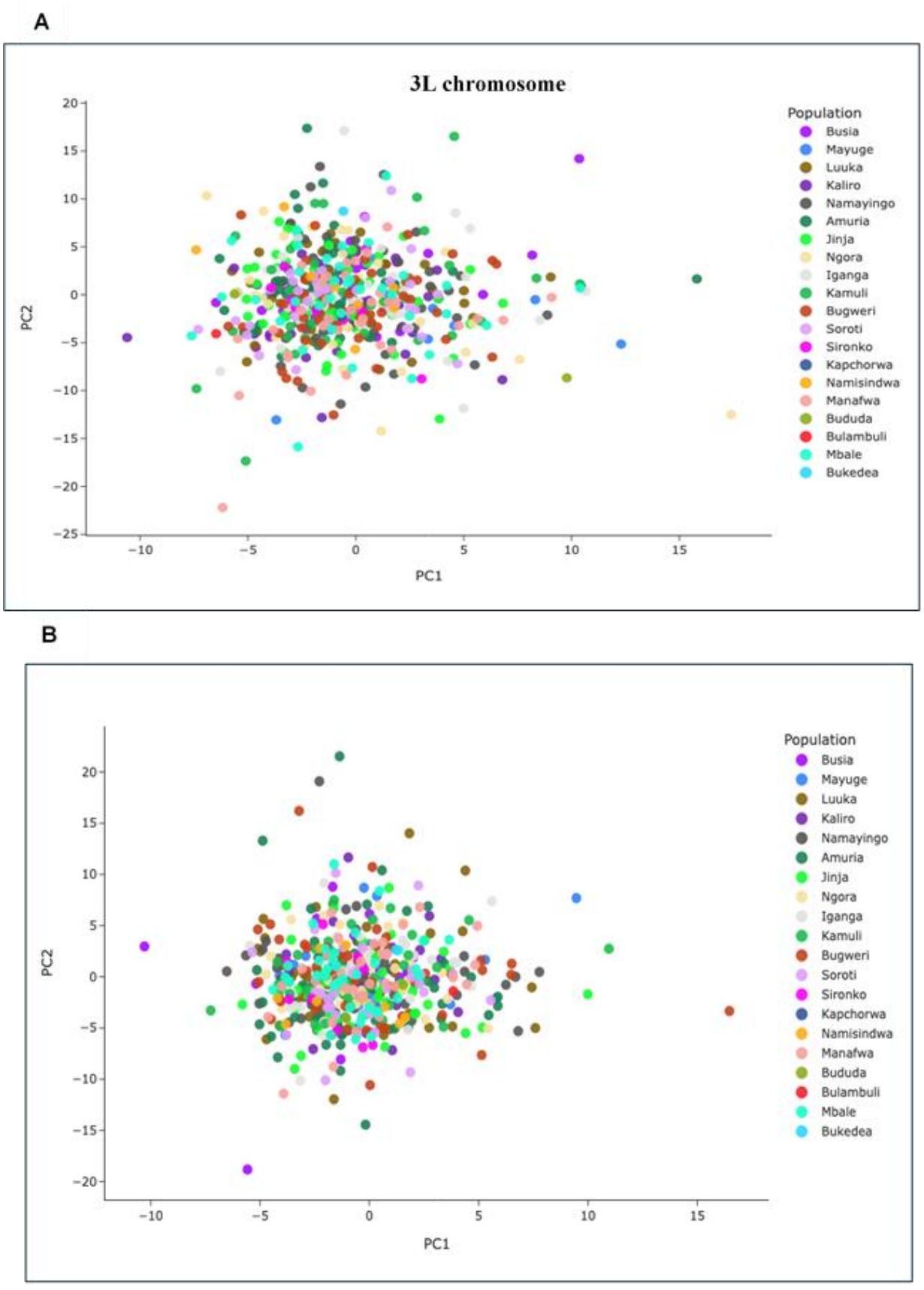


Figure 6.4: *PCA plots from the (A) 3L chromosome arm and (B) 3R chromosome arm*

Table 6.2: *Pairwise FST statistics*

	Amuria	Bugweri	Busia	Iganga	Jinja	Kaliro	Kamuli	Luuka	Manafwa	Mayuge	Mbale	Namayingo	Ngora	Soroti
Amuria	0	0.80186	0.45445	1.11237	0	2.21987	0	0	0	1.36228	1.63448	0	1.50724	0.92553
Bugweri	0.00066	0	2.50461	0	1.33534	2.67978	1.22872	0	1.45030	1.27087	2.45570	0	0.89978	1.70201
Busia	0.000340	0.00183	0	1.70213	2.13478	1.36204	0	0	1.22424	1.00366	1.70888	1.95943	0	0.99861
Iganga	0.00095	0	0.00109	0	0	1.66779	0	0.08737	0.30487	0	0	0.00004	0.62738	0.32786
Jinja	0	0.00106	0.00153	0	0	3.22400	0	0	0	0	1.54605	2.67983	1.40652	1.31750
Kaliro	0.00153	0.00167	0.00100	0.00111	0.00229	0	0	0	1.71760	1.20317	1.15082	1.83460	1.77006	0.50575
Kamuli	0	0.00095	0	0	0	0	0	0	0	0	0.46864	0.00005	0	0.34259
Luuka	0	0	0	0.00006	0	0	0	0	0	0.85023	0	0	0.49571	0.27048
Manafwa	0	0.00106	0.00085	0.00021	0	0.00114	0	0	0	0	1.52251	1.60915	1.13278	0
Mayuge	0.00094	0.00107	0.00069	0	0	0.00089	0	0.00060	0	0	0.32344	2.01864	1.85087	0.97697
Mbale	0.00113	0.00203	0.00109	0	0.00110	0.00082	0.00034	0	0.00104	0.00025	0	1.88598	1.82060	0.98968
Namayingo	0	0	0.00127	0.00004	0.00166	0.00131	0.00005	0	0.00105	0.00168	0.00122	0	1.28580	2.22343
Ngora	0.00103	0.00069	0	0.00044	0.00097	0.00120	0	0.00039	0.00081	0.00137	0.00139	0.00077	0	0.07404
Soroti	0.00074	0.00137	0.00076	0.00024	0.00101	0.00043	0.00024	0.00023	0	0.00073	0.00073	0.00153	6.000000E-05	0

Pairwise F_{ST} values for all the 14 districts in Eastern Uganda. The lower left triangle shows the average Hudson's F_{ST} values between each population pair, while the upper right triangle shows the z-scores for each F_{ST} value calculated via a block-jackknife method. The values in blue indicate non-significant F_{ST} values ($z < 1.96$, $p > 0.05$).

6.3.2 Temporal F and Effective Population Size (Ne) Analysis

The pairwise temporal F statistics were calculated and presented as a matrix. We observed finite drift (which indicates finite Ne) between certain population pairs. Negative temporal Ne values were replaced by infinity. Temporal F and Ne values computed for chromosomes 3R and 3L were consistent, showing finite Ne between population pairs, while those computed for chromosome 2R were inconsistent (Table 6.3 & Table 6.4).

6.3.3 Parameter estimation using the Eastern Uganda mosquito dataset

After adjusting for sample sizes, the first temporal samples were considered for March, April, May, June, July, August, September and October 2017, while the second group of samples considered were March, April, May, June, July, August, September, October, November 2017 and March 2018, as generation time was assumed to be 1 month (The Anopheles gambiae 1000 Genomes consortium, 2017). The dry season (DS) spans December to February and June to August, while the rainy season (RS) covers March to May and September to November. Seasonality was included to estimate aestivation and migration rates, assuming three generations per season. Specific time points considered were March 2017 (ERS), April 2017 (RS), July 2017 (DS), and September 2017 (ERS'), all of which have finite Ne values, making them suitable for parameter estimation. These time points are shown in Table 6.1. Six pairwise temporal *F*-values were calculated as summary statistics for simulations based on the observed *F*-values. Uniform prior distributions were assigned to the parameters (N_R, N_D, N_A, N_M), and simulations ran for six cycles with 5000 loci.

Table 6.3: *Temporal F statistics*

A

	3.2017	**4.2017**	**5.2017**	**6.2017**	**7.2017**	**8.2017**	**9.2017**	**10.2017**	**11.2017**	**3.2018**	**5.2018**	**9.2018**	**11.2018**
3. 2017	NA	0.00032	-0.00021	-0.00019	0.00043	6.00E-05	0.00072	NA	-0.00036	0.00167	7.00E-05	0.00406	9.00E-05
4.2017		NA	0.00039	-8.00E-05	0.00061	-0.00075	0.00013	NA	0.00071	0.00126	0.00012	0.00173	0.00019
5.2017			NA	-0.00019	-0.00026	-0.00024	0.00039	NA	2.00E-05	0.0016	6.00E-06	0.0036	-0.00025
6.2017				NA	-0.00034	-3.00E-05	-0.00011	NA	0.00042	0.00138	-0.00018	0.00469	-0.00037
7.2017					NA	-0.00043	0.00039	NA	0.00087	0.00109	0.00011	0.00361	-0.00055
8.2017						NA	0.00092	NA	-0.00078	0.00124	-0.00041	0.00187	-0.00013
9.2017							NA	NA	0.00186	0.00089	0.00035	0.00335	5.00E-05
10.2017								NA	NA	NA	NA	NA	NA
11.2017									NA	0.00179	-2.00E-06	0.00287	-0.00018
3.2018										NA	0.00193	0.00605	0.0017
5.2018											NA	0.00258	-0.00021
9.2018												NA	0.00149
11.2018													NA

B

	3.2017	**4.2017**	**5.2017**	**6.2017**	**7.2017**	**8.2017**	**9.2017**	**10.2017**	**11.2017**	**3.2018**	**5.2018**	**9.2018**	**11.2018**
3.2017	NA	0.00022	-5.00E-05	-0.00015	-0.00013	0.00057	-0.00038	NA	-0.00128	0.00122	9.60E-05	-0.00031	0.00011
4.2017		NA	0.00015	0.00039	0.00079	0.001898	-9.00E-05	NA	-0.00076	0.00164	0.00031	-0.00214	3.00E-05
5.2017			NA	8.00E-05	-4.00E-05	0.00079	-6.17E-06	NA	-0.00167	0.00148	4.00E-05	-0.00094	2.00E-05
6.2017				NA	-0.00023	0.00101	0.00045	NA	-0.00084	0.00139	7.00E-05	-0.0012	0.00031
7.2017					NA	0.0002	-0.00017	NA	-0.00135	0.00121	0.00024	-0.00122	0.00045
8.2017						NA	0.00057	NA	0.00107	0.00265	0.0013	0.00053	0.00089
9.2017							NA	NA	-0.00134	0.00172	0.00036	-0.00055	-2.00E-06
10.2017								NA	NA	NA	NA	NA	NA
11.2017									NA	0.0004	-0.00123	-0.00118	-0.00132

3.2018										NA	0.00152	0.00024	0.00155
5.2018											NA	-0.00149	-5.00E-05
9.2018												NA	-0.00082
11.2018													NA

C

	3.2017	**4.2017**	**5.2017**	**6.2017**	**7.2017**	**8.2017**	**9.2017**	**10.2017**	**11.2017**	**3.2018**	**5.2018**	**9.2018**	**11.2018**
3.2017	NA	0.004912	0.004751	0.00483	-9.00E-05	0.00161	0.00862	NA	0.00249	0.00793	0.00266	0.00350	0.00391
4.2017		NA	0.00219	-0.00070	0.00713	0.01095	0.02286	NA	-0.00023	0.02276	0.00198	-0.00209	0.00080
5.2017			NA	0.00070	0.00696	0.01268	0.02329	NA	-0.00056	0.02184	0.00077	0.00064	-0.00021
6.2017				NA	0.00701	0.01386	0.02260	NA	-0.00214	0.02230	0.00064	-0.00180	-0.00066
7.2017					NA	-0.00217	0.00382	NA	0.00645	0.00419	0.00334	0.00303	0.00595
8.2017						NA	-0.00120	NA	0.01334	0.00214	0.00879	0.00948	0.01257
9.2017							NA	NA	0.02351	0.00110	0.01758	0.01675	0.02279
10.2017								NA	NA	NA	NA	NA	NA
11.2017									NA	0.02176	0.00017	0.00045	-0.00182
3.2018										NA	0.01567	0.01487	0.02175
5.2018											NA	-0.00229	-3.00E-05
9.2018												NA	-0.00093
11.2018													NA

Temporal F statistics computed from (A) 3R, (B) 3L, and (C) 2R chromosome arms after adjusting for sample sizes. The number before the year represents the sampling month. For example, 3.2017 represents March 2017. October 2017 was excluded because of its small sample size. NA values indicate non-applicable or missing comparison: self-comparisons (diagonal) or cases where data were insufficient for calculations between time points.

A *Table 6.4: Ne estimates*

	3.2017	4.2017	5.2017	6.2017	7.2017	8.2017	9.2017	10.2017	11.2017	3.2018	5.2018	9.2018	11.2018
3.2017	NA	1556.725	Inf	Inf	4615.43	44049.6	4155.87	NA	Inf	3603.044	96760.97	2218.093	106102.6
4.2017		NA	1275.73	Inf	2448.78	Inf	19410.5	NA	4944.358	4369.379	54454.26	4919.421	49618.22
5.2017			NA	Inf	Inf	Inf	5176.41	NA	156380	3132.503	1072395	2223.103	Inf
6.2017				NA	Inf	Inf	Inf	NA	5942.99	3255.101	Inf	1598.734	Inf
7.2017					NA	Inf	2549.26	NA	2313.131	3672.278	43988.73	1938.246	Inf
8.2017						NA	545	NA	Inf	2821.145	Inf	3468.068	Inf
9.2017							NA	NA	537.1513	3372.157	11468.83	1791.798	140184.2
10.202								NA	NA	NA	NA	NA	NA
11.202									NA	1115.565	Inf	1744.802	Inf
3.2018										NA	519.0103	495.6518	2350.873
5.2018											NA	776.2769	Inf
9.2018												NA	671.1739
11.202													NA

B

	3.2017	4.2017	5.2017	6.2017	7.2017	8.2017	9.2017	10.2017	11.2017	3.2018	5.2018	9.2018	11.2018
3.2017	NA	2306.58	Inf	Inf	Inf	4377.6	Inf	NA	Inf	4911.23	73146.92	Inf	94263.63
4.2017		NA	3358.833	2578.288	1900.94	1053.57	Inf	NA	Inf	3360.13	21004.58	Inf	333066.5
5.2017			NA	6530.744	Inf	1889.17	Inf	NA	Inf	3390.75	159657.5	Inf	391026.5
6.2017				NA	Inf	993.466	3347.06	NA	Inf	3239.93	74430.35	Inf	27536.24
7.2017					NA	2469.3	Inf	NA	Inf	3320.52	21155.97	Inf	17997.47
8.2017						NA	878.055	NA	1400.08	1320.46	3426.387	12292.01	8466.701
9.2017							NA	NA	Inf	1744.57	11174.62	Inf	Inf
10.2017								NA	NA	NA	NA	NA	NA
11.2017									NA	4872.62	Inf	Inf	Inf
3.2018										NA	657.1476	12691.68	2582.93
5.2018											NA	Inf	Inf
9.2018												NA	Inf
11.2018													NA

C

	3.2017	4.2017	5.2017	6.2017	7.2017	8.2017	9.2017	10.2017	11.2017	3.2018	5.2018	9.2018	11.2018
3.2017	NA	101.694	210.478	310.31	Inf	1553.55	348.09	NA	1607.91	756.677	2633.5	2575	2555.435
4.2017		NA	228.198	Inf	210.409	182.712	109.37	NA	Inf	241.667	3288.4	Inf	11835.84
5.2017			NA	716.6	143.74	118.266	85.857	NA	Inf	228.975	7842.9	12585	Inf
6.2017				NA	71.3245	72.1505	66.363	NA	Inf	201.752	8599.7	Inf	Inf
7.2017					NA	Inf	261.61	NA	310.035	954.679	1496.5	2311	1343.454
8.2017						NA	Inf	NA	112.432	1636.55	511.79	685.86	596.5546
9.2017							NA	NA	42.5328	2718.75	227.58	358.24	307.122
10.202								NA	NA	NA	NA	NA	NA
11.202									NA	91.9227	17645	11184	Inf
3.2018										NA	63.834	201.72	183.886
5.2018											NA	Inf	Inf
9.2018												NA	Inf
11.202													NA

Ne estimates computed from the (A) 3R chromosome arm, (B) 3L chromosome arm, and (C) 2R chromosome arms after adjusting for sample sizes. NA indicates non-computable comparisons (self-comparisons or missing data) Negative temporal F's were translated into infinite Ne (Inf).

The inferred posterior distributions (Figure 5B, bottom panels) show a trade-off between the two dry-season survival strategies. Compared to the wide and uniform priors (Figure 6.5B, top panels), the posterior samples are more constrained, centering around lower values of both α and m, with a stronger concentration of α values above m, suggesting a slight dominance of aestivation over migration in the studied mosquito population.

To quantitatively interpret the ecological implications, we examined the posterior estimates of the effective population sizes during different seasonal states (Figure 6.5A). The median estimates are as follows:

- N_R (Early Rainy Season): ~5529
- N_D (Late Dry Season): ~2588
- N_A (Aestivators): ~1027
- N_M (Migrants): ~883.5

The median estimates were computed across simulation replicates to summarise central tendencies while minimising the influence of extreme stochastic outcomes. These values can be used to reconstruct seasonal population dynamics. The N_D estimate (~2588) represents the remnant population at the end of the dry season, individuals that survived locally, likely in marginal refugia. This group forms the resident dry-season survivors and is the baseline population carried into the Early Rainy Season (ERS).

During the onset of the ERS, this remnant N_D population is augmented by two influxes: (1) aestivators (N_A ~1027) who re-emerge from dormancy, and (2) migrants (N_M ~883.5) who arrive from other regions. These three groups collectively reconstitute the full Early Rainy Season population (N_R ~5529). In terms of proportions contributing to the ERS population:

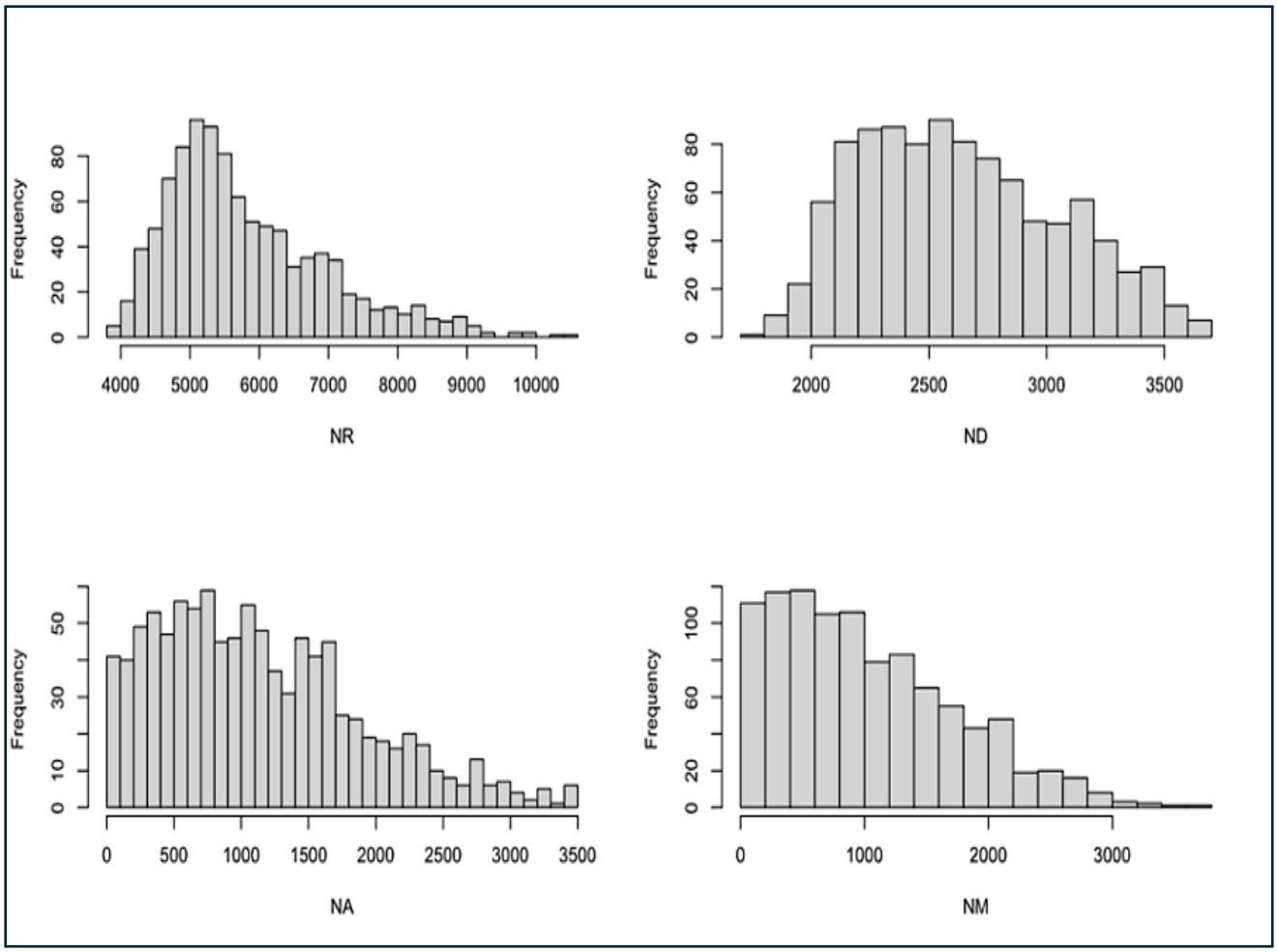
- Residents from N_D account for approximately 47% of N_R (2588 / 5529),
- Aestivators make up around 18.6% (1027 / 5529),

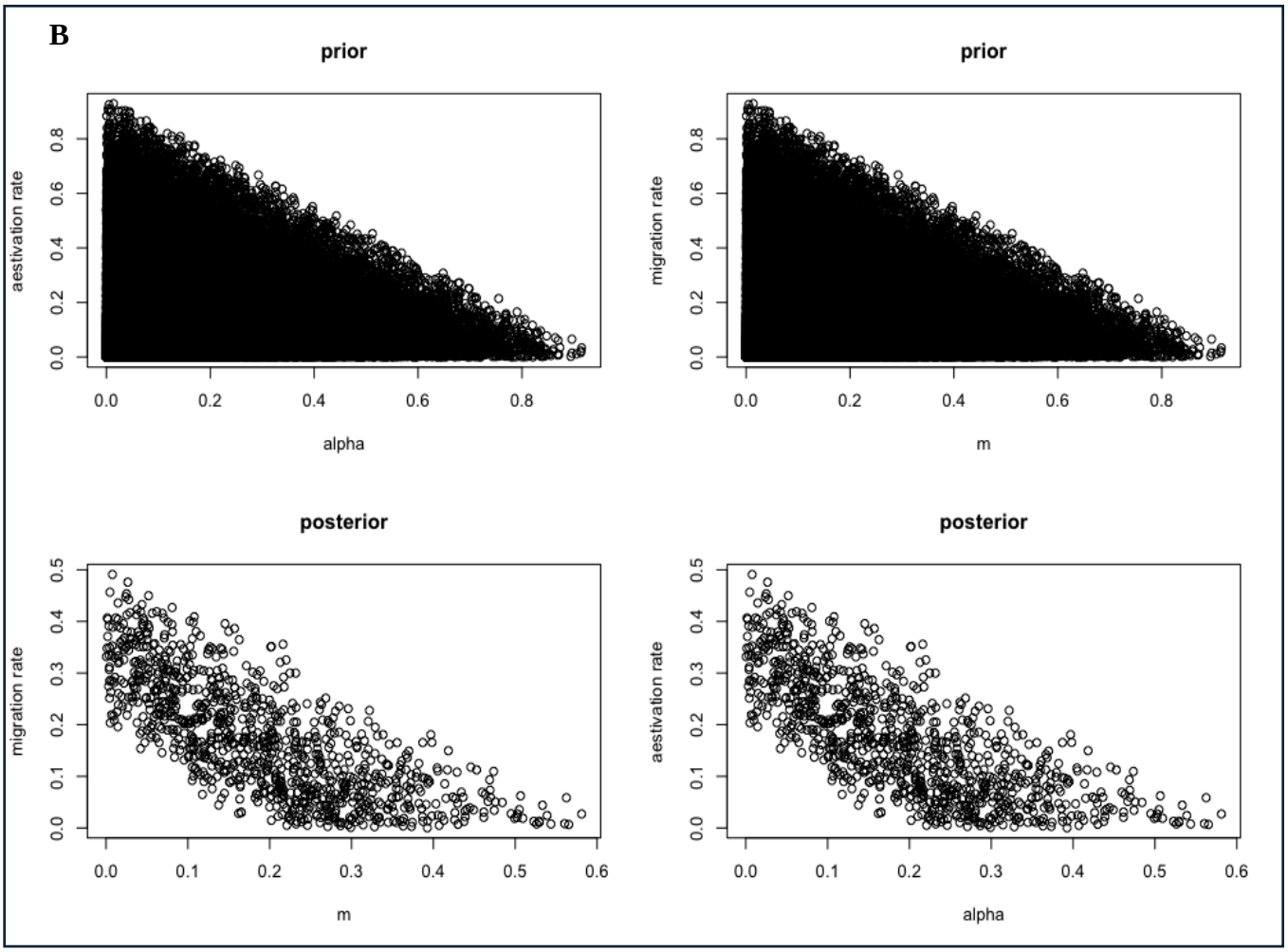
- Migrants contribute about 16% (883.5 / 5529).

This means that about 35% of the ERS population is new, derived from aestivation and migration, while the remaining 65% is composed of local survivors from the dry season.

Thus, the model suggests that both aestivation and migration are significant contributors to population rebound, but aestivation slightly exceeds migration, as supported by both the posterior densities in Figure 6.5B and the population estimates in Figure 6.5A.

A





A: Posterior histograms showing posterior Ne estimates, **B:** posterior plots that rule out zero migration/aestivation.

Figure 6.5 *Posterior plots*

6.4 Discussion

Population structure of *An. gambiae* mosquitoes collected in Eastern Uganda

Genetic differentiation among *An. gambiae* populations in Eastern Uganda is minimal, as shown by uniformly low pairwise F_{ST} values (all < 0.05). The very small magnitude of these values—most between 0.001 and 0.02 indicates high, ongoing gene flow, forming a well-connected metapopulation. Although some FST estimates were statistically significant (P < 0.05), their biological meaning is unchanged: the extremely low values

show that any genetic segregation is trivial and does not reflect real population subdivision. The overall pattern is one of genetic homogenization driven by sufficient gene flow to prevent divergence, not of isolated populations. Thus, the conclusion of high regional connectivity is based on the biological signal of low FST values, not on statistical significance alone.

The PCA plots generally suggest low genetic variation within the whole dataset, indicating genetic similarity, thus implying that the mosquito samples collected from the 14 districts are closely related and make up a homogeneous population (Novembre & Stephens, 2008). Closely related individuals could have a recent common ancestry, high gene flow from within populations (in this case within the districts considered), and a bottleneck effect or founder effect in the population (Patterson et al., 2006b).

Results from the PCA and pairwise F_{ST} therefore show no clear population structure, as a result, all mosquito samples were pooled together and considered as one population with thirteen time points between 2017 and 2018.

Temporal *F* and *Ne* values

Temporal *F*-statistics measure genetic drift between time points. In an ideal Wright-Fisher (WF) population, this drift would directly reflect *Ne*. However, our system involves two additional mechanisms, aestivation and migration, that modify this relationship. The two processes alter the expected drift patterns, which means that our observed temporal *F* values cannot simply be interpreted as *Ne* estimates given that aestivation reduces apparent drift by preserving genetic composition, while migration increases it through allele frequency changes. Thus, to disentangle these effects, we developed models that explicitly incorporate seasonal population size changes (N_R, N_D), an aestivation fraction and migration rate (m). Through ABC, we could simultaneously estimate these parameters by

comparing model simulations to empirical temporal *F* data and this approach allowed us to: (1) quantify the relative contributions of aestivation in contrast to migration, (2) estimate true *Ne* sizes during the different seasons, and (3) determine what proportion of the population persists through each mechanism.

The low temporal *F* values suggested low levels of genetic differentiation across time points, something expected given the short time between time points (Hartl & Clark, 1989; Wright, 1951). The *Ne* values computed from the 3R and 3L chromosome arms were consistent, while those from the 2R arm were not. This discrepancy in *Ne* estimates from the 2R arm could be because it contains multiple common inversions (for example 2La, 2Rb) that suppress recombination, may create localized selection pressures, or even distort neutral drift patterns, which structural variants violate WF assumptions, biasing Ne estimates (Lehmann, et al., 1998; White et al., 2007). As a result, we only considered the 3R and 3L arms in the subsequent analyses.

The lower temporal *Ne* estimates for the 2R chromosome in *An. gambiae* compared to 3R and 3L can be attributed to inversion polymorphisms, selection, reduced recombination, historical demographic events, and the presence of insecticide resistance alleles, which collectively reduce genetic diversity, strengthen linkage effects, and decrease effective population sizes in the 2R regions (Cheng et al., 2012; Fontaine et al., 2015; Hoffmann & Rieseberg, 2008; Kirkpatrick & Barton, 2006; White et al., 2010). In contrast, chromosomes 3R and 3L are less affected by such factors, leading to higher Ne estimates.

Checking temporal F statistics with Posterior medians

When the resulting temporal F statistics from posterior medians were compared with the observed values, they were close, suggesting that the model fits the observed data. By comparing the simulated to the observed temporal F statistics, we assessed how well the

model, and the estimated parameters explain the genetic differentiation observed in the mosquito population. These posterior distributions provided the range of plausible parameters that describe the data, thus enabling us to understand the biological processes that affect the malaria mosquito population dynamics, such as LDM and aestivation.

Using the posterior estimates of the number of aestivating (N_A), migrating (N_M), and total early rainy season mosquitoes (N_R), as shown in Figure 6.5A, we inferred the relative contributions of aestivation and migration to the population structure.

To rule out zero aestivation or migration, we examined the ratios of N_A/N_R and N_M/N_R—representing the population sizes of aestivators and long-distance migrants relative to the early rainy season population size, respectively. The results showed that $N_A/N_R \approx 18.6\%$ and $N_M/N_R \approx 16\%$, providing strong evidence for non-zero contributions from both aestivation and migration (Figure 6.5). This confirms that both strategies play a measurable role in population recovery at the onset of the rainy season.

The posterior distributions thus suggested that both aestivation and migration are non-zero, with moderate rates for both processes. To clarify this classification, we established reference categories based on the boundaries of the uniform prior distributions (0 to 1) and guided by biological plausibility: (i) A low rate of 0 to 0.1 indicates minimal contribution; unlikely to support population rebound alone, (ii) A moderate rate of 0.1 to 0.3 implies a substantial but not dominant contribution, consistent with mixed survival strategies, and, (iii) A high rate of values greater than 0.3 indicates dominant reliance on that process for population rebounds (which is biologically unlikely given field observations of spatial and seasonal variation in mosquito abundance and movement).

Assessing the posterior samples (Figure 6.5B), both aestivation rate (α) and migration rate (m) are concentrated in the 0.1 to 0.25 range, supporting their classification as moderate.

This was further supported by the estimated population sizes N_A and N_M (Figure 6.5A), which constituted roughly 18.6% and 16% of the early rainy season population (N_R), respectively, indicative of meaningful but not overwhelming contributions to population recovery. These findings align with empirical ecological expectations, where malaria mosquitoes are known to employ multiple dry-season persistence strategies rather than rely exclusively on one.

These findings are crucial for understanding how mosquito populations survive and maintain genetic diversity during the dry seasons in Eastern Uganda.

Implications and Further Considerations

This study aimed to detect seasonality signals in the temporal dynamics of the Eastern Uganda malaria mosquito populations by considering the alternating rainy and dry seasons. While the aestivation-migration model assumed two distinct dry seasons (June–August and December–February) and integrated them into the temporal estimates, the results show non-severe seasonality in the genetic data.

These results from Eastern Uganda differ markedly from those reported in Mali by Lehmann et al. (2017), where the dry season lasts 3 to 7 months and surface water is nearly absent, necessitating extreme survival strategies such as prolonged aestivation or long-distance migration. In contrast, the shorter and less severe dry season in Eastern Uganda, where intermittent or semi-permanent breeding habitats such as rice fields and irrigated farmland may persist, suggests a different ecological scenario. The presence of moderate levels of both aestivation ($N_A/N_R \approx 18.6\%$) and migration ($N_M/N_R \approx 16\%$) in our estimates (Figure 5A) indicates that while these strategies are clearly non-zero, they are not dominant. The lack of strong genetic bottlenecks and low temporal differentiation further support the possibility that a portion of the population continues to breed locally during the dry season.

We can therefore conclude that in Eastern Uganda, seasonality is less severe compared to Mali, resulting in the observed patterns which are consistent with a flexible, mixed-strategy survival response in which *An. gambiae* hedges across aestivation, migration, and local breeding depending on environmental conditions.

The observed temporal F-statistics were quite low across all seasonal comparisons, with values ranging from 0.0001 to 0.0009, indicating minimal genetic differentiation between time points. This suggests that population genetic structure remained largely stable across seasons, with only small changes detectable in allele frequencies. The minimal temporal *F* statistics indicate that migration does not introduce large allele frequency shifts between seasons, which can be explained by two model features. (1) LDM is not restricted to rainy seasons (RS). The model allows for migrants to arrive during both DS and RS, as neighboring populations may exhibit asynchronous breeding cycles due to microclimatic variation, an accidence that dampens abrupt genetic changes, and (2) Migrants likely originate from genetically similar populations. Low spatial F-statistics among districts (Table 2) implies that LDM primarily involves nearby populations with share allele frequencies, minimizing inflation of DS-RS differentiation.

Therefore, even with LDM, the combined effects of (a) continuous gene flow, (b) residual local breeding during DS and (c) aestivators re-entering the population maintain genetic stability. This aligns with the weak seasonal signal observed, contrasting with systems where LDM introduces pronounced differentiation (e.g. between isolated Sahelian and riparian populations in Mali; (Lehmann et al., 2017)).

Overall, the assumption that genetic drift would be more pronounced during dry seasons due to reduced population sizes (Berthier et al., 2002; Chakraborty & Nel, 1977; Wright, 1931) is not strongly supported by the observed data. Several factors could explain the

limited seasonal signal we observed in the data: (1) the relatively large posterior estimates for effective population sizes during both the rainy (N_R~ 5529) and dry (N_D~ 2588) seasons could reduce the impact of genetic drift across seasons, suggesting that mosquito populations may remain large enough during the dry season to prevent significant genetic bottlenecks (Charlesworth, 2009; Lehmann, Hawley, Grebert, & Collins, 1998), (2) both migration and aestivation are expected to buffer the population from large fluctuations during the dry seasons, however, given that the posterior estimates show non-zero N_A and N_M rates, with median values of N_A~ 1027 and N_M~ 883.5, these processes could allow a portion of the population to survive or recolonise areas during or after the dry season hence preserving genetic diversity by contributing to a more continuous genetic pool across seasons (Fontenille & Simard, 2004; Hartl & Clark, 1989; Lehmann et al., 2010; Slatkin, 1985; Wilson & Rannala, 2003), (3) the relatively short generation time (3 generations per season) means that genetic drift has a limited time to accumulate between sampling periods, which rapid turnover may reduce the potential to detect strong signals of seasonality (Charlwood et al., 1997; Hartl & Clark, 1989; Lanzaro & Tripet, 2003) and (4) the selected time points may not capture the full extent of genetic shifts related to seasonality, thus more granular or long-term sampling could be necessary to detect subtler shifts in allele frequencies related to dry season bottlenecks or rainy-season expansions (Berthier et al., 2002; Hartl & Clark, 1989; Hendry & Kinnison, 1999; Lehmann et al., 1998; Waples, 1989).

The implications of the weak seasonal signals on understanding the population dynamics of the Eastern Uganda malaria mosquitoes are that; (a) genetic diversity is relatively stable across seasons, which could indicate that mosquito populations are resilient to seasonal changes, maintain sufficient size, and use migration/aestivation dynamics to avoid significant genetic drift, and (b) given that mosquito populations remain large and

genetically stable throughout both dry and rainy seasons, this could in turn result in the persistence and spread of malaria.

These small observed F-statistics and the posterior parameter estimates suggest that genetic drift is minimal over the sampled time points, possibly due to the large *Ne* value during the rainy season (Charlesworth, 2009; Wright, 1931). These results also indicate that aestivation and migration play important roles in sustaining genetic diversity during the dry seasons.

The findings from this study also have critical implications for the design and deployment of gene drive systems that are particularly aimed at controlling malaria mosquito populations, and thus provide key insights into the potential challenges and opportunities for their (gene drive) success in this context:

1. The minimal genetic differentiation between seasons suggests that the Eastern Uganda *An. gambiae* populations are genetically stable year-round, with high effective population sizes and continuous migration, maintaining genetic diversity. The positive implication of this on gene drive propagation is that the lack of significant seasonal genetic bottlenecks could facilitate the continuous spread of a drive throughout the population because high population connectivity and migration ensure that the drive element is not confined to isolated subpopulations (Alphey et al., 2010; Eckhoff et al., 2017; Lehmann et al., 1998; North et al., 2013; Turelli & Hoffmann, 1991; Unckless et al., 2017). The ability of mosquitoes to maintain stable populations during the dry season will increase the likelihood that a gene drive will persist and spread even during periods of low mosquito density(Eckhoff et al., 2017; Hancock et al., 2011; Huestis & Lehmann, 2014; Lehmann & Diabate, 2008; A. North et al., 2013). On the other hand, the negative implication

is that population stability also slows down its fixation so that large effective population sizes, particularly during the dry season (ND≈2588), reduce the impact of genetic drift, meaning that natural selection alone could take longer to drive the engineered allele to fixation, which could delay the efficacy of gene drive interventions, especially if additional evolutionary factors, such as resistance development or fitness costs associated with the gene drive, come into play (Deredec et al., 2008; Eckhoff et al., 2017; Hammond et al., 2016; Marshall et al., 2017; Min et al., 2017; Unckless et al., 2017).

2. The non-zero estimates of aestivation and migration rates have implications for the dynamics of gene drive spread: (a) aestivating individuals could act as a reservoir of wild-type alleles that may reintroduce genetic variation after the drive element has spread, which could in turn, slow down the drive's progress, particularly if aestivating individuals have lower exposure to the gene drive during its peak spread in the rainy season (Gantz & Bier, 2016; Noble et al., 2017; Unckless et al., 2017; Yakob & Walker, 2016), (b) long-distance migrants could, on one hand, facilitate the spread of gene drive elements across different mosquito populations, promoting their propagation over a larger geographical area, while on the other hand, they (migrants) could also reintroduce wild-type mosquitoes from other areas into gene-drive targeted populations, reducing the overall efficacy of the drive (Eckhoff et al., 2017; Fontaine et al., 2015; Lehmann et al., 1998; North et al., 2013; Turelli & Hoffmann, 1991).
3. (a) Targeting mosquitoes during the dry season when population sizes are smaller could increase the relative impact of gene drive-induced population suppression, given that with fewer individuals and potentially reduced genetic diversity during this season, the gene drive could spread more effectively; (b) a gene drive strategy

that instead spans both the dry and rainy seasons (multi-season deployment) could be critical in ensuring sustained drive propagation such that the continuous cycling of the population through distinct dry and rainy periods means that any seasonal gaps in the drive's effectiveness could allow wild-type alleles to recover, suggesting that a gene drive system that remains active all year round or designed to perform optimally across different seasonal conditions may be more successful (Burt, 2003b; Huestis & Lehmann, 2014; Turelli & Hoffmann, 1991; Yakob & Walker, 2016).

4. Gene drive systems face the challenge of potential evolution of resistance alleles capable of preventing the drive from achieving fixation (Champer et al., 2016; Hammond et al., 2017; Marshall & Akbari, 2018; Noble et al., 2019; Unckless et al., 2017). The large effective population sizes observed in this study, especially during the rainy season (N_R~ 5529) create, a higher likelihood of resistance alleles emerging and spreading before the gene drive can fully propagate, which could be particularly problematic in regions where mosquito populations are consistently large and interconnected (Champer et al., 2016; Eckhoff et al., 2017; Hammond et al., 2017; Noble et al., 2019; Unckless et al., 2017). This could be countered by designing drive systems that incorporate mechanisms to delay or prevent resistance evolution, such as using multiplexed CRISPR-Cas9 systems that target multiple genes simultaneously in malaria mosquito populations like those in Eastern Uganda, where resistance can arise due to large population sizes (Champer et al., 2018; Gantz et al., 2015; Noble et al., 2019b).
5. Given that migration plays an important role in maintaining genetic continuity across different subpopulations, this implies that (a) for gene drives intended to suppress mosquito populations, localized releases in high-density regions may lead

to faster propagation, even though, migration from untreated areas could weaken the effect, necessitating larger-scale or repeated releases to ensure that the gene drive is not overwhelmed by wild-type mosquitoes migrating into the area (Eckhoff et al., 2017; Gantz et al., 2015; Hammond et al., 2017; Marshall & Akbari, 2018; North et al., 2013), (b) on the contrary, for gene drives aimed at modifying populations without causing extinction (such as those targeting malaria transmission without reducing population size), the observed migration rates suggest that it may be difficult to confine the gene drive to specific regions, and result in the gene drive spreading beyond the intended area, raising concerns about its impact on non-target populations (Eckhoff et al., 2016; Marshall & Akbari, 2018).

Designing gene drive technologies for malaria vector control should, therefore consider factors such as targeting specific seasons, incorporating mechanisms to mitigate resistance, or adjusting spatial deployment strategies, which will be crucial to ensuring their success.

Challenges and limitations of the study

Even though our results indicate minimal genetic differentiation across seasons, several factors could influence the interpretation of these findings:

- The temporal samples in this study (March, April, July, and September 2017) may not fully capture the fine-scale dynamics of genetic changes across the seasons; hence, more frequent or longer-term sampling, especially during critical transition periods between seasons, may provide greater resolution in detecting seasonal shifts (Hartl & Clark, 1989; Huestis & Lehmann, 2014; Lehmann, Hawley, Grebert, & Collins, 1998).

- Our model assumes no sampling error and uses a fixed generation time across seasons. However, environmental conditions such as temperature or rainfall could affect mosquito generation time and alter the strength of genetic drift between seasons. Therefore, we could incorporate environmental covariates such as microhabitat availability or local weather conditions to enhance the explanatory power of the model (Baylis & Rawlings, 1998; Gaggiotti et al., 2009; Hartl & Clark, 1989; D. L. Huestis & Lehmann, 2014; Lyimo & Takken, 1993).
- With relatively low temporal F values, the power to detect weak but biologically meaningful differentiation may be limited, and yet, genetic differentiation may occur at a scale that is too subtle for our current statistical framework to capture, particularly in populations that maintain large effective sizes (Berthier et al., 2002; Charlesworth, 2009; Kalinowski, 2005; Waples, 1998; Whitlock & Mccauley, 1999).

6.5 Conclusion

In this study, we successfully estimate the temporal dynamics of aestivation and migration in Eastern Uganda mosquito populations with reasonable fits between observed and simulated F-statistics. The aestivation-migration model provides insights into population size fluctuations and the role of seasonality in maintaining genetic diversity, with potential applications in understanding mosquito-borne disease transmission dynamics. Our results suggest that Eastern Uganda *An. gambiae* populations exhibit minimal genetic differentiation across seasons, with large Ne values, and non-zero migration, and aestivation rates likely playing a critical role in maintaining genetic stability.

With insights into the temporal dynamics of mosquito malaria vector populations in Eastern Uganda, the results highlight the role of temporal drift and seasonality in shaping genetic

variation. The finite Ne values noted across time points indicate that temporal genetic structure exists, and at the same time, the seasonal model suggests a balanced interplay between aestivation, long-distance migration, and population size fluctuations.

Future work could include a more comprehensive analysis of seasonality and dry season persistence mechanisms across sub-Saharan Africa, with an emphasis on correlating these patterns with climatic variables. It could also focus on incorporating sampling error into the model and exploring more complex scenarios, such as varying migration rates or different seasonal dynamics across years. Furthermore, testing these estimates against data from subsequent years, such as 2018, could provide more robust insights into temporal dynamics in these mosquito populations.

More investigations can be done on understanding the role of seasonality in mosquito populations by focusing on incorporating data from additional years or sampling more frequently within each season and exploring other environmental factors like microhabitat availability, temperature, humidity and vector control measures, which could play a role in shaping mosquito population dynamics, given that including these factors in the model could help refine models of seasonal differentiation, explore potential genetic adaptations related to aestivation or migration processes that provide insights into long-term persistence of populations under seasonal pressures and improve our understanding of the forces that drive population structure.

To have a more comprehensive view of aestivation and LDM, the next steps could also include a more comprehensive analysis of seasonality and dry season persistence mechanisms across Africa, with an emphasis on correlating these patterns with climatic variables.

CHAPTER SEVEN: THE POPULATION GENETIC STRUCTURE AND DEMOGRAPHIC HISTORY OF ANOPHELES GAMBIAE AND ANOPHELES ARABIENSIS AT ISLAND AND MAINLAND SITES IN UGANDA

Preamble

Understanding the population structure and connectivity of malaria vector populations is fundamental to the safe and effective development of genetic vector control tools, such as gene drive systems (Ambrose et al., 2024; Hancock et al., 2024). This knowledge is also critical for defining appropriate release sites, anticipating allele spread, and ensuring ecological and regulatory containment (Burt, 2003; James et al., 2018; National Academies of Sciences, Engineering, 2016). Islands have been suggested as ideal sites for conducting gene drive field trials (National Academies of Sciences, Engineering, 2016; Scott et al., 2002), given their relatively small size and distinct boundaries which can help limit the unintended spread of modified organisms and support more contained and monitored experimental releases (Lanzaro, et al., 2021).

This study therefore investigates the genetic structure and connectivity between *An. gambiae* and *An. arabiensis* populations from selected island and mainland sites in Uganda using targeted amplicon sequencing data. By leveraging genome-wide amplicon sequencing on samples collected from selected sites at the Lake Victoria island and adjacent mainland sites, the study provides critical insights into vector population connectivity, effective population sizes and gene flow dynamics. The findings emphasize the higher isolation, greater genetic differentiation and smaller effective population sizes of island mosquito populations compared to nearby mainland populations, underlining the suitability

of islands as strategic sites for contained field evaluations of gene drive and other genetic control technologies, thereby, forming a critical component of the baseline evidence required to inform site selection, ecological risk assessment**,** and predictive modeling for gene drive development efforts. By evaluating how genetically isolated or connected these mosquito populations are, this work contributes directly to ensuring that any future interventions are context-sensitive, geographically appropriate**,** and grounded in a clear understanding of local vector population dynamics.

The detailed genetic assessments presented here contribute valuable knowledge towards site selection and trial design for genetically-based malaria control strategies and support the overarching goal of developing sustainable, safe, and effective interventions against malaria transmission.

This work has been submitted to the Malaria Journal and is under review

Assessing the population genetic structure and demographic history of *Anopheles gambiae* and *An. arabiensis* at island and mainland sites in Uganda: Implications for testing novel malaria vector control approaches.

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Disclosures

None

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Conflict of Interest Statement

The authors declare that there is no conflict of interest

Abstract

Even with enormous effort put into malaria control, it remains a global burden, disproportionally affecting people residing in areas such as Uganda and many other countries in sub-Saharan Africa. Innovative tools for malaria control are being developed, such as a gene drive system to suppress the vectors' populations, requiring knowledge of the population genetics of malaria vectors, including their population structure, size and genetic diversity to ensure effective and targeted implementation.

This study collected 2918 *Anopheles gambiae* and 173 *Anopheles. arabiensis* across six populations from both the islands on Lake Victoria and mainland Uganda for amplicon sequencing. Large pairwise F_{ST} values were observed between the two species, indicating their divergence. We observed low but often significant F_{ST} values between the 6 *An. gambiae* populations, while between the *An. arabiensis* mainland populations, F_{ST} values were not significant. Principal Component Analysis also revealed strong genetic structure between the two species but did not provide a clear picture between populations within each species. We also found that mainland *An. gambiae* populations had higher within population genetic diversity than the islands', while *An. arabiensis* had the lowest nucleotide diversity. Tajima's D values were all negative, suggesting a recent population expansion. The islands *An. gambiae* populations had very low contemporary effective population sizes in the tens and hundreds, as estimated from linkage disequilibrium, while the mainland population sizes were consistently higher, in the thousands.

This study revealed that there is significant genetic differentiation between *An. gambiae* and *An. arabiensis*, as well as between island and mainland populations of *An. gambiae*, with mainland populations showing higher connectivity and island populations exhibiting greater isolation. In contrast, *An. arabiensis* mainland populations displayed no significant

differentiation, suggesting panmixia. These findings thus highlight the influence of geographic and ecological factors on population structure and provide critical insights for selecting candidate sites and designing field trials for genetic-based malaria control strategies.

Keywords: *An. gambiae*, *An. arabiensis*, population genetics, genetic structure, Single Nucleotide Polymorphisms (SNPs).

Background

Even with enormous progress over the years, malaria remains a global burden, especially in the sub-Saharan Africa because of the exceptionally adaptive vector dynamics of the *Anophelines* among other factors such as insecticide resistance, drug resistance, limited access to healthcare, and environmental and socio-economic challenges (Bhatt, et al., 2015; Lehmann et al., 2010).The World Health Organisation (WHO) reported about 200 million cases and 600,000 deaths in 2023, (WHO, 2024) 249 million malaria cases in 2022 and 608,000 deaths from 85 malaria-endemic countries in 2022 (WHO, 2023), and similarly 247 million cases and 619,000 deaths in 2021 (WHO, 2022). Sub-Saharan Africa carries the greatest burden globally and accounts for 94% of all malaria cases and about 96% of all deaths (WHO, 2023, 2024). Uganda is one of the most highly burdened countries in sub-Saharan Africa and ranked third in the number of malaria cases worldwide in 2022, contributing 5% of the global burden (WHO, 2023).

The fight against malaria is increasingly hampered by the evolution of resistance in both the parasite and vectors, and the partial efficacy of current control methods (Hemingway, 2014; Mushtaq et al., 2024; Plowe, 2022; Thu et al., 2017; WHO, 2024). Achieving malaria elimination and eradication will thus require new products and interventions to be used in tandem with the already existing tools (Kaddumukasa et al., 2020b). One promising strategy is a mosquito gene drive system, capable of spreading into an entire population from low initial frequencies (Hammond et al., 2021; Marshall & Akbari, 2016) suppressing or replacing mosquito populations (Burt, 2014). Significant advancements have been made so far in target gene identification, gene construct development, and genome sequencing of the major malaria mosquito populations (Gantz et al., 2015; Hammond & Galizi, 2017; Makunin et al., 2022).

Despite the proof-of-concept mosquito transformation studies done in laboratories that show promising results and have resulted in identification of candidate gene drive systems (Galizi et al., 2014; Gantz & Bier, 2015; Hammond et al., 2016), there is need to advance to further field trials such that product feasibility and efficacy can be evaluated. Therefore, detailed population genetic assessments must be done systematically before deploying any proposed trial or intervention. This involves understanding the relationship between malaria vectors and the environment they occupy, which provides information about gene flow, and in turn, aids in understanding species evolution (Ng'habi et al., 2011). Information on population genetic structure and gene flow within a vector species provides a basis for modeling the impact of trial intervention and in trial design and helps select suitable locations for field trials, given that, ideally, these locations should have limited gene flow with surrounding populations (Lanzaro et al., 1998; Lanzaro & Tripet, 2003).

In Uganda, the geographic and genetic isolation of lacustrine *An. gambiae* island populations from the mainland render them suitable candidate sites for initial testing of mosquito gene drive systems (Hammond & Galizi, 2017). However, this isolation could eventually limit the spread of a gene drive during broader implementation (Makunin et al., 2022).

Knowledge of A*n. gambiae* and *An. arabiensis* genetic diversity, structure, and population sizes at island and mainland sites will certainly inform potential variations at the target field sites and efficacy monitoring (Kayondo et al., 2005; Lanzaro & Tripet, 2003; Wiltshire et al., 2018). Several studies have examined the structure of *An. gambiae* at the island and mainland populations in Uganda (Kayondo et al., 2005; Lukindu et al., 2018; Wiltshire et al., 2018), but there are no studies that compare the population structure of both *An. gambiae* and *An. arabiensis*, despite their known sympatric occurrence in many regions

(Della Torre et al., 2005). *An. arabiensis* from the lacustrine islands is often excluded from further analysis due to their limited numbers in the mosquito collections done to date (Kayondo et al., 2005; Lukindu et al., 2018; Wiltshire et al., 2018), and anecdotal observations suggest that the islands on Lake Victoria may have minimal complexity of malaria vector species, with *An. gambiae* as the predominant vector (personal communication).

Also, microclimate variation is suggested to be an important ecological factor that affects mosquito survival and fitness and could therefore affect population numbers and hence malaria transmission potential (Ngowo et al., 2017; Okech et al., 2003). Mosquito survivorship is strongly influenced by microclimatic conditions in a given area (Zhong et al., 2016) and could result in increased density of a given species in an area whose numbers were previously very low (Ngowo et al., 2017; Okech et al., 2003; Zhong et al., 2016).

With these points in mind, this study aims to provide an up-to-date overview of the population structure, size, and diversity of the two malaria vector species on three islands sites and three mainland sites of Uganda using an amplicon panel designed to work across the whole *Anopheles* genus to identify species.

Methods

Mosquito sampling

Mosquito collections were conducted from six sites; three mainland sites (Kayonjo, Katuuso, Kibbuye) and three island (Bugiri, Kiimi, Kansambwe) populations (Figure 7.1). Kibbuye and Katuuso villages are situated in Mukono district and Kayonjo village in Kayunga district (Figure 7.1), all about 50km Northwest of the capital city, Kampala. The lacustrine island sites are situated within Lake Victoria in Uganda and are connected by ship and ferry services to the Ugandan mainland.

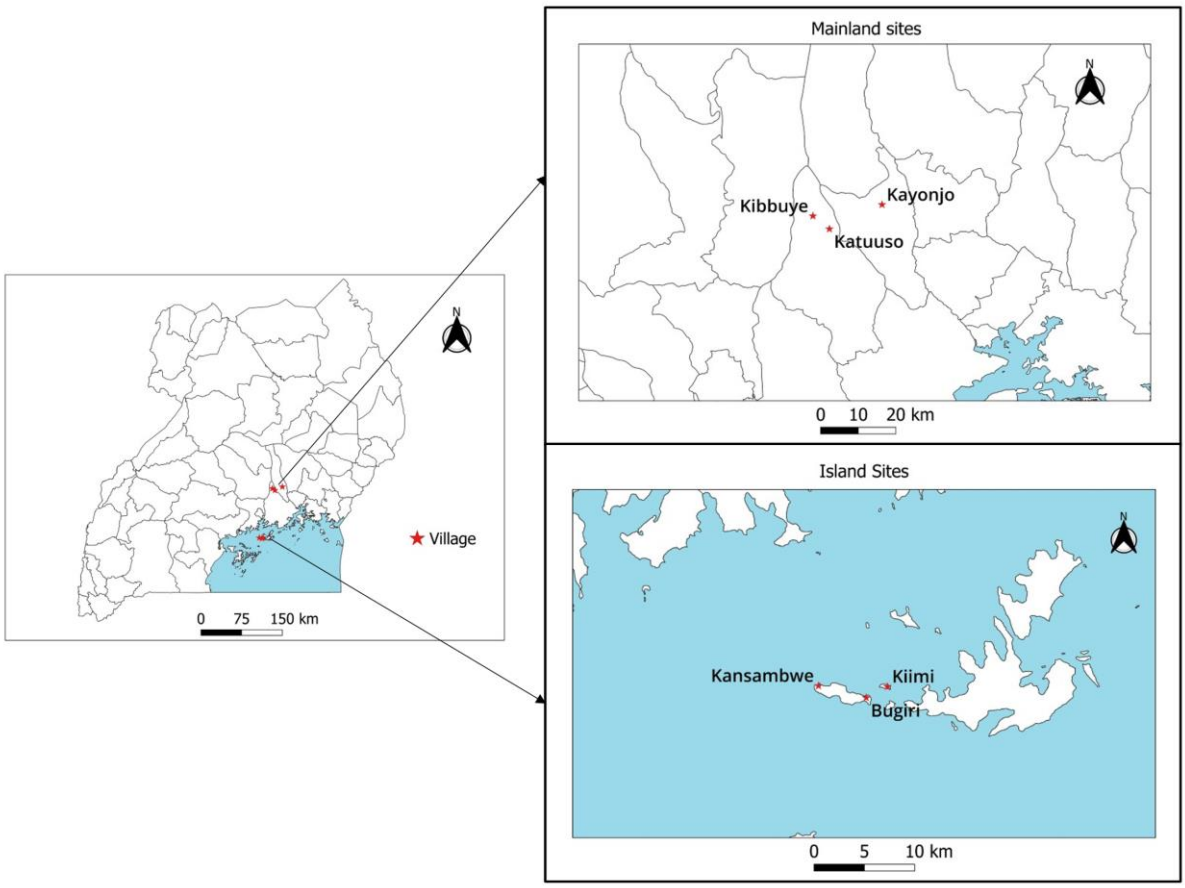


Figure 7.1: *Map showing the location of the 3 mainland and 3 island study sites in Uganda*

On the islands, collections were done from April 2013 to April 2016, and on the mainland from January 2016 to December 2018. Adult mosquitoes were sampled indoors and outdoors using human landing catches (HLC), pyrethroid spray catches (PSC), indoor and outdoor aspirators, and catch basin traps (CBT). All mosquitoes were morphologically identified using the *Anopheles* morphological identification keys (Gillies et al., 1987) and then stored in 80% ethanol in a -20 ^{0}C freezer for subsequent molecular analysis. For this study, we kept only *An. gambiae* and *An. arabiensis* mosquitoes, although some *An. funestus*, *An. maculipalpis*, and *An. coustani* were also collected (but excluded from this analysis).

Genomic DNA extraction

A total of 3515 whole mosquito samples, each stored in a 96-well plate of 80% ethanol, were shipped to the Wellcome Sanger Institute, United Kingdom, for DNA extraction and amplicon sequencing. For details regarding the sequencing techniques used and the amplicon regions considered, refer to the original publication (Makunin et al., 2022). In brief, DNA extraction was completed by removing the ethanol from each specimen, adding 100 uL of lysis buffer C to each, and incubating the plates at 56 ^{0}C for overnight (Makunin et al., 2022). The DNA from each mosquito was then subjected to a single Polymerase Chain Reaction (PCR) reaction containing a 64-primer pair plex (Makunin et al., 2022). Each reaction then went into a second PCR to add indexing primers. Eight plates of mosquitoes were pooled for a single MiSeq library (Makunin et al., 2022).

Using the **ANO**pheles **SP**ecies and **P**lasmodium (ANOSPP) panel of 64 phylogenetically informative and highly variable "amplicon loci" of which 62 target the *Anopheles* nuclear genome and the other 2 targeting the *Plasmodium* mitochondria (note: these amplicon loci are of ~160 base pairs or "sites" long in downstream analyses), the generated sequence data enabled species identification, detection of *Plasmodium*, hybrid or contaminated samples, and identification of cryptic species (Makunin et al., 2022) (For all other details regarding the sequencing techniques and the functions of the 62 amplicon loci, please refer to the original publication (Boddé et al., 2022; Makunin et al., 2022)).

Using a Variational Autoencoder (VAE), a machine learning approach that identifies patterns in high-dimensional data by compressing it into a lower-dimensional representation (Kingma & Welling, 2013) to distinguish between closely related species (Boddé et al., 2022).

Bioinformatics Analysis

The raw reads from the generated sequence data for the confirmed *An. gambiae* and *An. arabiensis* were aligned to the reference genome file (VectorBase-59_AgambiaePEST_Genome.fasta). They were genotyped to produce variation data using AmpSeeker, a computational pipeline used to analyse amplicon-sequenced data (Nagi, 2023). The pipeline is built on the Snakemake workflow management system, is highly customisable, and allows users to specify the kinds of data operations, visualizations, and analyses they want to perform on their data (Quality control, Sequence Alignment visualizations, PCA plots, allele frequency calculations).

The resulting raw variant call format (VCF) file contains variant calls across all samples and comprises a total of 12,412 sites (inclusive of insertions and deletions (indels), primer regions, fixed and polymorphic sites). Subsequent subsetting was performed on the VCF file based on the metadata information, which provided details about the mosquito samples, including individual sample IDs, species, collection location, collection period, and season, among others. In the filtering process, indels were removed, resulting in 9,890 remaining sites. These sites were distributed across the genome as follows: X (n = 952), 2L (n = 1881), 2R (n = 2815), 3L (n = 1615), 3R (n = 2627). The minor allele frequency (maf) spectrum between 0 and 2% is shown in Figure 7.2, and the number of alleles per locus (from fixed to quadriallelic) is in Figure 7.3. Our downstream analyses were based on these 9,890 sites with potential further filtering, such as according to maf and missing values.

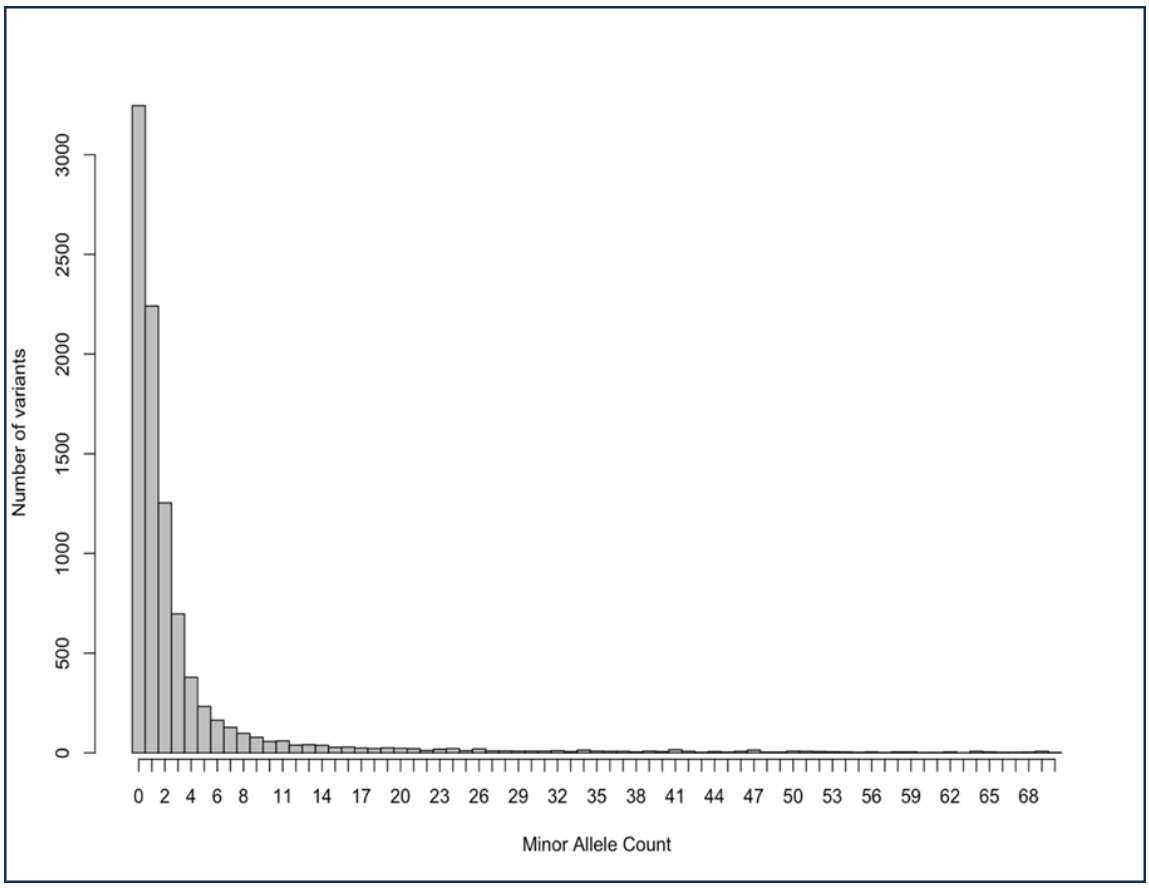


Figure 7.2: *Minor allele frequency (MAF) spectrum between 0 and 2% (for the whole dataset)*

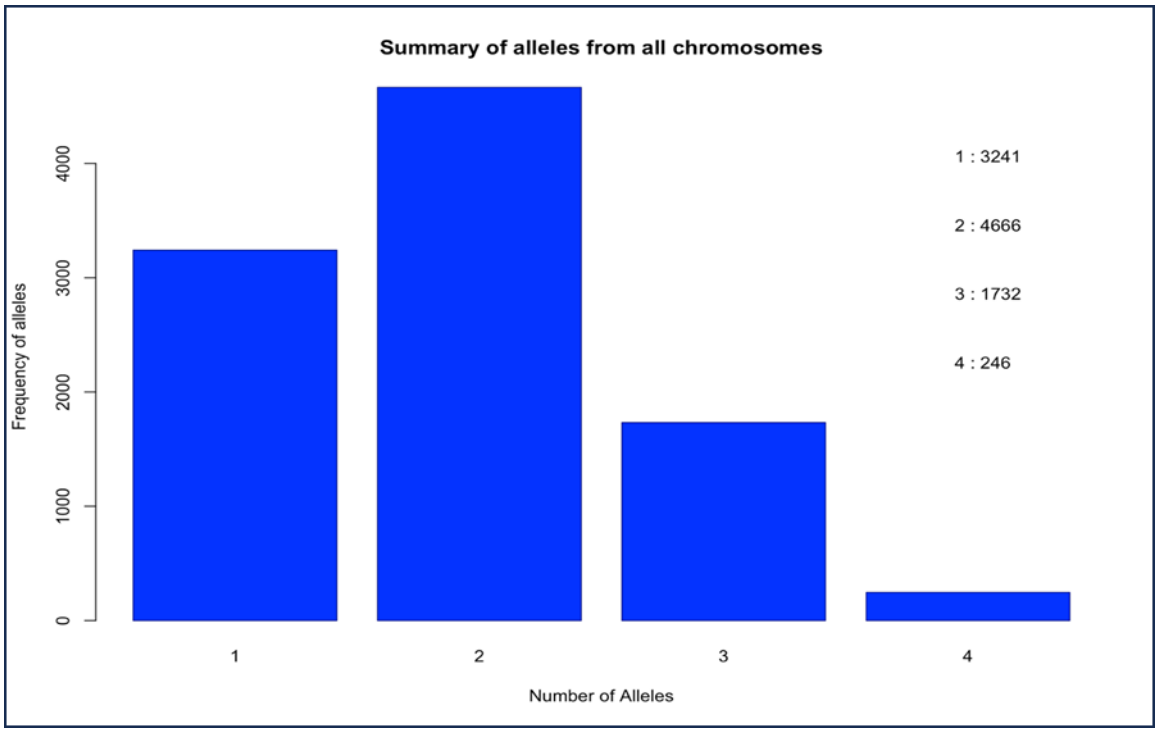


Figure 7.3: *Summary of the number of alleles per site from all chromosomes.*

9890 sites are included. The 5 sites with 0 alleles are missing values in all our *An. gambiae* and *An. arabiensis* samples and were excluded from the graph.

Population genomic analysis

Population structure

The following methods were used to describe population structure: (1) F_{ST} between and within pairs of populations using version 1.3.23 of the Popkin package in R (Ochoa & Storey, 2016, 2021), (2) Principal Components Analysis (PCA) using version 3.6.2 of the prcomp package in R (Ochoa & Storey, 2016, 2021), and (3) Bayesian clustering analysis with STRUCTURE v2.3.4 (Pritchard et al., 2000). Average pairwise Hudson's F_{ST} was calculated using the fst_hudson_pairwise function of the Popkinsuppl package (Ochola, 2022), after which standard errors were computed using the leave-one-out approach that is a specific form of the jackknife resampling method (Arnold et al., 2013; Bhatia et al., 2013; Efron & Tibshirani, 1994). Z-scores were then computed by dividing the estimated FST by its standard error. The Z-scores were used to assess the significance (P-values) of the F_{ST} values. The P-values are derived from the Z-scores by comparing them against the standard normal distribution, which allows for determining the probability of observing such F_{ST} values under the null hypothesis of no differentiation between populations (Efron & Tibshirani, 1994). All the *An. gambiae* collected from the same location were considered as a population, while all 173 *An. arabiensis* were grouped as the seventh population.

For pairwise F_{ST}, we used biallelic sites with 10% missing data from within the 3L chromosome with maf ≥1%, while for PCA, we first considered all chromosomes and thereafter only the 3L arm. To investigate the role of geographic distance in shaping genetic differentiation, we ran a Mantel test to test for isolation by distance (IBD) between populations by combining Euclidean geographic distances (calculated from geographic

coordinates) and genetic distances based on 9999 permutations using GenAlEx 6.51b2 (Diniz-Filho et al., 2013; Peakall & Smouse, 2012). For *An. arabiensis* populations, the same F_{ST} and Mantel tests were also conducted using the 3L chromosome arm biallelic SNPs but only for the three mainland populations. The islands were left out because of their small sample sizes (Table 7.1). PCA was performed in R v4.3.1 with the prcomp() function (Patterson et al., 2006b; Team, 2023), first for biallelic SNPs from all chromosomes and then for the 3L chromosome arm.

We analyzed doubletons, defined as loci with exactly two copies of the minor allele in a biallelic site. A contingency table of observed counts of heterozygous doubleton pairs across populations was constructed, and the expected counts were calculated based on sample size proportions to assess population structure. Populations were grouped into three categories: *arabiensis* (*An. arabiensis*), *mainland_gam* (mainland *An. gambiae*), and *Island_gam* (island *An. gambiae*), and a chi-square test was conducted to compare observed and expected counts to detect significant population structure.

Table 7.1. *The number of confirmed An. gambiae and An. arabiensis samples across six collection sites*

	Bugiri †	Kiimi †	Kansambwe †	Kayonjo	Katuuso	Kibbuye	Total
An. gambiae	28	258	294	1178	450	710	2918
An. arabiensis	2	0	3	51	39	78	173
Total	30	258	297	1229	489	788	3091

† Denotes island populations.

To find the optimal number of clusters (K) in STRUCTURE, we conducted 20 independent runs for each K (from 2-8), using a burn-in value of 100,000 iterations followed by 100,000 repetitions (Pritchard et al., 2000). Then K was determined using the Delta K method of Evanno et al (2005), following which CLUMPAK was used to construct a graphical

representation of the genetic structure of the 3091 (both *An. gambiae* and *An. arabiensis*) mosquito samples (Kopelman et al., 2015b). In this analysis, we randomly chose one site per amplicon locus from the 2R, 3R, and 3L chromosome arms to minimise genetic linkage, with the constraint that maf≥1%.

Genetic diversity and Ne

To assess the genetic diversity per population, we calculated the overall nucleotide diversity (p) as well as individually for the 6 *An. gambiae* and 3 mainland *An. arabiensis* populations for each chromosome arm (Nei & Miller, 1990b) using VCF files without missing data. Deviation from the standard neutral model was tested using Tajima's D, which was performed for each population and chromosome to examine population expansion (Tajima, 1989). Tajima's D and nucleotide diversity were computed using the Pegas package in R (Paradis, 2010b). Estimates of contemporary Ne were attained using the linkage disequilibrium (LD) based method LDNe (Waples & Do, 2008) of NeEstimator v.2.01 (Do et al., 2014). We randomly chose one site per amplicon locus from chromosomes 3R and 3L with maf≥1% to avoid tight linkage. We similarly individually computed nucleotide diversity, Tajima's D and contemporary Ne for individual mainland *An. arabiensis* populations.

Results

Genetic differentiation (F_{ST})

2918 samples were confirmed as *An. gambiae* and 173 as *An. arabiensis*. These individuals and their resulting amplicon data were our subjects for downstream population genetic analyses, and Table 7.1 summarises their distribution across the six collection sites. The average pairwise Hudson's F_{ST} is found in Table 7.2A. All pairwise comparisons produced low F_{ST} values (0.00054-0.028), except between the "outgroup" *An. arabiensis* and any *An.*

gambiae populations. Among *An. gambiae* mainland populations, pairwise F_{ST} values were very low (0.00054-0.0016, indicating minimal genetic differentiation, and similarly, low F_{ST} values were observed between Bugiri and the other two islands (Kiimi and Kansambwe), suggesting significant gene flow. The highest F_{ST} values occurred between Kansambwe and all the mainland sites (island vs mainland), and between Kiimi and Kansambwe (island vs island), implying restricted gene flow in these comparisons (Table 7.2A). The pairwise F_{ST} values between Bugiri and all the other sites, Kayonjo and Katuuso, Kiimi and Katuuso and Kiimi and Kibbuye were low and non-significant ($z < 1.96$, $P > 0.05$). We then calculated pairwise F_{ST} values among the mainland *An. arabiensis* populations (Table 7.2B), and low and statistically nonsignificant pairwise F_{ST} values were mostly recorded. Also to note, the F_{ST} value between Katuuso and Kibbuye was negative.

Table 7.2: *Pairwise FST statistics*

A. *An. gambiae* & *An. arabiensis*

	BUGIRI †	**KIIMI †**	**KANSAMBWE †**	**KAYONJO**	**KATUUSO**	**KIBBUYE**	***An. arabiensis***
BUGIRI †	0	0.6796089	0.5914296	1.762527	1.628254	1.47858	4.361302*
KIIMI †	0.00287	0	4.631849*	1.977574*	1.943631	1.78073	4.360677*
KANSAMBWE †	0.00272	0.02123	0	3.189523*	3.041091*	3.916723*	4.041615*
KAYONJO	0.00995	0.01199	0.02715	0	1.204753	1.991724*	3.779667*
KATUUSO	0.01201	0.01483	0.02841	0.00054	0	2.439927*	3.826917*
KIBBUYE	0.01033	0.0174	0.02433	0.00109	0.00163	0	3.737203*
An. arabiensis	0.2171	0.20209	0.26279	0.22654	0.2333	0.20902	0

B. *An. arabiensis*

	KAYONJO	**KATUUSO**	**KIBBUYE**
KAYONJO	0	1.817574	0.09362245
KATUUSO	0.00785541	0	-1.070926
KIBBUYE	0.00018109	-0.0014994	0

(A) Pairwise FST from the 3L chromosome arm for the six *An. gambiae* populations, with the 173 *An. arabiensis* grouped as the seventh population. **(B)** Pairwise FST among the

three *An. arabiensis* populations. For both tables, the lower left triangle in each table shows average Hudson's F_{ST} values between each population pair, while the upper right triangle shows the z-scores for each F_{ST} value calculated via a block-jackknife method. The values with * indicate significant F_{ST} values ($z > 1.96$, $p < 0.05$). † Denotes island populations.

The pairwise F_{ST} values for *An. gambiae* were plotted against geographic distances to investigate the strength of isolation by distance (Diniz-Filho et al., 2013), and a marginally significant correlation was detected (Figure 7.4, P = 0.052), suggesting a potential trend towards significance. Therefore, according to the IBD hypothesis, there may be a meaningful association between the genetic and geographical distances among populations.

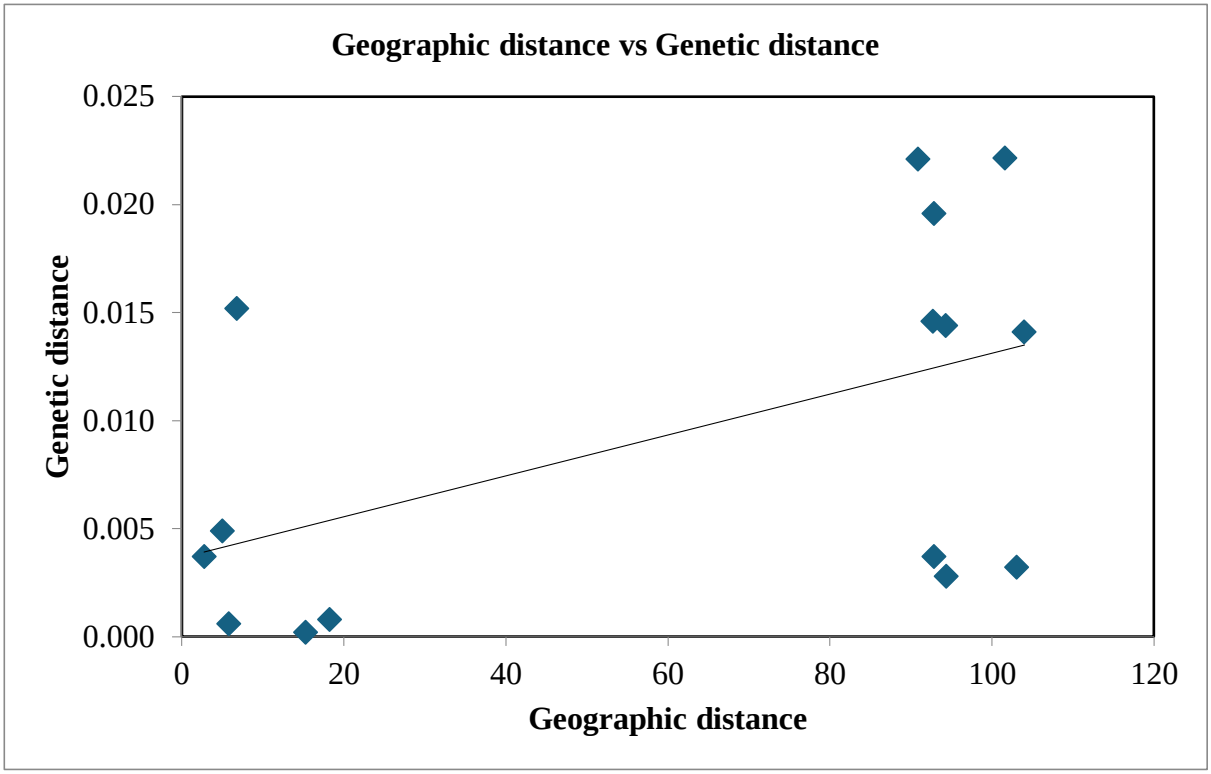
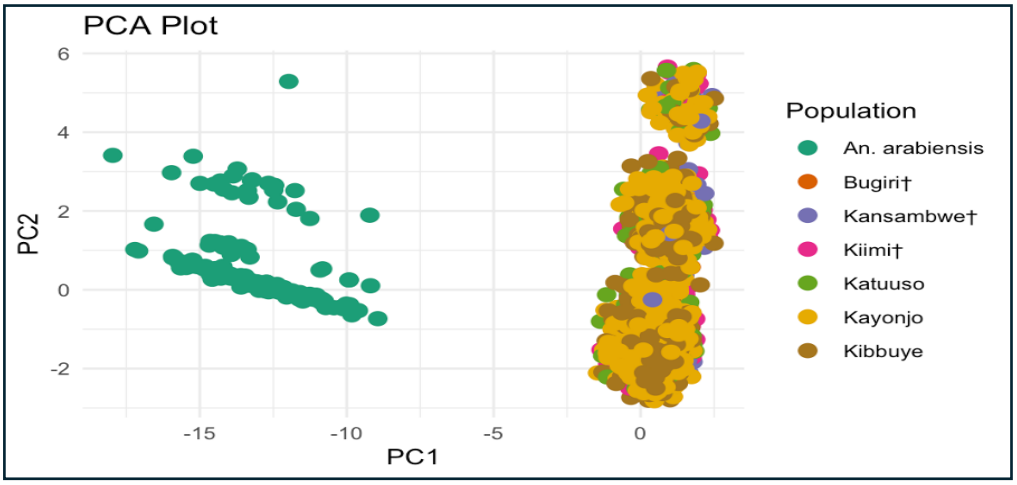


Figure 7.4: *FST versus geographical distance (km) for An. gambiae.* FST values were extracted from Table 2. The figure shows a positive slope with no significant correlation between the genetic and geographic distances, P = 0.063

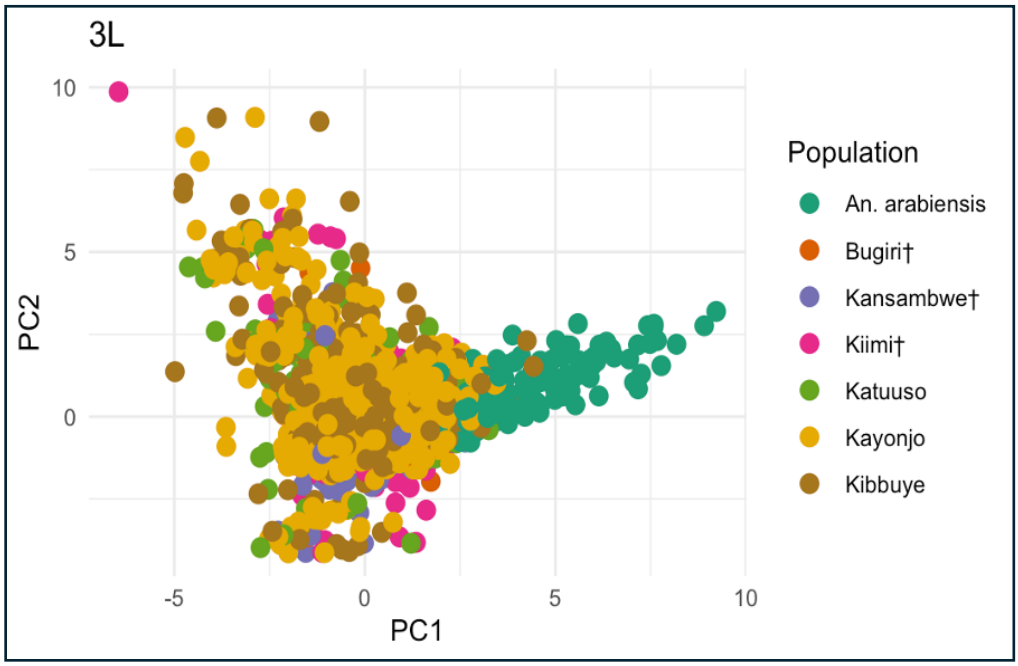
PCA

PCA visualises how the individuals were genetically clustered within and between collection sites. Analysis using all biallelic sites of all chromosome arms of all *An. gambiae* and *An. arabiensis* samples showed clustering into two distinct groups in accordance with each species' genetic difference (Figure 7.5A). The 3L chromosome arm from both *An. gambiae* and *An. arabiensis* also showed species-specific clustering (Figure 7.5B) and using chromosome 3L sites of only *An. gambiae*, no geographical clustering of individuals was observed (Figure 7.5C).

A



B



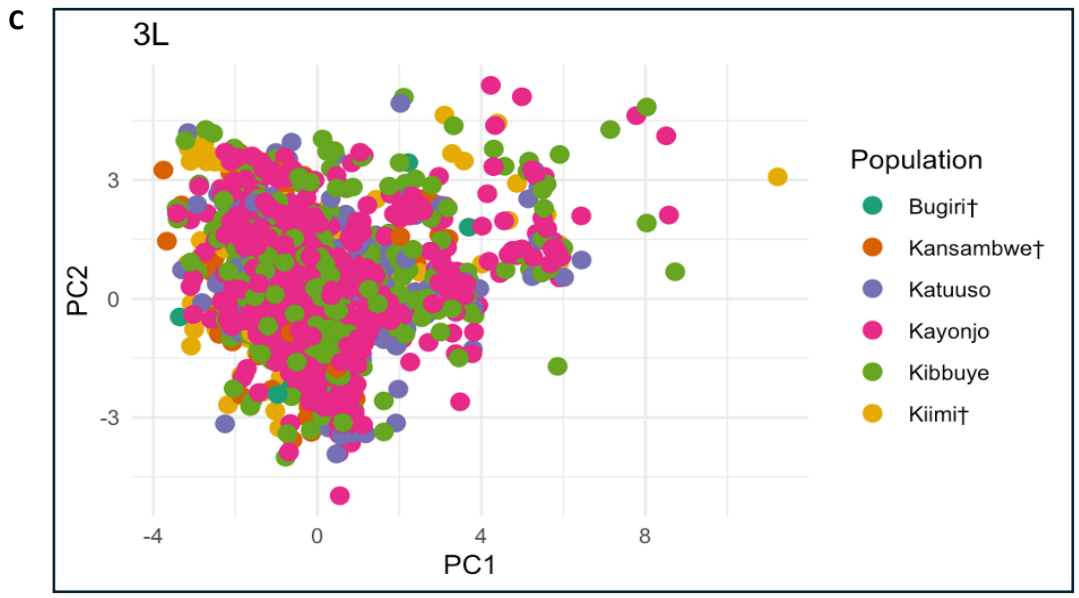


***Figure 7.5** PCA Plots*

A: Principal component analysis of all chromosome arms of An. gambiae (island and mainland populations) and An. arabiensis**, B:** The PCA was calculated from the 3L chromosome arm of An. gambiae and An. arabiensis individuals, and **C**: The PCA was calculated from the 3L chromosome arm of only An. gambiae individuals. † Denotes island populations.

Allele sharing in doubleton variants

839 pairs of doubletons were discovered. We calculated the expected counts based on sample size proportions to account for the distribution of the observed heterozygote doubleton pairs under a null hypothesis assuming no population structure. Because of the limited resolution available to distinguish between individual populations in our dataset, we reduced the original 6+1 (Bugiri, Kiimi, Kansambwe, Kayonjo, Katuuso, Kibbuye, and *An. arabiensis*) population groups into three broader categories: *arabiensis*, *island_gam*, and *mainland_gam*. In this way, more robust statistical comparisons and interpretations of allele sharing across major population segments could be made. The reduction from 6+1 populations to three groups improved statistical power and provided insights into gene flow dynamics across the major geographic and species categories of interest.

There were significant deviations between observed and expected pairwise counts of doubleton pairs in the three groups: *arabiensis*, *island_gam*, and *mainland_gam*. The observed pairwise counts of heterozygotes within and between population groups highlight actual genetic similarities, while the expected pairwise counts reflect the hypothetical distribution of pairings in the absence of genetic structuring.

When we considered within-group genetic structuring (Table 7.3), the *mainland_gam* group showed a significantly higher within-group count of heterozygous pairs than expected (682 observed vs. 480 expected), which suggests a higher-than-anticipated allele sharing or pairing frequency within this group. The *island_gam* group showed a lower-than-expected level of allele sharing (2 observed vs. 29.54 expected), suggesting limited gene flow with other populations, while the *arabiensis* group showed moderated within-group genetic isolation (15 observed versus 2.63 expected counts), suggesting that it is a relatively closed genetic pool with reduced gene flow to other populations.

Table 7.3: *Allele sharing in doubleton variants*

A. Observed counts

	arabiensis	*island_gam*	*mainland_gam*
arabiensis	15	4	102
island_gam	NA	2	34
mainland_gam	NA	NA	682

B. Expected counts

	arabiensis	*island_gam*	*mainland_gam*
arabiensis	2.628186	17.62252	71.03699
island_gam	NA	29.54064	238.1587
mainland_gam	NA	NA	480.01296

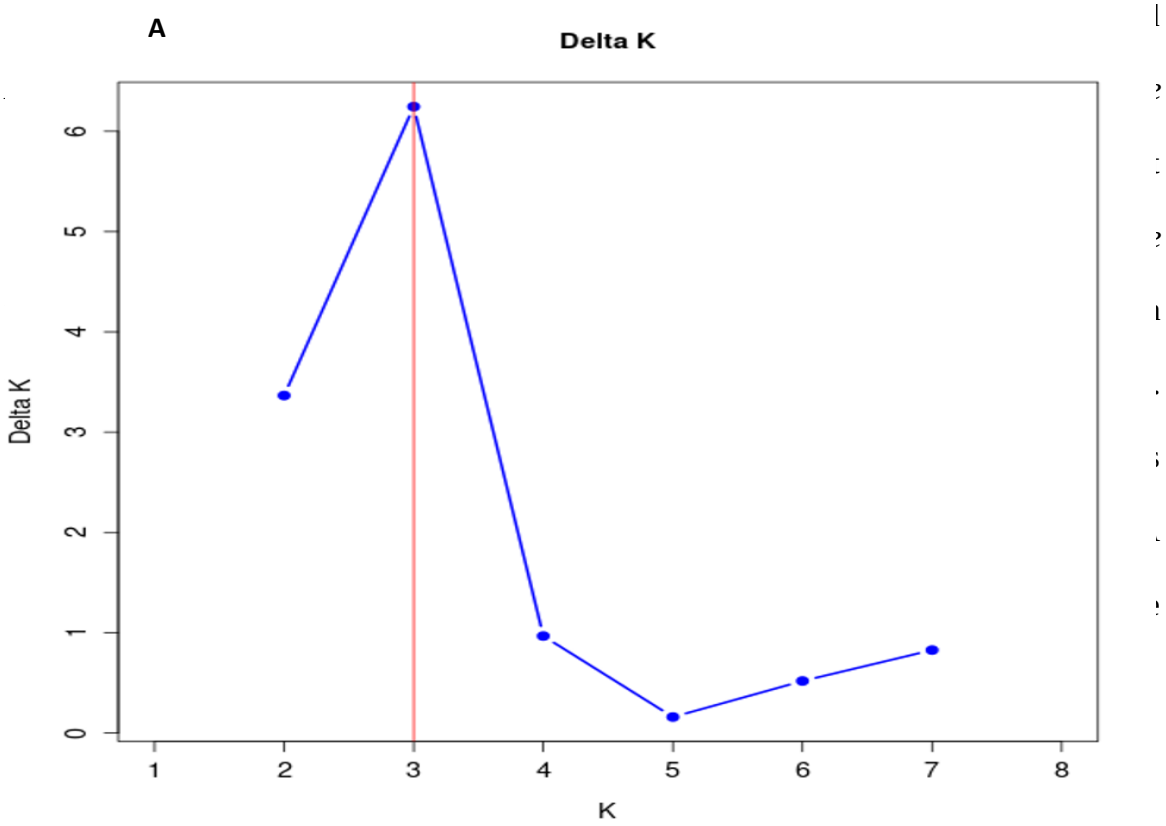
Within and between group counts

When we considered between-group genetic structuring, the observed gene flow between *arabiensis* and *mainland_gam* groups (102 observed versus 71.04 expected counts)

indicates occasional genetic exchange, which could be because of ancestral similarities. *island_gam* versus *mainland_gam* have very significantly fewer pairs than expected (34 pairs versus an expectation of 238.16 pairs), suggesting restricted gene flow between the two geographic populations. The *arabiensis* versus *island_gam* groups had very significantly fewer pairs than expected (4 pairs versus an expectation of 17.62252 pairs), suggesting restricted gene flow between the two groups.

Bayesian clustering analysis

Based on the log-likelihood values and the DeltaK plot, K=3 was the optimal number of evolutionary clusters (Figure 7.6A). Therefore, the six *An. gambiae* populations and pooled *An. arabiensis* were substantially admixed at K = 3 (K = 3 best explains the genetic variance present between all the populations). Upon close inspection of the results from the Bayesian



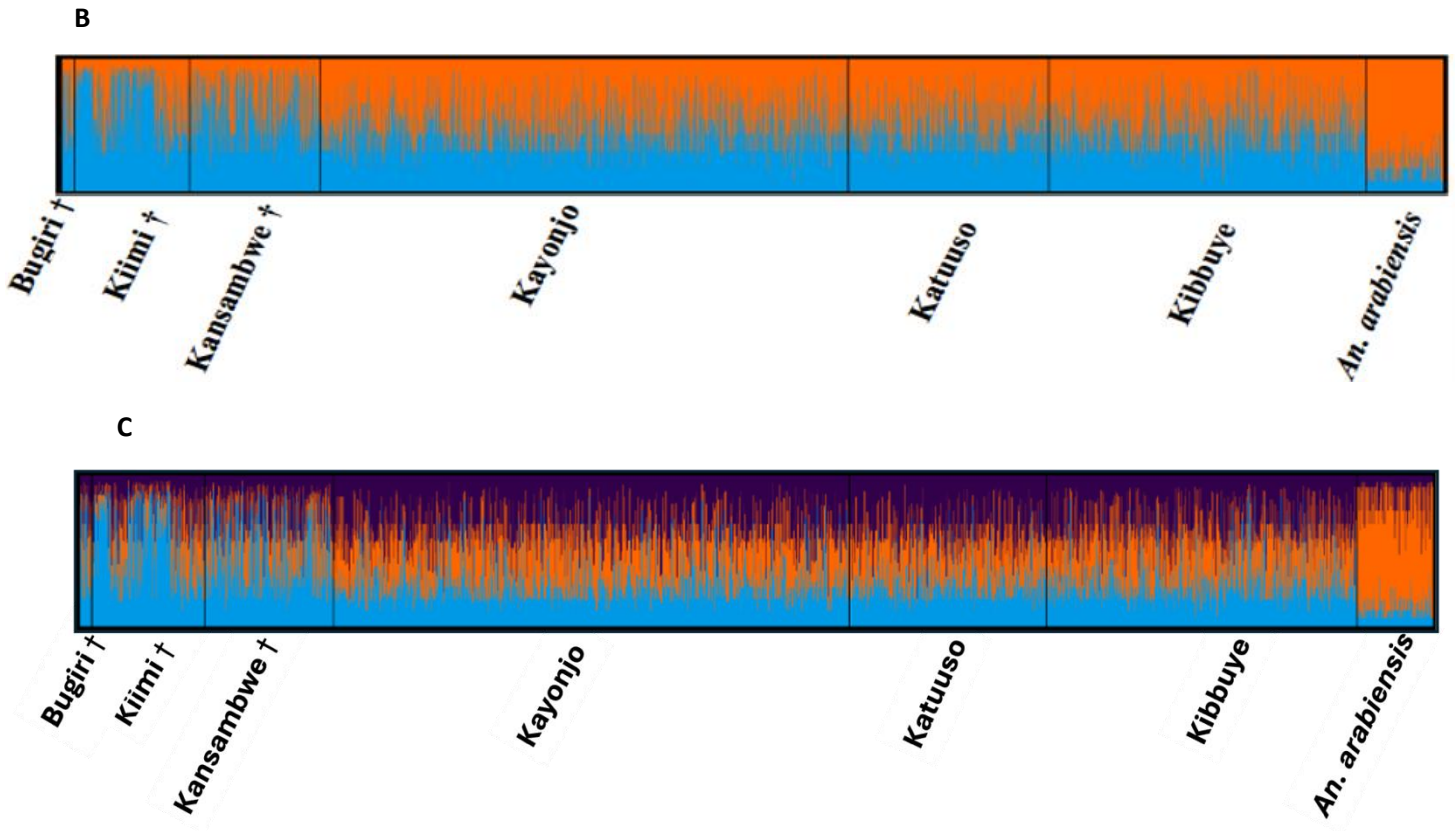


Figure 7.6: *Outputs from Bayesian clustering analysis for the 2918 An. gambiae and 173 An. arabiensis*. **(A)** The optimal number of clusters (K=3) based on the Evanno et al (Evanno et al., 2005c) method showing deltaK **(B)** Bar plot representing K=2 of 3091 mosquitoes, each represented by a thin vertical bar colored in proportion to their estimated ancestry within each cluster (**C**) Bar plot representing K=3 of 3091 mosquitoes. A vertical black line bounds each population and the labels with † for islands while those without are for mainland samples.

Genetic diversity and neutrality tests

Nucleotide diversity (p) and Tajima's D were calculated for each *An. gambiae* population (SI Table S1), then further for each chromosome arm (Figure 7.7A). The overall nucleotide diversity for *An. gambiae* was 0.0115 (range from 0.0107 to 0.01169, SI Table S1) and that of *An. arabiensis* was 0.00859 (ranging from 0.00829 to 0.00859; SI Table S2). Generally, higher diversity values were reported for mainland *An. gambiae* populations (0.01143 to 0.01169; SI Table S1) compared to those for island populations (0.01071 to 0.01103; SI

Table 1). On the other hand, the diversity values for *An. arabiensis* mainland populations were lower compared to the mainland *An. gambiae* populations. The pattern of nucleotide diversity compared between chromosomes for different populations (Figure 7.7B) was consistent across all populations and chromosome arms (mainland sites' p > island sites' p).

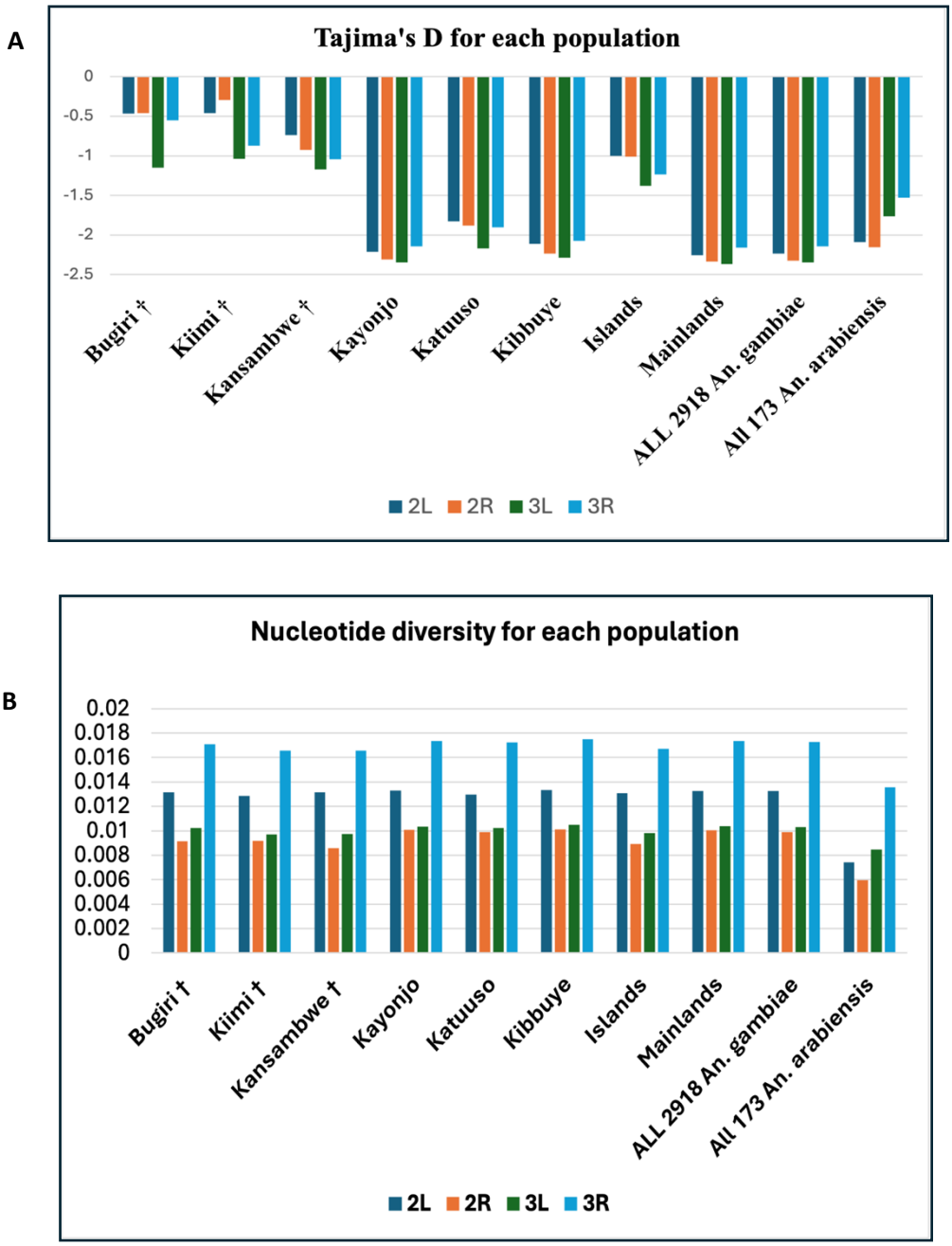


Figure 7.7: *Neutrality tests*

(A) Tajima's D for the six An. gambiae populations per chromosome arm. In addition, we calculated the same statistic for the following subsets: pooled An. gambiae from the island and the mainland sites, then all An. gambiae, and all An. arabiensis (**B)** Nucleotide diversity (π) for the six An. gambiae populations per chromosome arm. † Denotes an island population.

In each population, mean chromosomal nucleotide diversity was highest on the 3R chromosome and lowest on the 2R arm. However, pooled *An. arabiensis* (from all locations) had an overall low diversity compared to the different *An. gambiae* populations, with the lowest diversity in the 2R chromosome arm (P = 0.031, Figure 7.7B). Overall, the pooled island and mainland diversity falls between the values for each sample site across chromosome arms (Figure 7.7B).

Mean Tajima's D was negative for all populations, an indication of an excess of rare alleles and thus a deviation from the neutral model of a well-mixed population of constant size. However, unlike the *An. gambiae* mainland populations, Tajima's D values for *An. gambiae* island populations and all *An. arabiensis* populations were not statistically significant from zero (SI Table S1, S2). When tested for each chromosome and population, all Tajima's D values were negative. The same was done for the *An. arabiensis* mainland populations (SI Table S2). The Tajima's D value of the pooled *An. arabiensis* chromosome 2R arm was significantly more negative than other arms of the same population (P = 0.031), and the 2R arm of the individual and pooled populations.

Contemporary effective population size (*Ne*)

Generally, for *An. gambiae* populations, smaller Ne values were observed for the island populations (between 146 and 249), compared to mainland populations (between 4054 and 8190) (Table 7.4A). The point estimate for Bugiri was inconclusive but came with a very small lower bound. For the *An. arabiensis* populations, we only estimated Ne for the

mainland sites, and their estimates were comparable to the mainland *An. gambiae* populations (between 3402 and 3587, see Table 7.4B).

Table 7.4: *Contemporary Ne estimates*

A. *An. gambiae*

Population	No. of individuals	Number of sites	Overall r^2	Estimated Ne
Bugiri†	28	37	0.035383	∞ (15.1 – ∞
Kiimi†	258	32	0.006168	146.4 (61.7–640.2)
Kansambwe†	294	32	0.004764	249.3 (146.7– 538.5)
Kayonjo	1178	15	0.000901	6723.1 (515.9 - ∞)
Katuuso	450	27	0.002181	4054.1 (640.9 - ∞)
Kibbuye	710	21	0.001416	8190.9(521.5 - ∞)

B. An. *arabiensis*

Population	No. of individuals	Number of sites	Overall r^2	Estimated Ne
Kayonjo	51	31	0.018910	∞ (49.4- ∞)
Katuuso	39	35	0.027836	3402.8 (36.3 - ∞)
Kibbuye	78	31	0.013438	3587.4 (95.9 - ∞)

Site choice is described in the main text. † Denotes island populations. 0.01 was the lowest allele frequency used. The values in parentheses represent the jackknife range.

Discussion

Polymorphic sites and population structure

From the 62 amplicon loci we successfully mapped 9890 sites onto the reference genome, many of which were fixed sites (Figure 7.3) or with low polymorphism (Figure 7.2). We recognise that the primary objectives of ANOSPP are for species identification of any *Anopheline* and parasite detection. Primer binding sites were selected to be extremely conserved because the panel was designed to work across the entire genus, representing more than 100 million years of divergence. Thus, these 62 loci lack highly polymorphic sites outside of the inversions and this posed some challenges to the downstream population genetic analyses. Nonetheless, this study provided a lot of useful insights into the

population structure and diversity among the chosen island and mainland *An. gambiae* and *An. arabiensis* populations in Uganda.

In this study, the level of genetic differentiation among the *An. gambiae* populations was mostly low, regardless of the types of populations ($F_{ST} < 0.05$). Many of these low F_{ST} values, however, were statistically larger than zero with small P-values, meaning that these populations were not entirely panmictic but rather segregated while maintaining certain levels of connectivity. All pairwise F_{ST} values between Bugiri and the rest of the *An. gambiae* populations were very low and insignificant, which could either be the consequence of high gene flow to/from Bugiri (Slatkin, 1987) or the small sample size of Bugiri that could have culminated in insufficient power to detect true genetic differentiation (due to large sampling variance) (Kalinowski, 2005).

High genetic differentiation was observed between *An. gambiae* and *An. arabiensis* populations, concurring with the fact that they are two distinct species. When the *An. arabiensis* mainland populations were independently considered, all the pairwise F_{ST} values were generally low and non-significant. It is noteworthy that the F_{ST} value between Katuuso and Kibbuye was negative, implying extremely low levels of genetic differentiation. The Hudson's method allows negative F_{ST} values when the observed heterozygosity within populations exceeds heterozygosity between populations, therefore, negative values indicate that populations are more similar than expected, reflecting low or zero differentiation, with no detectable genetic structure between them (Bhatia et al., 2013; Charlesworth, 1998; Hudson et al., 1992).

The genetic differentiation between *An. gambiae* populations was varied with inter-island pairwise comparisons having slightly higher values compared to the inter-mainland pairwise values. Higher differentiation values between island populations were comparable

to that reported in the Kayondo et al (Kayondo et al., 2005) study and could be because of their separation by water, small populations and local adaptation. The highest pairwise F_{ST} values were recorded between Kansambwe and all three mainland sites ($P<0.05$), while the lowest values were recorded for Katuuso and Kayonjo (0.00054 $p>0.05$). These pairwise F_{ST} results attained in this study were consistent with previous studies which showed lower differentiation within mainland populations compared to differentiation within island populations and between island and mainland populations (Kayondo et al., 2005; Lukindu et al., 2018; Wiltshire et al., 2018).

In comparison, looking at the pairwise F_{ST} values for mainland *An. arabiensis* populations, low and non-significant genetic differentiation was observed, implying unrestricted gene flow between them. The negative F_{ST} values (between Katuuso and Kibbuye) could be because of an excess of within-population genetic diversity over the between-population genetic diversity (Smaragdov et al., 2018).

The genetic differences between the island and mainland *An. gambiae* populations and within island populations could be a result of their physical separation by water, small population size and local site-specific ecological adaptation (Kayondo et al., 2005). The low and non-significant F_{ST} values between Bugiri and all the other sites, Kayonjo and Katuuso, Kiimi and Katuuso and Kiimi and Kibbuye could be indicative of high levels of gene flow between them (mainland vs mainland), or low sample size of the Bugiri population (low statistical power) (Bortolotto et al., 2011).

We noted that low point estimates of pairwise F_{ST} yielded high Z_scores, suggesting the presence of subtle yet statistically significant population structure despite low apparent differentiation. This occurrence can largely be attributed to the statistical power afforded by a large sample size, which can detect even minor genetic differences across populations

(Das et al., 2023; Fumagalli, 2013). For example, when sample sizes are substantial, there is an increased sensitivity to detect structure, enabling the identification of subtle allele frequency shifts that might otherwise be masked in smaller datasets (Fumagalli, 2013). Between Kiimi and Katuuso, the low pairwise F_{ST} value and non-significant P-value could be because of temporal sampling variability, which could result in capturing different allele frequencies due to temporal fluctuations (Bi et al., 2019; Machado et al., 2021; Sandoval-Castellanos, 2010). Comparing the relative amounts of gene flow taking place between populations, in turn, aids in predicting the trajectory of the alleles introduced by gene drive into the wild-type population (Lanzaro & Tripet, 2003).

Given that the P-value from the Mantel test is slightly above the conventional threshold of 0.05 (P=0.052), it is very close to the threshold and therefore suggests a potential trend toward a significant correlation between pairwise F_{ST} values and geographical distances among populations. This could, therefore, suggest a relevant relationship that deserves further investigation into the relationship between genetic structure and geographical distribution. This value could, however, indicate a weak or suggestive association between genetic differentiation and spatial separation, potentially hinting at IBD in the population (Diniz-Filho et al., 2013). Therefore, the geographic distance between sites could be responsible for part of the differentiation observed.

Although *An. gambiae* F_{ST} results showed reasonable differentiation between mainland and island populations, such patterns were not observed from PCA. The first PC separated the two species, but this could be the only definitive conclusion from the plots (Figures 7.4A, 7.4B). We further observed a clustering that is not geographically distinct, with individuals from different populations intermixed and showing no clear separation or clustering by location (Figure 7.4C). This could suggest that these mosquito populations are not

genetically distinct from one another on the 3L chromosomal arm, thus implying minimal genetic differentiation between these populations and that individuals from different locations share very similar genetic profiles for the loci analyzed on this chromosome arm.

PCA generally captures the largest sources of genetic variation, which may not reveal distinct clusters if other factors dominate the variation represented by the PCs (Lenz et al., 2016). Therefore, ecological and seasonal influences that contribute to the observed structure warrant further investigation into assessing the relative contribution of these factors to the differentiation observed between these populations.

We investigated population structure using doubleton analysis that showed considerable genetic connectivity within the mainland populations compared to island populations (Table 7.3). This suggests that the *island_gam* group is somewhat genetically isolated from other groups and aligns with observed patterns in many malaria vector studies where isolated populations exhibit unique genetic signatures due to limited allelic exchange with surrounding groups (Chen et al., 2004; Marshall et al., 2008). The moderate within-group isolation of the *arabiensis* group (Table 7.3) indicates more frequent genetic interactions within this group than expected, potentially due to localized genetic structures, ecological factors, or other selective pressures driving allele frequencies. This could reflect a localized population structure and its adaptability to arid environments, which fosters more frequent allele pairings within the group than across more widely distributed populations (Lehmann et al., 2003).

In the between-group comparisons, the *island_gam* versus *mainland_gam* counts showed a rather marked deficit in inter-group pairwise counts (Table 7.3), suggestive of distinct separation or limited interaction between the island and mainland populations, probably brought about by geographical or ecological barriers. The counts between *arabiensis* and

mainland_gam groups indicate a degree of gene flow, implying occasional genetic exchange, which could have direct implications for vector control, especially given the differential patterns of gene flow and inbreeding detected across these populations.

This pattern of observed versus expected doubleton pairs sides with the broader view of population structure in malaria vectors, where poor gene flow across ecological boundaries frequently results in discrete genetic clusters (The Anopheles gambiae 1000 Genomes consortium, 2017a), and has implications on both gene drive strategies and management of insecticide resistance.

The strong genetic differentiation between *An. gambiae* and *An. arabiensis* populations was also observed from the outputs from STRUCTURE (Figure 6). STRUCTURE software was used to detect clustering between individuals using a Bayesian algorithm, based on local Hardy-Weinberg equilibrium of genotypes at multiple loci (Pritchard et al., 2000), and found that K= 3 clusters were the optimum assignment (Figure 7.6B). This means that three clusters are sufficient to represent the major genetic differentiation within the dataset, and adding more clusters only subdivides existing groups but does not add biologically meaningful sub-structure. This is often observed when there's low genetic differentiation, where minor allele frequency differences can create additional "clusters" in STRUCTURE without reflecting true population separation (Guillot, 2008).

STRUCTURE output for the *An. gambiae* and *An. arabiensis* species of each population showed sharing of ancestry between each individual at differing proportions (with differing consistency between all island, mainland *An. gambiae* and *An. arabiensis* individuals). The three genetic clusters corresponded to first species (*An. arabiensis*) then to geographical (mainland and island *An. gambiae*) differences, with relatively more similarity between island compared to mainland *An. gambiae* individuals (Figure 7.6B) These results are

comparable to the F_{ST} and PCA results that show less diversity between island and mainland *An. gambiae* individuals and more diversity (clear separation) between *An. gambiae* and *An. arabiensis* individuals (Do et al., 2014; Waples & Do, 2008).

Despite using very few polymorphic sites, STRUCTURE was able to outline the three clusters with visible differences. Therefore, using more sites could produce clearer and more distinct clustering. It is also notable that because pairwise F_{ST} is sensitive to allele frequency differences between populations and works best when populations are distinct and genetically isolated, it may not always capture subtle population differentiation, while STRUCTURE, on the other hand, assigns individuals to clusters based on their multilocus genotypes, and is thus more sensitive to subtle population differentiation (Holsinger & Weir, 2009; Jombart et al., 2010; Pritchard et al., 2000; Weir & Cockerham, 1984). It is important to note that Both PCA and Bayesian clustering approaches consistently identified similar numbers of genetic clusters, strengthening confidence in the inferred population structure.

Genetic diversity and neutrality tests

The Tajima's D test is used to distinguish between DNA sequences evolving neutrally from the ones that are evolving under a non-random process or non-constant demography. The statistic compares two measures of genetic diversity; the number of segregating sites (S) and the average number of pairwise differences (π) in a population, and infers evolutionary processes like selection, population expansion, or contraction (Tajima, 1989). Tajima's D values were generally negative (SI Tables S1&S2), in line with previous findings (The Anopheles gambiae 1000 Genomes consortium, 2017a). More negative Tajima's D values reported at mainland sites compared to the island sites was an indication of an abundance of an excess of low-frequency alleles at the mainland sites, potentially due to [66,67] more

recent or stronger evolutionary events such as purifying selection, more recent population expansion, or selective sweep that affected the genetic diversity in the populations at the mainland (Kreitman, 2000; Nielsen, 2005; Tajima, 1989). Given that *An. gambiae* thrives in human-dorminated environments, its population and range will thus expand most in areas where human population density is high and growing, whether on island or mainland sites (O'Loughlin et al., 2014).

Tajima's D for *An. gambiae* island populations and all *An. arabiensis* populations were not statistically significantly different from zero (SI Table S1, S2); which supported the fact that these populations are in genetic and demographic equilibrium with no population bottleneck or expansion, an occurrence consistent with the neutral mutation hypothesis (Wei et al., 2022). This suggests that these populations are at mutation-drift equilibrium, maintaining a stable population (Dharmarathne et al., 2020; Parimittr et al., 2018). This non-deviation from the neutral mutation hypothesis could be because of the relatively small sample sizes at the island sites (SI Table S1) and *An. arabiensis* (SI Table S2) populations compared to those at mainland sites, a quantity that Tajima's D is said to be highly sensitive to (Larsson et al., 2013).

Nucleotide diversity (π) is a useful measure of genetic diversity since it directly quantifies the number of differences in genotypes between all possible pairs of alleles in a population, and its variation is influenced by a species' life history (Nei & Li, 1979). Using the patterns of nucleotide diversity, we can infer aspects of long-term population demography by considering deviations from the null model of neutral variation in isolated populations of constant size (O'Loughlin et al., 2014). It was known that *An. arabiensis* has a lower diversity than *An. gambiae* (Besansky et al., 2003). The estimates of the overall nucleotide diversity for each *An. gambiae* population were within the range of values reported in previous studies which utilised four loci (white, tox, G6pd, xdh) across several African

locations (Besansky et al., 2003) and immune-related (LRIM1, CTL4, CTLMA2, APL2) and housekeeping genes (Obbard et al., 2007), and were consistent with the fact that island populations display marginally lower nucleotide diversity compared to the mainland (Bergey et al., 2020; Campos, Hanemaaijer, et al., 2021). The explanation for this could be inbreeding, or higher degree of isolation with small neighbourhood size (Campos, et al., 2021; Ellegren & Galtier, 2016; Fischer et al., 2017; Lanzaro, et al., 2021). Small population sizes experiencing stochastic genetic losses due to genetic drift. Also limited migration from meta-populations from the mainland due to the geographical barriers.

On chromosome arm 2R, the pooled *An. arabiensis* population has lower nucleotide diversity than individual or pooled *An. gambiae* populations. This could be because chromosome 2R contains many segregating inversions, some of which are polymorphic in *An. gambiae* and fixed in *An. arabiensis,* which inversions can limit recombination in certain regions, leading to reduced genetic diversity over time (Lobo et al., 2010; O'Loughlin et al., 2016). This phenomenon is especially pronounced in species like *An. arabiensis*, where these inversions (2La, 2Rb and 3Ra) could be fixed, and further decrease genetic variation along the 2R chromosome relative to *An. gambiae* populations (Petrarca et al., 2000; White et al., 2009).

Additionally, the frequencies for inversions segregating in both species could be/are different for the two species (Lobo et al., 2010). This could also suggest that there is a decline in population size and/or balancing selection, a feature of the maintenance of segregating 2Rb, c, d, u and j inversions (O'Loughlin et al., 2014; Peter Andolfatto, Frantz Depaulis, 2001).

Contemporary effective population size

Estimates of Ne attained using the LDNe method (Waples & Do, 2008) in NeEstimator v.2.01 (Do et al., 2014) were based on its performance that is superior to other single-sample estimators (Gilbert & Whitlock, 2015; J. Wang, 2016). While nucleotide diversity contains demographic information over a much longer timeframe, our Ne estimates from unlinked LD are local and recent, focusing only on one to a few generations ago. Ne estimates of the *An. gambiae* Island populations were generally smaller compared to mainland populations and were comparable to those reported in the Kayondo et al. and Wiltshire et al. studies (Kayondo et al., 2005; Lukindu et al., 2018; Wiltshire et al., 2018). These low Ne estimates recorded for island sites indicate their small neighbourhood size and could be the cause of higher levels of genetic differentiation (presented as pairwise F_{ST} estimates) shown in Table 4A (Do et al., 2014), given that random genetic drift which affects the stability of allele frequencies in a population is more variable in smaller populations (Wiltshire et al., 2018).

The low Ne values reported for island populations could also be influenced by factors such as the pronounced effect of seasonality on islands, leading to fluctuations in vector abundance, particularly during dry seasons when population size is suppressed, resulting in increased genetic drift. Furthermore, anthropogenic events that result in habitat fragmentation and patchy populations together with isolation through geographical barriers such as lakes restrict gene flow between subpopulations, leading to reduced genetic diversity and lower effective population size (Ne) within each isolated group (Multini et al., 2020). The infinity values of point estimates such as those recorded for Bugiri, was almost certainly due to insufficient sample size compared to the magnitude of genetic drift. The infinite jackknife upper bound in the mainland populations suggests that based on the LD observed, it's statistically feasible that Ne could be extremely large, although the exact

size cannot be pinpointed with certainty (Hare et al., 2010; Wang, 2005; Waples & Do, 2009).

Therefore, Confidence intervals extending toward infinity reflect high uncertainty due to limited information or weak identifiability rather than biologically implausible estimates.

Larger Ne values observed in the mainland *An. gambiae* populations suggest greater genetic diversity and better connectivity, making them more resilient to control measures and more difficult to eliminate compared to smaller island populations (Athrey et al., 2012; Hoffmann & Willi, 2008; Neafsey & Waterhouse, 2015; Rousset, 1997; Weetman et al., 2018). High Ne thus implies slower genetic drift which allows for sustained genetic variation that enhances adaptation to environmental changes, moreover, studies on dry season persistence indicate that seasonality influences Ne estimation, as dry season surviving populations can rebound and maintain genetic diversity across seasons, further complicating efforts to control malaria vector populations (Charlesworth, 2009; Kamdem et al., 2017; Waples, 2016; White et al., 2011; Yaro et al., 2012).

This knowledge of the size of the host population informs the introduction threshold for gene drive releases (minimum number of mosquitoes required to transform a population), and therefore, in this case, a small and reproductively isolated population could effectively reduce this introduction threshold and enable the successful integration of the introduced gene (Lanzaro & Tripet, 2003)

Conclusion

The results from this study show that the mainland *An. gambiae* populations were more diverse and differentiated compared to the mainland *An. arabiensis* populations. On the other hand, the *An. gambiae* populations collected from the three islands considered in this study were relatively genetically differentiated from the mainland populations, and even

from one another, we suspect primarily driven by water as a physical barrier to gene flow, smaller effective population sizes and potentially local ecological adaptations. Due to the ease of access to the islands which allows easy monitoring, the fact they comprised a smaller proportion of non-target malaria vectors in Uganda (very few *An. arabiensis*), higher genetic differentiation, genetic structure, smaller effective population sizes compared to mainland sites, and their small geographical size, islands are promising sites for field trials to test the effectiveness of mosquito gene drive systems.

As mentioned at the start, *An. arabiensis* individuals are often excluded from further analysis due to their limited numbers in the mosquito collections done to date (Kayondo et al., 2005; Lukindu et al., 2018; Wiltshire et al., 2018), which could be because of the sampling methods used. Therefore, studies that target the exophilic and zoophagic characteristics of this species should be carried out to give a better picture of its density, specifically at the islands which are the potential sites for field trials to test mosquito gene drive systems. This will help us to confirm the proportion of the non-target malaria vector species at the island sites.

As much as we would like to provide a comprehensive perspective of the overall spatial and temporal variation of the two species in Uganda, the latter cannot be investigated here because of the different sampling times in the island and mainland sampling. We welcome further studies on other biological factors, such as the mosquitoes' seasonal dynamics and dry season persistence mechanisms (Mwima et al., 2023, 2024), as these contribute to our observed population structure.

Future works should include a dataset using whole genome sequenced data, exploring temporal sampling, further investigations into the specific contribution of the suggested factors we have used, as well as the influence of other suggested biotic (e.g., passive

transport) and abiotic factors (e.g., seasonality and chromosome inversions) that may contribute to the observed differentiation. These will add a valuable body of information required for evaluating these Islands as potential release sites for mosquito gene drive systems.

Supplementary Tables

Table S1.*Nucleotide diversity and Tajima's D results*

Population	**Nucleotide diversity (p)**	**Tajima's D**
Kayonjo	0.01163	-2.3069*
Katuuso	0.01143	-2.0219*
Kibbuye	0.01169	-2.2356*
Kiimi †	0.01081	-0.8223
Kansambwe †	0.01071	-1.0827
Bugiri †	0.01103	-0.6954
All *An. gambiae* (Mainland & Island)	0.0115	-2.3073*

For the six *An. gambiae* populations. The last row is all *An. gambiae* (2918) combined for comparison. Site choice was as described in the main text. The asterisk * denotes statistical significance, ($p < 0.05$). † Denotes an island population.

Table S2. *Nucleotide diversity and Tajima's D for the three An. arabiensis populations*
The last row is all *An. arabiensis* (173) combined for comparison. Site choice was as described in the main text.

Population	**Nucleotide diversity (π)**	**Tajima's D**
Kayonjo	0.00859	-1.17475
Katuuso	0.00829	-0.98993
Kibbuye	0.00847	-1.73386
All *An. arabiensis*	0.00859	-1.91156

CHAPTER EIGHT: GENERAL DISCUSSION

Preamble

The malaria burden globally remains high, with an estimated 200 million malaria cases and 600,000 deaths reported annually (WHO, 2024). With the continued rise in insecticide resistance, current vector control tools such as ITNs, and IRS are becoming less effective especially when used singly, which has contributed to stagnation in malaria control gains in the recent years (Bhatt, Weiss, Cameron, Bisanzio, Mappin, Dalrymple, Battle, Moyes, Henry, Eckhoff, et al., 2015; Hemingway et al., 2016; WHO, 2023). To achieve malaria elimination and eradication will require new products and interventions to be used in tandem with the already existing tools (Kaddumukasa et al., 2020a), and one promising strategy is a mosquito gene drive system, capable of spreading desirable vector control traits such as resistance to malaria parasite transmission or reduced mosquito fertility into an entire population from low initial frequencies (Burt, 2014; Hammond, et al., 2021; Marshall & Akbari, 2016). But before these tools are rolled out, there is a need to carry out field trials to test their efficacy under natural conditions, building on prior laboratory and contained studies.

Information about mosquito bionomics, population genetic structure, gene flow, and dry season survival is therefore required to select suitable locations for field trials (Carlson et al., 2004; Govoetchan, et al., 2014; Lanzaro et al., 1998; Lanzaro & Tripet, 2003; Lehmann et al., 2017). This thesis examined the dry season survival mechanisms of malaria mosquitoes in sub-Saharan Africa, their population genetics at selected island and mainland sites in Uganda and utilised mathematical models and simulations to determine the seasonal population genetics of *An. gambiae* in Eastern Uganda. The results from this study are then used to make predictions and informed recommendations about how gene drive, as a novel malaria vector control approach could be used effectively in the future.

8.1 Dry Season Survival Mechanisms of Malaria Mosquitoes

The objective was to examine how the malaria vectors that belong to the *An. gambiae* species complex persist during the dry season and re-establish at the onset of the rainy season. Four hypotheses, namely, aestivation, local refugia, local migration, and LDM, were suggested by previous studies and evaluated, highlighting the challenges that come with validating these mechanisms because of the limitations in methods used. This review emphasized the need to integrate ecological experiments with genetic studies to provide conclusive evidence on these mechanisms. Understanding these persistence mechanisms is, however, important for the effective application of the novel and existing vector control approaches.

This study was undertaken because malaria vector populations generally exhibit large fluctuations in abundance that are a result of seasonal changes, especially in the transition from the dry to rainy seasons (Charlwood, et al., 2000; Govoetchan, et al., 2014; Hidalgo et al., 2014; Jawara et al., 2008; Magombedze et al., 2018; Omer et al., 1968; Omer & Cloudsley-Thompson, 1969; Wagoner et al., 2014; Yaro et al., 2012). This study considered the different mechanisms malaria mosquitoes use to persist during the dry season, something critical of malaria vector control strategies, following which several hypotheses were proposed. Four hypotheses, namely, aestivation (a dormancy-like state), local refugia (hidden habitats), local migration (short-range movement), and LDM (wind-aided or other long-range dispersal) were explored.

This review, differentiated between the persistence mechanisms used by malaria mosquito species from the Equatorial region where surface water is available nearly all year round or the dry seasons last for less than 2 months, and the Sahelian region where there is hardly surface water in vast areas for between 3 and 8 months.

This review involved compiling findings from ecological and genetic studies across sub-Saharan Africa, assessed the strengths and limitations of existing methods and considered the evidence that supports each persistence hypothesis. Our key findings were that:

(1) For aestivation, ecological and genetic studies suggested that mosquitoes could enter a state of dormancy during the long dry season that usually spans up to 7 months, but due to the inconsistencies that are a result of the challenges in sampling hidden or dormant individuals, there is limited evidence of aestivating individuals. However, the evidence of one marked mosquito that was captured at the start of the rainy season, 7 months after being marked and released (see Lehmann et al., 2010 study) is proof that mosquitoes can persist throughout the dry season in the Sahel (Lehmann et al., 2010). For local refugia, some populations could survive in hidden habitats such as tree holes, animal burrows, abandoned buildings, or water-filled scrap tyres that are not considered by conventional sampling methods (Charlwood, et al., 2000).This hypothesis, therefore, requires that further studies that consider sampling outside the conventional areas be done.

(2) Concerning local migration, the review established that movement from the nearby areas with better conditions is possible. However, identifying the exact sources and pathways remains challenging, particularly in regions like the Sahel, where surface water is largely absent during the long dry season. In contrast, however, some areas in the tropical regions could retain limited surface water, potentially supporting more localized persistence.

(3) With LDM, there was evidence of windborne migrations among *An. gambiae* species for up to 250m above the ground, even though previous studies showed that mosquito dispersal did not go beyond 5km (Constantini et al., 1996; Service, 1997b; Y. T. Touré et al., 1998). Recent studies using aerial sampling captured live

mosquitoes flying at altitudes between 40-290 metres above ground level, travelling tens to hundreds of kilometers per night (Atieli et al., 2023b; D. L. Huestis et al., 2019). More than 90% of the captured *Anopheline* females were blood-fed or gravid, indicating that they were not only viable upon arrival but also capable of oviposition and contributing to the next generation (D. L. Huestis et al., 2019). Even though these findings support the plausibility of windborne LDM playing a role in population re-establishment, the extent of its contribution to post-dry season rebounds remains an active area of research.

The limitations of this review were that (1) techniques like MRR though valuable for testing aestivation and local migration, suffer from very low recapture rates, especially in the dry season when mosquito densities are minimal, thus reducing their accuracy (Epopa et al., 2017c; Faiman et al., 2022a; Lehmann et al., 2010; Y. T. Touré et al., 1998), (2) field sampling methods aiming to identify local refugia face challenges in distinguishing between true absence and failure to detect mosquitoes hiding in cryptic habitats (Charlwood, Vij, & Billingsley, 2000), and (3) genetic methods, used to test aestivation, local migration and LDM hypotheses suffer from limited marker coverage, insufficient temporal data and assumptions that may not hold in natural populations, resulting in inconclusive results when trying to detect signals of population bottlenecks or gene flow (Hoban et al., 2013; R. S. Waples & Do, 2009). These methodological constraints water down the support for any one hypothesis as the definitive mechanism behind post-dry season mosquito rebounds.

This review emphasizes the ecological plasticity of malaria vectors and informs models of seasonal dynamics and the design of malaria vector control interventions. For example, in the Sahelian region, aestivation has been strongly supported by multiple field and laboratory studies as a key survival mechanism for *An. coluzzii* populations, while in the

more humid tropical regions, evidence suggests that local refugia and local migration could be more prominent strategies. Targeting dry-season refugia or exploiting vulnerabilities in diapause-associated metabolic pathways may thus offer new avenues for disrupting the lifecycle of these vectors, tailored to regional ecological contexts.

Understanding dry season survival strategies like aestivation, local refugia and migration is critical because these processes shape the seasonal population structure and dispersal patterns of malaria vectors. This has direct implications for the success of novel vector control approaches like gene drive technologies which rely on predictable population connectivity and movement to spread effectively. These insights into dry season survival dynamics are therefore essential for informing the design, target and evaluation of gene drive-based interventions.

Overall, this study reviewed evidence supporting multiple persistence mechanisms such as aestivation, local refugia, and migration in the *Anopheles gambiae s.l* complex, highlighting the species- and region-specific nature of dry season survival strategies. However, it did not conclusively determine which single persistence mechanism predominates across sub-Saharan Africa due to variability in ecological contexts and methodological limitations.

Each of the four hypotheses had some supporting evidence but also faced specific methodological limitations; (1) aestivation was supported by physiological and reproductive suppression observed in *An. coluzzii* during the dry season in the Sahel, even though direct detection of aestivating females remains elusive because of the limitations of current sampling methods, (2) local refugia was suggested by occasional dry season captures in hidden habitats, yet conventional sampling often fails to detect these cryptic sites making it difficult to distinguish true absence from detection failure, (3) local

migration was inferred from recolonisation patterns near permanent water sources, however confirming source populations and exact migration pathways remains a challenge due to limited spatial sampling and (4) LDM was strongly supported by aerial sampling of live, viable mosquitoes at high altitudes, yet establishing the contribution of these migrants to population rebounds requires further studies, particularly those integrating genetic tracking, reproductive fitness assessments and long-term population monitoring.

This study thus recommends the need for further studies that integrate ecological experiments and advanced genetic analyses to resolve uncertainties, develop standardised sampling protocols, and increase the resolution of genetic studies to capture dry-season dynamics. By addressing these gaps, this review highlights how clarifying mosquito persistence mechanisms can enhance vector control strategies, particularly in regions with prolonged dry seasons.

8.2 Evaluating Dry Season Persistence Mechanisms Using Population Genetics

Malaria vector populations in seasonal environments must survive extended dry periods with minimal or no breeding opportunities. As mentioned earlier, several mechanisms have been proposed to explain how these populations persist through the dry season and rebound with the onset of rains, including aestivation (a form of adult-stage diapause), survival in local refugia, local migration**,** and long-distance migration**.**

This study particularly focused on aestivation**,** using a novel population genetic framework to assess its contribution to dry season persistence and its influence on allele frequency change and effective population size (*Ne*). Rather than assuming aestivation as the sole mechanism, we used the model to evaluate whether population genetic signals from temporal genotype data of *Anopheles coluzzii* in Thierola, Mali**,** an area where aestivation

has been previously hypothesized, were consistent with expectations under partial reproductive arrest (A. S. Dao et al., 2014; Huestis et al., 2014; Huestis & Lehmann, 2014; Yaro et al., 2012).

Aestivation, a form of adult mosquito diapause, is hypothesized as the major survival strategy employed by *An. coluzzii* during the dry season in the Sahel region (Hidalgo et al., 2016a; Lehmann, Weetman, Diana L Huestis, et al., 2017; Yaro et al., 2012). During the aestivation process, mosquitoes undergo reproductive arrest and survive in hidden or inaccessible habitats, making direct sampling extremely challenging. This combined occurrence significantly impacts population dynamics, dampens genetic drift signals and poses important considerations for the design and timing of malaria vector control interventions.

This study, therefore, presents a novel genetic framework used to estimate the proportion of aestivating individuals and its influence on Ne using mathematical modeling, simulations, and temporal allele frequency data. The approach was applied to *An. coluzzii* populations from the Sahelian region. This method is generalizable and can be extended to assess dry season survival mechanisms in other ecological regions.

We developed and applied a discrete generation forward simulation model to replicate the genetic evolution of a mosquito population across wet and dry seasons, incorporating both breeding and dry-season aestivation compartments. While the model itself is general and adaptable to other species with similar ecological dynamics, this study applied it specifically to *An. coluzzii* populations from the Sahel. This decision was based on availability of high-resolution temporal allele frequency data from Thierola, Mali, as published in Lehmann et al., (2017), and on existing field and genetic evidence suggesting that *An. coluzzii* in this region relies on aestivation for dry season survival. Future work

could apply the same modeling framework to other populations or regions once comparable genetic data becomes available. In the aestivation compartment, individuals are in a dormant state and not reproducing (Adamou et al., 2011a; Jiang et al., 2023; Lehmann et al., 2010).

The breeding compartments were used to track changes in allele frequencies, given that no genetic drift happened in the aestivation compartment. Including an aestivation compartment was an eye-opener into how populations maintain genetic continuity through harsh environmental conditions, and our findings showed that aestivation serves as a genetic buffer by preserving allele frequencies and reducing the impact genetic drift has during the dry season. Results from this model confirmed that when aestivating individuals re-combine with the breeding population at the onset of the rainy season, a remarkable reset in genetic variance (V_t) occurs and significantly affects Ne estimates, resulting in an overestimation of the dry-season population size if not accounted for. Therefore, by incorporating the temporal change in allele frequencies, the proportion of aestivating individuals as well as the effective breeding sizes during the dry and rainy seasons were inferred. This framework was then applied to *An. coluzzii* populations from Thierola in Mali, where results showed a high proportion of aestivating adults, approximately 79% in the first year and 39% in the second year, thus supporting the role of partial diapause as a key dry season survival mechanism in this Sahelian region.

Applying the developed model to a Malian *An. coluzzii* dataset revealed a high degree of aestivation, such that, depending on the season, its proportions ranged from 20% to 80%. These findings agreed with the dry season ecological observations that occur in *An. coluzzii* populations (Lehmann, Weetman, Diana L Huestis, et al., 2017; Magombedze et al., 2018; Mwima et al., 2023), and confirmed the critical role aestivation plays in maintaining genetic

diversity and facilitating population rebounds in subsequent generations. They indicate that aestivating populations maintain a degree of genetic connectivity with active populations, enabling genetic flow across seasonal bottlenecks. They also contribute to the general understanding of diapause its evolutionary implications, drawing attention to how aestivation influences not only genetic drift but could also influence potential adaptation through preserving alleles that could be crucial for malaria vector fitness under changing environmental conditions. The buffering capacity of aestivation could also explain how *An. coluzzii* populations survive in areas with extreme seasonal conditions and emphasize the merits of including dormancy dynamics in population genetics models. Understanding aestivation also provides an opportunity to interrupt the mosquito population dynamics more effectively.

From this study, further exploration could include (1) developing innovative sampling techniques to locate and characterize aestivators to further validate and refine the model, (2) the model framework used could be applied to other malaria vector species to elucidate species-specific survival strategies and their genetic outcomes and (3) investigating how patterns in climate change could affect the dynamics of aestivation and its genetic impact, and in turn aid in predicting the long-term sustainability of malaria vector populations.

This work also has significant implications for gene drive strategies because they rely on population connectivity for efficient spread, and the persistence of genetically isolated or partially dormant populations could slow the dissemination of drive constructs. Integrating population genetics, mathematical modeling, and ecological observations provides a robust framework for understanding the role of aestivation as a dry season survival mechanism. These results not only advance the field of mosquito population genetics but also highlight practical ways in which our knowledge of malaria vector control can be improved and if

dry season survival mechanisms like aestivation could become a target for the development of more focused and effective interventions to prevent and eliminate malaria in regions where marked seasonality occurs. For example, the inclusion of diapause dynamics in mathematical models will be important for predicting gene drive success and determining optimal times and locations of release.

8.3 The Population Genetics of Malaria Mosquitoes at Island and Mainland Sites in Uganda

Genetic and ecological factors shape mosquito populations and are important in understanding their dynamics and designing and effectively rolling out vector control strategies, such as gene drives. These results derived from pairwise Fst comparisons and PCA indicate high interspecific variation between *An. gambiae* and *An. arabiensis*, consistent with their distinct species status. In contrast, the low intraspecific Fst values, particularly among mainland *An. gambiae* and *An. arabiensis* populations reflect high gene flow within each species. On the other hand, higher differentiation was reported between island and mainland populations, which might be attributed to physical barriers like Lake Victoria. Island populations had more genetic isolation and low values of Ne, making them suitable candidate sites for specific intervention testing, such as gene drive systems.

Higher genetic diversity was reported amongst the mainland *An. gambiae* populations compared to those at the islands, indicative of larger Ne and possibly higher connectivity. However, among the *An. arabiensis* populations, generally low nucleotide diversity was recorded particularly on the 2R chromosome, likely affected by fixed inversions that reduce recombination (M. B. Coulibaly et al., 2007; Lulu & Asfaw, 1999). Tajima's D values across all populations suggested either recent population expansion or selective sweeps, with higher values among mainland populations, which could be indicative of higher allele turnover and adaptation potential (M. J. Donnelly et al., 2001; Kamdem et al., 2017). The

small Ne values were recorded for island *An. gambiae* populations were indicative of their reduced genetic variability, likely due to isolation and bottlenecks that could be caused by geographical or ecological barriers. This again renders them ideal sites for carrying out field trials of targeted genetic interventions, given that because they are isolated, there is limited gene flow to other areas.

The higher Ne values at the mainland sites are indicative of better connectivity and resilience, even though this could pose challenges for effective gene drive due to slower genetic drift and greater adaption capacity (Athrey et al., 2012; Kirkpatrick & Barton, 2006; Nolan, 2021).

Given that these results are pivotal in using genetic-based malaria vector control methodologies, this study on *An. gambiae* and *An. arabiensis* gave deep analysis into the genetic diversity and population structure and the effective population size across the island and mainland populations in Uganda. Therefore, they emphasize the great necessity of choosing suitable sites where the gene drive field test trials should be conducted. And because the island sites show genetic isolation, low values of Ne, and comparatively clear differentiation from the mainland, they could be suitable candidate sites for gene drive field trials.

On the other hand, knowledge about their genetic diversity and population dynamics may help in refining malaria vector management strategies such as monitoring insecticide resistance and gene drive system design (Kientega et al. 2024; Selvaraj et al. 2020). These future studies would thus incorporate temporal sampling for a deeper understanding of the seasonal dynamics, investigate the ecological and climatic factors that drive mosquito population persistence, especially in the dry season, and make use of WGS in refining genetic diversity and structure analyses.

In summary, this study contributes significantly to the population genetics of *An. gambiae* and *An. arabiensis* in Uganda, emphasizing major differences in genetic diversity, structure, and connectivity among island and mainland populations. Such results will help inform the design and evaluation of new genetic tools for malaria vector control and support global efforts toward malaria elimination.

8.4 Modeling Dry Season Survival of *An. gambiae* in Eastern Uganda

Given the significant malaria burden Uganda carries (WHO, 2023), understanding the genetic structure and seasonal dynamics of malaria vector populations is critical when designing effective control strategies (Athrey et al., 2012; Katusi et al., 2022; Kientega et al., 2024; McDonough & Connallon, 2023; Ørsted et al., 2019). The pronounced fluctuations in population size experienced by the primary malaria vectors are driven by the alternating rainy (RS) and dry (DS) seasons, which fluctuations shape genetic diversity and connectivity, influencing how mosquito populations respond to environmental pressures and vector control measures (M. J. Donnelly et al., 2001; Krajacich et al., 2018b; Mawejje et al., 2021; C. M. Williams et al., 2017).

This study explored how *An. gambiae* populations in Eastern Uganda persist through the dry season and re-establish during the rainy season. The relative contributions of aestivation (a dormancy strategy that allows mosquitoes to survive harsh dry conditions) and LDM (where individuals disperse to or from more favorable environments) as persistence and dry season survival mechanisms were examined, given that they are essential for understanding how genetic diversity is maintained, how populations adapt to seasonal variability, and how they might respond to interventions like gene drives (Hidalgo et al., 2015; Kabbale et al., 2013b; Leung et al., 2022; Niang et al., 2015; Tauber et al., 1986; C. M. Williams et al., 2017). By using both spatial and temporal genetic data, this

work helps to better understand population size fluctuations, connectivity, and dry-season survival strategies in Eastern Uganda and has critical implications for malaria control, particularly for the deployment of gene drive.

The genetic analyses revealed low spatial differentiation among the *An. gambiae* populations across 14 districts in Eastern Uganda. This was supported by PCA results that showed large genetic clustering, an indication of high levels of gene flow likely facilitated by a lack of significant physical or ecological barriers. This high connectivity between populations from all 14 districts implies that; gene drive spread can be facilitated for the efficient dissemination of constructs across large areas; however, the downside is that migratory connectivity could undermine site-specific interventions, as untreated populations may quickly repopulate treated areas (Beeton et al., 2022; Carrasco-Escobar et al., 2024; Hancock et al., 2024).

Temporal genetic analyses provided insights into population size and persistence in that low temporal Fst values indicated limited genetic drift between RS and DS, suggesting large Ne and stable allele frequencies all year round (Angst et al., 2024; Taylor & Manoukis, 2004). At the same time, consistent Ne estimates from the 3L and 3R arms reflected stable genetic diversity. Still, inconsistencies on the 2R arm were due to structural variations such as inversions known to reduce recombination (The Anopheles gambiae 1000 Genomes consortium, 2017a). These findings point out the plasticity of *An. gambiae* populations in Eastern Uganda to seasonal bottlenecks, with both aestivation and migration buffering against significant genetic drift during the DS. By incorporating temporal allele frequency changes, joint estimation of the proportions of aestivating individuals and long-distance migrants were estimated, revealing their complementary roles in sustaining genetic diversity and ensuring population persistence across seasons. This analysis, focused

specifically on aestivation as a mechanism of dry season persistence and LDM, as a contributor to early wet season rebounds. These two mechanisms were chosen for initial investigation based on their theoretical contrast and the modeling framework developed. However, this does not imply exclusion of other plausible strategies. These are preliminary results, and future work will aim to extend the model to also incorporate local migration from nearby areas and survival in cryptic local refugia, which may further explain the observed seasonal dynamics in *An. gambiae* populations from Eastern Uganda.

This genetic diversity and high connectivity observed in Eastern Uganda has significant implications for gene drive technologies in that (1) gene drives could propagate effectively due to high gene flow and the absence of strong genetic bottlenecks, (2) Large Ne values during the RS may slow the fixation of gene drives, increasing the likelihood of resistance alleles emerging, thus, multiplexed gene drive systems that target multiple loci may mitigate this risk, and (3) smaller DS population sizes provide an opportunity to maximize the impact of gene drive constructs, though aestivating individuals may act as reservoirs of wild-type alleles, slowing drive progress.

The weak seasonal genetic signals in Eastern Uganda contrast studies from the Sahel, where the harsh dry season results in stronger bottlenecks and more pronounced genetic shifts (A. S. Dao et al., 2014; D. L. Huestis et al., 2014; D. L. Huestis & Lehmann, 2014; Lehmann et al., 2010). This difference emphasizes the significance of tailoring malaria vector control strategies to local ecological conditions. Future studies that investigate the population dynamics of mosquitoes should focus on finer-scale temporal sampling within and across seasons to capture finer-scale genetic shifts. Climatic and ecological variables could also be integrated with habitat availability to further enhance the modeling of seasonal persistence into inter-annual variability in malaria vector population genetics.

This study generally advances the understanding of *An. gambiae* population genetics in Eastern Uganda and informs dry-season persistence mechanisms and gene drive technologies. Combining genetic data with temporal and spatial analyses lays the groundwork for developing targeted malaria control strategies that integrate ecological insights with innovative genetic control tools, to reduce the malaria burden.

8.5 Concluding Remarks

The integration of ecological and genetic studies, mathematical modeling, and simulations is significant for enhancing our understanding of malaria mosquito population persistence and provides key insights into the deployment of novel vector control strategies, like gene drives.

It would, therefore, be of interest to further optimize malaria control strategies using high-resolution temporal sampling over several years to capture finer-scale genetic dynamics, adding variables like rainfall and temperature to a model that will provide more realistic modeling of dry season persistence. Such findings should be applied to other regions and vector species to enable their generalization and refining of control strategies.

This research thus helps to understand the genetic dynamics and dry season persistence strategies of malaria vectors in Uganda and provides deeper insights into how these populations go through seasonal bottlenecks to inform the design of more targeted and efficient interventions. The findings will contribute to the development and deployment of new tools, like gene drives, toward global efforts for malaria elimination and eradication.

CHAPTER NINE: CONCLUSIONS AND RECOMMENDATIONS

9.1 Conclusion

9.1.1 Dry Season Survival Mechanisms of Malaria Mosquitoes

The review highlights that the mechanisms enabling the *An. gambiae* complex species to persist through dry seasons and re-establish during rains remain unresolved and context-dependent across sub-Saharan Africa. While multiple hypotheses including aestivation, refugia use, and migration have been proposed, methodological and data limitations have hindered their conclusive validation. Evidence suggests that different persistence strategies operate across ecological zones, underscoring the need for integrated ecological, genetic, and modeling approaches to inform seasonally adaptive malaria control strategies.

9.1.2 Evaluation of Dry Season Persistence mechanisms using Population Genetics

This objective introduced a novel population genetic framework for indirectly estimating the proportion of malaria mosquito populations undergoing aestivation without directly sampling dormant individuals. By extending the Wright–Fisher model to incorporate partial aestivation, the approach uses seasonal allele frequency dynamics to infer rainy- and dry-season breeding sizes and the contribution of aestivating individuals. Simulations and empirical application to *Anopheles coluzzii* demonstrate that aestivation reduces apparent genetic drift and biases temporal effective population size estimates, with important implications for interpreting seasonal population dynamics. Overall, the framework provides a robust genetic tool for quantifying dormancy and persistence mechanisms in highly seasonal environments, with direct relevance for evaluating vector control strategies, including gene drive interventions.

9.1.3 Modeling Dry Season Survival of *An. gambiae* in Eastern Uganda

This study demonstrated that seasonal population rebounds in *Anopheles gambiae* populations in Eastern Uganda are driven by a combination of aestivation and long-distance migration mechanisms, both occurring at measurable and moderate levels. The limited temporal genetic differentiation and consistently large effective population sizes observed across seasons indicate a resilient population buffered against strong genetic drift by these complementary persistence mechanisms. These findings provide a region-specific ecological and genetic framework for understanding mosquito population persistence and have important implications for interpreting the spread of adaptive traits and designing vector control strategies that account for both local persistence and population connectivity.

9.1.4 The population Genetics of Malaria Mosquitoes at Island and mainland Sites in Uganda

Population genomic analysis revealed clear genetic differentiation between *An. gambiae* and *An. arabiensis*, as well as significant though low population structure within *An. gambiae* between island and mainland populations in Uganda. Mainland populations exhibited higher genetic diversity, connectivity, and effective population sizes, while island populations were more genetically isolated with smaller effective population sizes, reflecting restricted gene flow across Lake Victoria. These findings provide a critical genetic baseline and demonstrate that island populations, due to their isolation and demographic characteristics, are suitable candidate sites for controlled field evaluation of gene drive–based vector control interventions.

Overall, this thesis gives insights into the genetic structure, dry season survival strategies, and seasonal dynamics of selected Ugandan and Sahelian malaria mosquito populations. The integration of genetic analysis with ecological observations and mathematical modeling sets the stage for a detailed understanding of mosquito population persistence in

support of developing and deploying novel malaria vector control tools. Integrating ecological data, improved malaria vector sampling techniques and long-term vector monitoring will be crucial in improving these tools to ensure effective malaria control. These insights will contribute to global efforts toward malaria elimination by offering actionable knowledge to optimize vector control strategies and advance the fight against malaria.

9.2 Recommendations

9.2.1 Dry Season Survival Mechanisms of Malaria Mosquitoes

Future research should adopt integrated, multi-method approaches that combine ecological monitoring with advanced genetic and genomic analyses to better resolve malaria vector persistence mechanisms. Longitudinal, high-resolution sampling across seasons and ecological zones, supported by whole-genome data and forward-time population genetic simulations, is recommended to capture hidden or transient populations and regional variability in persistence strategies. Controlled laboratory and semi-field experiments, alongside cross-disciplinary collaboration, will be essential for identifying the ecological and physiological drivers of dormancy and migration and for informing the development of targeted, seasonally adaptive vector control interventions.

9.2.2 Evaluation of Dry Season Persistence mechanisms using Population Genetics

Future studies should adopt multi-season, high-resolution genetic sampling designs to robustly estimate diapause and seasonal persistence parameters, ideally integrating complementary genetic analyses like linkage disequilibrium, kinship inference, and population structure with extended stochastic models that incorporate migration, overlapping generations, and state switching between dormancy and reproduction. Where feasible, genetic inferences should be validated using direct ecological approaches such as mark–release–recapture or isotopic tracing to strengthen mechanistic interpretation.

Expanding this framework to other diapausing taxa and applying it within vector control programmes, particularly those involving gene drive or resistance management will improve prediction accuracy and intervention design. Interdisciplinary collaboration will also be essential to reduce parameter uncertainty and advance the management of highly seasonal vector populations.

9.2.3 Modeling Dry Season Survival of *An. gambiae* in Eastern Uganda

Future research and malaria control strategies should explicitly account for the coexistence of aestivation and migration as drivers of seasonal mosquito population rebound. Vector control programmes should intensify dry-season interventions targeting local refugia while ensuring regional coordination to limit re-invasion through migration. For gene drive development and other genetic control tools, models must incorporate empirically estimated migration rates, aestivation proportions, and large effective population sizes to generate realistic predictions of spread and persistence. Further studies should employ finer temporal and broader spatial genetic sampling, integrate environmental and climatic data to identify migration corridors, and validate genetic inferences using complementary ecological approaches such as mark–release–recapture and physiological assays. Finally, the modeling framework developed here should be extended through robust field-based evaluation using longitudinal entomological and genomic data, enabling direct comparison between model predictions and observed dynamics and strengthening the application of these tools for context-specific malaria elimination strategies across Africa.

9.2.4 The population Genetics of Malaria Mosquitoes at Island and mainland Sites in Uganda

Based on the findings from this study, lacustrine islands in Uganda should be prioritized as candidate sites for initial, contained field trials of gene drive systems, given the demonstrated genetic isolation and small effective population sizes of *An. gambiae*. To

refine non-target risk assessment, targeted sampling of *An. arabiensis* using methods suited to its exophilic and zoophagic behavior is recommended to clarify its presence and abundance on these islands. Future studies should incorporate temporal and seasonal sampling to better capture population dynamics and dry-season persistence, which are critical for predicting intervention durability. Advancing from amplicon-based to whole-genome sequencing will improve resolution of population structure, gene flow, and selection signatures. And finally, integrating ecological and microclimatic data with robust pre- and post-intervention genomic monitoring, alongside interdisciplinary and community-engaged research frameworks, will be essential for the safe, ethical, and effective deployment of genetic control tools in support of malaria elimination in Uganda.

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APPENDICES

UNHRO
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REPUBLIC OF UGANDA

Our Ref: GC/127/19/01/600

Your Ref:

January 25, 2019

UVRI REC APPROVAL NOTICE

To: Dr Jonathan Kayondo, Principal Investigator

Re: Application Title: **"Target Malaria: Entomological studies to characterize field sites in Uganda to support the evaluation of novel mosquito-related malaria-control methods"**

Type: [] Initial Review

[ ] Protocol Amendment

[ ] Letter of Amendment (LOA)

[✓] Continuing Review

[ ] Material Transfer Agreement

[ ] Other, Specify:_____

Thank you for submitting your progress report for the above study dated January 3, 2019 to the UVRI Research Ethics Committee (REC).

This is to inform you that after review of your report, UVRI REC continuation approval has been granted for you to continue with this study up to January 19, 2020.

At that time, REC would expect you to submit a progress report and request for renewal, prior to the expiry date, to allow timely review.

Yours sincerely,

Dr. Tom Lutalo
Chair, UVRI REC
C.C Secretary, UVRI REC

Uganda National Council for Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

Our Ref: HS 2378 **25th February 2019**

Dr. Jonathan Kayondo
Uganda Virus Research Institute
Kampala

Dear Dr. Kayondo,

Re: Research Approval: Target Malaria: Entomological Studies to Characterize Field Sites in Uganda to Support the Evaluation of Novel Mosquito – Targeted Malaria – Control Methods

I am pleased to inform you that on **06/03/2018**, the Uganda National Council for Science and Technology (UNCST) approved the above referenced research project. The Approval of the research project is for the period of **06/03/2018** to **06/03/2021.**

Your research registration number with the UNCST is **HS 2378.** Please, cite this number in all your future correspondences with UNCST in respect of the above research project.

As Principal Investigator of the research project, you are responsible for fulfilling the following requirements of approval:

1. All co-investigators must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the research protocol or the consent form (where applicable) must be submitted to the designated Research Ethics Committee (REC) or Lead Agency for re-review and approval **prior** to the activation of the changes. UNCST must be notified of the approved changes within five working days.
3. For clinical trials, all serious adverse events must be reported promptly to the designated local IRC for review with copies to the National Drug Authority.
4. Unanticipated problems involving risks to research subjects/participants or other must be reported promptly to the UNCST. New information that becomes available which could change the risk/benefit ratio must be submitted promptly for UNCST review.
5. Only approved study procedures are to be implemented. The UNCST may conduct impromptu audits of all study records.
6. An annual progress report and approval letter of continuation from the REC must be submitted electronically to UNCST. Failure to do so may result in termination of the research project.

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Below is a list of documents approved with this application:

	Document Title	Language	Version	Version Date
1.	Research proposal	English	N/A	N/A
2.	Information sheets	English and Luganda	N/A	N/A
3.	Acceptance forms	English and Luganda	N/A	N/A
4.	Individual consents	English and Luganda	N/A	N/A

Yours sincerely,

Isaac Makhuwa
For: Executive Secretary
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

Copied to: Chair, Uganda Virus Research Institute, Research Ethics Committee

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Malaria Journal

REVIEW Open Access

Potential persistence mechanisms of the major *Anopheles gambiae* species complex malaria vectors in sub-Saharan Africa: a narrative review

Rita Mwima[1,2], Tin-Yu J. Hui[3], Ann Nanteza[2], Austin Burt[3] and Jonathan K. Kayondo[1*]

Abstract

The source of malaria vector populations that re-establish at the beginning of the rainy season is still unclear yet knowledge of mosquito behaviour is required to effectively institute control measures. Alternative hypotheses like aestivation, local refugia, migration between neighbouring sites, and long-distance migration (LDM) are stipulated to support mosquito persistence. This work assessed the malaria vector persistence dynamics and examined various studies done on vector survival via these hypotheses; aestivation, local refugia, local or long-distance migration across sub-Saharan Africa, explored a range of methods used, ecological parameters and highlighted the knowledge trends and gaps. The results about a particular persistence mechanism that supports the re-establishment of *Anopheles gambiae*, *Anopheles coluzzii* or *Anopheles arabiensis* in sub-Saharan Africa were not conclusive given that each method used had its limitations. For example, the Mark-Release-Recapture (MRR) method whose challenge is a low recapture rate that affects its accuracy, and the use of time series analysis through field collections whose challenge is the uncertainty about whether not finding mosquitoes during the dry season is a weakness of the conventional sampling methods used or because of hidden shelters. This, therefore, calls for further investigations emphasizing the use of ecological experiments under controlled conditions in the laboratory or semi-field, and genetic approaches, as they are known to complement each other. This review, therefore, unveils and assesses the uncertainties that influence the different malaria vector persistence mechanisms and provides recommendations for future studies.

Keywords *Anopheles*, Persistence mechanisms, Dry season survival, Malaria

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Background

Malaria vector populations exhibit strong seasonal fluctuations in abundance and are present in large numbers during the rainy season, but drop to extremely low levels when the larval habitats dry up [1–3]. This has been observed within members of the *Anopheles gambiae* species complex (or *Anopheles gambiae* sensu lato) (Diptera: Culicidae) and beyond, and across diverse ecological or geographical set-ups, including the West-African Sahel and East Africa Savanna. Prevailing hypotheses suggest that the possible ways that could explain the seasonal

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RESOURCE ARTICLE

MOLECULAR ECOLOGY RESOURCES WILEY

The population genetics of partial diapause, with applications to the aestivating malaria mosquito *Anopheles coluzzii*

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Abstract

Diapause, a form of dormancy to delay or halt the reproductive development during unfavourable seasons, has evolved in many insect species. One example is aestivation, an adult-stage diapause enhancing malaria vectors' survival during the dry season (DS) and their re-establishment in the next rainy season (RS). This work develops a novel genetic approach to estimate the number or proportion of individuals undergoing diapause, as well as the breeding sizes of the two seasons, using signals from temporal allele frequency dynamics. Our modelling shows the magnitude of drift is dampened at early RS when previously aestivating individuals reappear. Aestivation severely biases the temporal effective population size (N_e), leading to overestimation of the DS breeding size by $1/(1-\alpha)^2$ across 1 year, where α is the aestivating proportion. We find sampling breeding individuals in three consecutive seasons starting from an RS is sufficient for parameter estimation, and perform extensive simulations to verify our derivations. This method does not require sampling individuals in the dormant state, the biggest challenge in most studies. We illustrate the method by applying it to a published data set for *Anopheles coluzzii* mosquitoes from Thierola, Mali. Our method and the expected evolutionary implications are applicable to any species in which a fraction of the population diapauses for more than one generation, and are difficult or impossible to sample during that stage.

KEYWORDS
aestivation, diapause, genetic drift, persistence, temporal allele frequency

1 | INTRODUCTION

Many insect species have evolved the mechanism of diapause, a form of dormancy, which halts development and reproduction under unfavourable environmental conditions (Cloutier et al., 2021; Ojima et al., 2018). Some forms of diapause may also include reduced activity such as flying and feeding. Metabolism is supressed, and most energy resources are diverted towards survival. Diapause is pre-programmed and genetically coded, then activated by changing of environments (Ojima et al., 2018). Depending on the species, known stimuli include day length, temperature and access to water. Examples of species that undergo diapause include *Drosophila suzukii*, *Phormia regina* (Queen blow flies), *Danaus plexippus* (Monarch butterfly), multiple moth species, tropical walking-stick insects and many more (Herman & Tatar, 2001; Salman et al., 2019; Tatar & Yin, 2001;

Rita Mwima and Tin-Yu J. Hui should be considered joint first authors.

Malaria Journal

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Article in Press

Assessing the population genetic structure and demographic history of *Anopheles gambiae* and *Anopheles arabiensis* at island and mainland sites in Uganda: implications for testing novel malaria vector control approaches

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Rita Mwima, Tin-Yu Hui, Edward Lukyamuzi, Marilou Bodde, Alex Makunin, Krystal Birungi, Martin Lukindu, Ann Nanteza, Dennis Muhanguzi, Mara Lawniczak, Austin Burt & Jonathan Kayondo