

The effect of silencing immunity related genes on longevity in the naturally occurring *Anopheles arabiensis* mosquito population of southwest Ethiopia

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Thesis submitted in fulfilment of the requirements for the degree of Doctor in Veterinary Sciences (UGent) and Biomedical Sciences (UA)

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22 April 2022

This PhD research was made possible with the financial support of VLIR-UOS Institutional University Cooperation with Jimma University (IUC-JU) and the NASCERE program.

In memory of my father
with love and eternal appreciation

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List of Abbreviations

ACT: Artemisinin-based Combination Therapy

AMPs: Antimicrobial Peptides

ASDR: Age-Standardized Death Rate

ATSBs: Attractive Toxic Sugar Baits

cDNA: complementary DNA

CSP: Circumsporozoite Protein

Ct-values: threshold cycle values

DALY: Disability Adjusted Life Years

DNA: Deoxyribonucleic Acid

DO: Dissolved Oxygen

dsRNA: double stranded RNA

EIP: Extrinsic Incubation Period

FMOH: Federal Ministry of Health

FN3D1: Fibronectin type III Domain- protein 1

FN3D2: Fibronectin type III Domain- protein 2

FN3D3: Fibronectin type III Domain- protein 3

GM: Genetically Modified

GPRGR9: G Protein coupled Receptor Protein 9

HR: Hazard Ratio

IMD: Immune deficiency

IPTP: Intermittent Preventive Treatment in pregnant women

IRS: Indoor Residual Spraying

JAK-STAT Janus Kinase-Signal Transducers and Activators of Transcription

LLINs: Long Lasting Insecticide Treated Nets

NF- κ B: NF-kappaB

PBS: Phosphate-buffered Saline

PCR: Polymerase Chain Reaction

PFA: Paraformaldehyde

PGRPLC3: Peptidoglycan Recognition Protein LC3

qPCR: quantitative real-time PCR

RNA: Ribonucleic Acid

SM1: Salivary gland and Midgut peptide 1

SSA: Sub-Saharan Africa

TBV: Transmission Blocking Vaccines

TDS: Total Dissolved Solid

VC: Vectorial Capacity

WHO: World Health Organization

YLL: Years of Life Lost

Chapter 1 Introduction

1.1 Overview of malaria

1.1.1 Malaria disease and etiology

Malaria is a life-threatening, infectious disease caused by a protozoan blood parasite belonging to the genus *Plasmodium*. There are five *Plasmodium* parasite species that can cause malaria in humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and the recently emerged *Plasmodium knowlesi*. Infections with two of these species, *P. falciparum* and *P. vivax*, represent the most common form of malaria in different parts of the world (WHO, 2021). Almost all severe forms and deaths of malaria are caused by *P. falciparum* (WHO, 2021). Cerebral malaria, pulmonary edema, acute renal failure and severe anemia are the major complications of severe malaria. Jaundice with hepatic dysfunction (Abro et al., 2009) and metabolic complications including acidosis and hypoglycemia are the most common complications that can lead to death within hours or days (Planche et al., 2005; Trampuz et al., 2003). Neurological complications due to *P. falciparum* also occur (Van der Wal et al., 2005). Rarely, *P. vivax* causes serious acute lung injury and acute respiratory distress syndrome (Mohan et al., 2008). *P. knowlesi* infections have also led to severe complications (Kotepui et al., 2020).

1.1.2 The epidemiology of malaria

Geographically, malaria is mainly found in the tropical and subtropical countries of the world. Africa is the most affected area because of the presence of the most efficient and long-lived mosquitoes, the *Anopheles gambiae* complex, and the most severe form of malaria species, *P. falciparum* (CDC, 2018; Guerra et al., 2008). The presence of suitable local weather conditions results in transmission throughout the year in many locations. On top of that, insufficient resources and socio-economic instability hold back the effectiveness of the malaria control strategies (CDC, 2018; WHO, 2015). In some countries of America and South Asia malaria is considered a considerable disease but it is not a major cause of death as compared to Africa (WHO, 2021) (Figure 1.1).

Plasmodium falciparum contributes to the majority of the malaria cases, i.e., accounting for 99.7% of the cases in the World Health Organization (WHO) African Region, 50% in the WHO Southeast

Asia Region, 71% in the Eastern Mediterranean and 65% in the Western Pacific, while *P. vivax* is the main malaria parasite for the WHO Region of the Americas, representing 75% of cases (WHO, 2021).

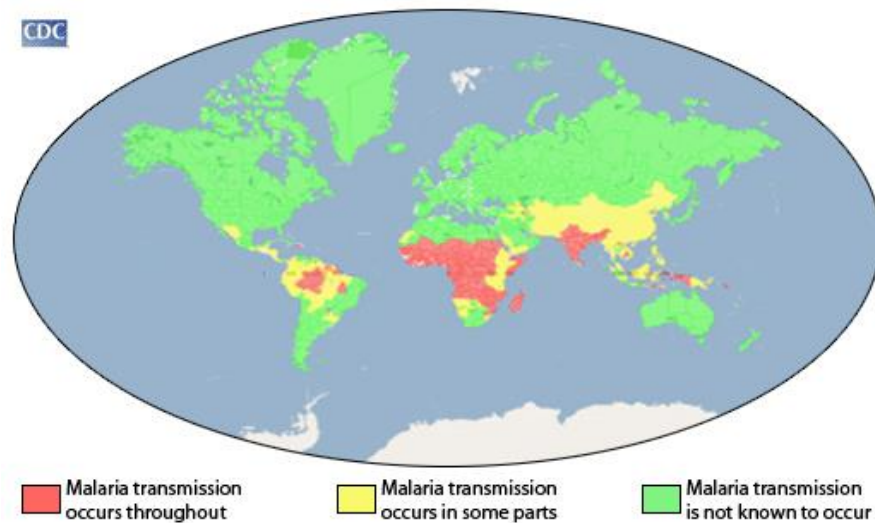


Figure 1.1. Malaria risk map of districts by annual parasite incidence, Ethiopia (Source [FMOH NSP: 2017-2020](#)).

1.1.3 Global burden of malaria

Malaria is a leading cause of illness and death worldwide. In the past, sub-Saharan Africa (SSA) was characterized by the highest global malaria prevalence and the highest malaria related morbidity and mortality (WHO, 2019; WHO, 2020). Recently, in 2020, there were an estimated 241 million malaria cases and 627,000 deaths across the globe. Nearly all these malaria cases (95%) and deaths (96%) are in African countries. Most of them are children under five years which accounted for 80% (272,000) of overall malarial mortalities (WHO, 2021). Six sub-Saharan African countries including Nigeria (31.9%), the Democratic Republic of Congo (13.2%), Uganda, Cote d'Ivoire, United Republic of Tanzania (4.1%) and Mozambique (3.8%) contributed more than half of the malaria deaths of the world (WHO, 2021). On the other hand, the WHO European region has been free of malaria (WHO, 2021). In general, the trend of malaria cases and deaths has shown an increase in both the incidence of cases and death rate in 2020. For example, malaria case incidence (cases per 1,000 population at risk) reduced from 81 (241 million) in 2000 to 59 (224

million) in 2015 and 56 (227 million) in 2019 and increased again to 59 (241 million) in 2020. Likewise, the global malaria deaths reduced gradually over the period of 2000-2019 from 896,000 in 2000 to 562,000 in 2015 and 558,000 in 2019 and increased to 627,000 in 2020 (WHO, 2021) (Figure 1.2).

The significant malaria reduction that has been observed between 2000-2015 (WHO, 2016a) was due to a wide distribution of long-lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS) (Bhatt et al., 2015; Cibulskis et al., 2016). Since 2000, globally the incidence and mortality rates of malaria have been reduced by 37% and 70% respectively. An estimated 70% of the reduction in the number of cases in Africa was due to malaria interventions. In 2015, more than half of the population in SSA slept under insecticide treated nets (ITNs), compared with 2% in 2000 (Cibulskis et al., 2016). In Africa, between 2000 and 2015 the prevalence of *P. falciparum* infection reduced by half, the incidence of clinical disease fell by 40% and 663 million cases were averted (542-753 credible interval). Interventions, particularly ITNs, contributed most (68%) to the averted cases (Bhatt et al., 2015). Moreover, improvement in the availability of rapid diagnostic tests and anti-malaria drugs has also facilitated the people to access timely and appropriate treatment (Cibulskis et al., 2016).

However, as of 2015-2019 (WHO, 2021), there was no considerable progress in decreasing malaria cases in the WHO's African region. This could be due to insecticide resistance (Djouaka et al., 2016; Wat'senga et al., 2018), residual malaria transmission (Sherrard-Smith et al., 2019), host behavior changes like staying outdoor during early night or sleeping outdoor without using protective measures (Gari and Lindtjørn, 2018). On top of that, in some areas bed nets are not always well accepted, sometimes due to practical reasons. The use of a bed net might increase the presence of bedbugs (and the associated irritability) and it might become uncomfortably warm underneath especially during the dry season (Ingabire et al., 2015).

In 2020, malaria deaths increased to 627,000 which corresponds to an increase of 12% compared to 2019. Of the extra 69,000 malaria deaths occurring in 2020, about two thirds or an estimated 47,000 (68%) were due to the service distribution during the COVID-19 pandemic (WHO, 2021). In addition, misdiagnosis of malaria due to similarity in symptoms of malaria and COVID-19

might also have increased malaria deaths (Hussein et al., 2020). Overall, WHO reports show that the trend in malaria reduction stalled since 2015 (WHO, 2021) (Figure 1.2).

In endemic regions, malaria causes a significant health impact on young children and pregnant women. Pregnant women are at risk of severe malaria illness due to placental infection, and threatens the lives of over 24 million women living in SSA (Fondjo et al., 2020). An estimated 200,000-500,000 pregnant women suffered from severe malaria related anemia which has led to 10,000 maternal deaths. Globally, from 75,000 to 200,000 infant deaths are attributed to malaria each year (Fondjo et al., 2020). Malaria during pregnancy is responsible for intrauterine growth retardation, still birth, premature delivery, low birth weight, perinatal and neonatal morbidity and mortality and post-partum morbidity (Fondjo et al., 2020; Guyatt and Snow, 2001). Malaria has also a devastating effect on the birth weight in the WHO African region. In 2018, about 11 million pregnant women who live in moderate-to-high malaria transmission areas in Africa have delivered 872,000 children with low birth weight (WHO, 2019).

Malaria also has a significant health impact on children as it results in severe anemia. For example, between 2015 and 2018 the prevalence of anemia in children under 5 with malaria was the double of that in children without malaria in 21 WHO African malarious countries (WHO, 2019).

In addition to the health burden, malaria has negative social and economic consequences on the individual, society and government (RBM, 2011; Sarma et al., 2019). It is a major constraint for economic development and causes poverty. Estimated costs of malaria to Africa is \$12 billion every year (RBM, 2011).

Malaria has both direct and indirect economic costs. The direct costs include expenditures covered by both individual and government related with transport to health facilities, medicine, diagnosis, maintaining health facilities and health care infrastructure, prevention, education and research (Alonso et al., 2019; Dalaba et al., 2018; RBM, 2011; Tefera et al., 2020). In high burdened countries, malaria may contribute for about 40% of public health expenditure, 30% to 50% of inpatient admissions, and up to 50% of outpatient visits (RBM, 2011).

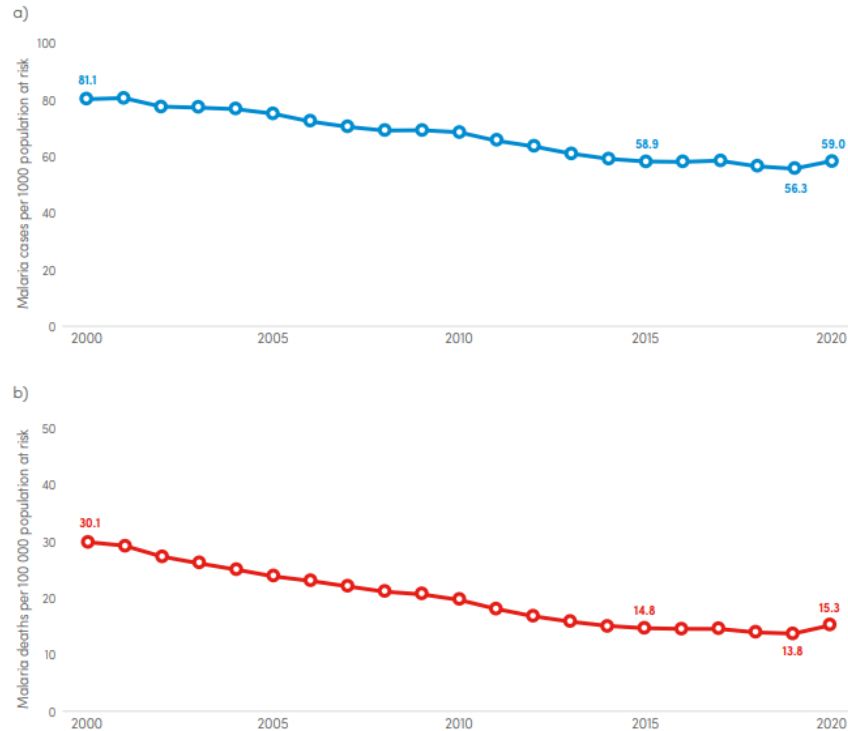


Figure 1.2. Global trends in a) malaria case incidence rate (cases per 1,000 population at risk), b) mortality rate (deaths per 100,000 population at risk), 2000-2020. (Source: WHO 2021) Available at <https://apps.who.int/iris/rest/bitstreams/1398397/retrieve>.

The indirect costs of malaria include human suffering, pain and death caused by the disease and productivity losses due to absenteeism from formal employment, loss of workdays and the price of unpaid work done at home by both men and women. Malaria also hampers children's schooling and social development through both absenteeism and permanent neurological and other damage associated with severe episodes of the disease (Breman, 2001; RBM, 2011).

Moreover, malaria affects the development of the tourist industry, investments and agricultural activities. Malaria in a community or country also obstructs individual and national growth of prosperity due to its influence on social and economic decisions. The risk of contracting malaria in endemic areas can deter investment. It further affects household decision making in many ways with a negative impact on economic productivity and growth. Economically, countries with a high burden of malaria have growth rates that are 1.3% less per person per year than countries with a low burden and malaria-free countries (RBM, 2011).

1.1.4 Malaria burden in Ethiopia

In Ethiopia, approximately 68% of the population is at risk of malaria (WHO, 2021). The transmission of the disease occurs mainly at altitudes below 2,000 m above sea level (EPHI, 2016; Yalew et al., 2017). However, endemic malaria regions at higher altitudes above 2,000 m have also been reported (Daygena et al., 2017; Delil et al., 2016; Tesfaye et al., 2011) (Figure 1.3). In most of the regions, the major transmission season is from September to December, following the main rainy season from July to September. In some regions there is a short transmission season from April to May, following the short rainy season (EPHI, 2016). However, in some area's transmission occurs all year round (Hawaria et al., 2020; Woyessa et al., 2012). *Plasmodium falciparum* and *P. vivax* are the major parasite species in Ethiopia with a high proportion of *P. falciparum* (more than 60%) (Kalil et al., 2020; Esayas et al., 2020).

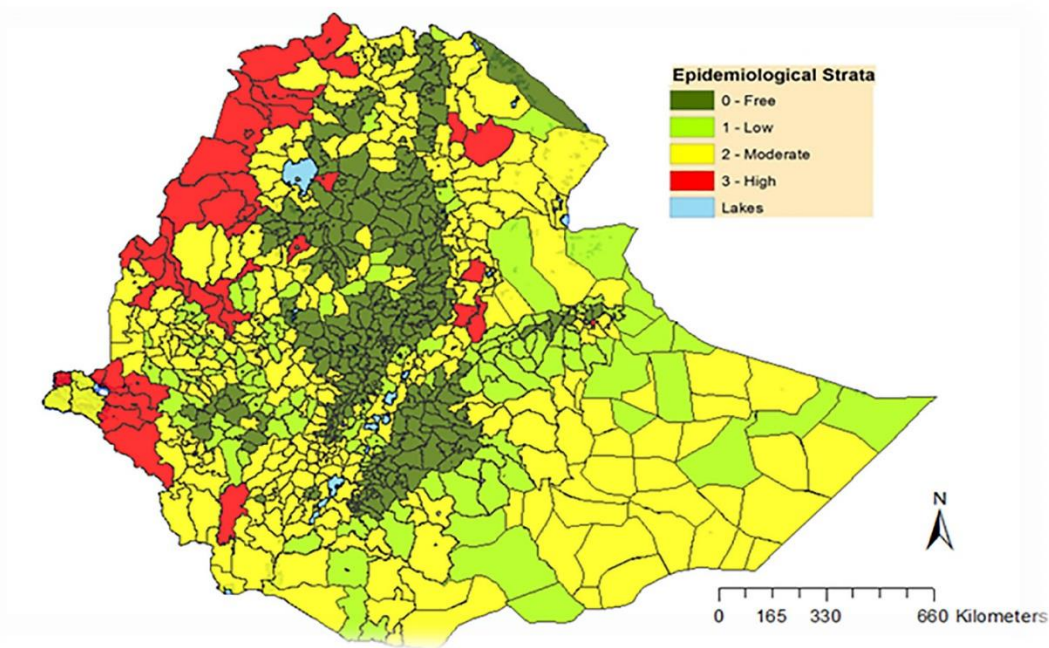


Figure 1.3. Malaria risk map of districts by annual parasite incidence, Ethiopia (Source [FMOH NSP: 2017-2020](#)).

In Ethiopia reduction in the burden of malaria has been reported from 2000 to 2016. However, it is still one of the major public health problems. In 2016 an estimated 2,927,266 new malaria cases and 4,782 deaths have been reported. In the same year the level of mortality and disability adjusted life years (DALY) due to malaria was high at 365,900 years. About 332,100 life years have been lost (YLL) and about 35,200 years have been lived with disability. High mortality and DALY have been reported among children under 5 (Girum et al., 2019). In 2020, 1,743,755 malaria cases and 173 deaths have been reported (WHO, 2021).

1.2 Malaria transmission and vector species in Africa

1.2.1 Malaria primary vector species in Africa

Malaria is transmitted mostly by *Plasmodium* infected female *Anopheles* mosquitoes. There are about 462 *Anopheles* species worldwide, of which only 70 are considered to possess the ability to transmit *Plasmodium* (Hay et al., 2010). In Africa the main vector species involved in malaria transmission include *An. arabiensis*, *An. gambiae*, *An. funestus*, *An. melas*, *An. merus*, *An. moucheti* and *An. nili* (Sinka et al., 2012). Recently, a new species, *An. stephensi* has been identified in the Horn of Africa including Ethiopia and Sudan (Sinka et al., 2020). The vectorial capacity of these species is determined by their abundance, their feeding tendency on humans and their adult longevity (Takken and Lindsay, 2003). These characteristic features of African malaria vector species make that Africa is the most malaria burdened continent of the world. *An. gambiae*, *An. funestus* and *An. arabiensis* are the primary vector species that sustain malaria transmission in SSA (Sinka et al., 2012). *An. coluzzii* is also another important vector species and it is widely distributed in Africa (Coetzee et al., 2013). In Ethiopia, more than 46 species of the *Anopheles* mosquito have been recorded, of which *An. gambiae* and *An. arabiensis* are the most common primary vectors. *An. arabiensis* is the dominant species in most parts of the country (Animut et al., 2013; Animut et al., 2012).

Studies have shown that a major shift in malaria vector species composition occurred following the intensive application of insecticide-based malaria control tools, such as LLINs and IRS, with *An. arabiensis* replacing *An. gambiae*. For example, in Kenya the scale-up of ITNs correlated with

a proportional decrease in *An. gambiae* from 85 % in 1970–1998 to 1 % in 2009. In 2009 *An. gambiae* s.s. comprised 1% of the indoor collections and *An. arabiensis* the remaining 99% (Bayoh et al., 2010). In southern Kenya, Mutuku et al. (2011) also reported a significant decline in the relative proportion of *An. gambiae* s.s. with proportionate increase of *An. arabiensis*. A similar trend was reported in southern Tanzania where a 79% reduction in *An. gambiae* was observed compared with 38% for *An. arabiensis* following high LLIN coverage (Russell et al., 2010). *An. arabiensis* occupies over 70% of SSA. This species dominates in arid zones and some of the highland areas (Mutero et al., 2004; Onyabe and Conn, 2001).

1.2.2 Feeding and resting behavior of *An. arabiensis*

Both male and female mosquitoes feed on sugar for their survival, flight activity and reproduction. In addition to sugar, female mosquitoes need a blood meal in order to provide nutrients for the maturation of their eggs. *Anopheles arabiensis* exhibits varying feeding and resting behavior due to a great behavioral plasticity. Host odor is one of the factors influencing host preference behavior of mosquitoes (Becker, 2010). Based on their host preference behavior, *An. arabiensis* mosquitoes are zoophagic vectors, i.e., they mainly feed on animals. They are more attracted by odor from cattle than that from human and other animals (Habtewold et al., 2004; Mahande et al., 2007; Masebo et al., 2013).

The mosquito resting behavior can be classified into endophilic and exophilic behavior. Endophilic mosquitoes are characterized by resting indoors, inside the human dwelling during the time between the end of blood feeding and the onset of searching for a suitable oviposition site whereas exophilic mosquitoes prefer to rest and spend this time period outside the human dwelling (Pates and Curtis, 2005). *Anopheles arabiensis* exhibit both exophilic and endophilic behavior (Kent et al., 2007; Mendis et al., 2000; Muriu et al., 2008). In malaria endemic African countries *Anopheles* vectors have developed insecticide avoidance behavior (Carrasco et al., 2019). In response to insecticide exposure, *An. arabiensis* exhibit strict exophilic behavior (Derua et al., 2012; Kitau et al., 2012; Sougoufara et al., 2016).

1.3 The sporogonic life cycle of the malaria parasite

The life cycle of the malaria parasite is composed of a series of complex steps involving the two hosts, the human host and the mosquito vector. Mosquitoes are the definitive hosts where the sexual phase of the lifecycle takes place. The life cycle of the vector begins when a female *Anopheles* mosquito bites and ingests male and female gametocytes together with the blood from an infected person (Figure 1.4 (no. 8)). In the mosquito midgut, gametocytes differentiate into male and female gametes through a process known as gametogenesis and next they fuse together into zygotes (Figure 1.4 (no. 9)). The zygotes subsequently differentiate into motile ookinetes (Figure 1.4 (no.108)). The ookinetes must then penetrate the peritrophic matrix that completely surrounds the blood meal and invade the midgut epithelium, about 18 to 36 hours after the ingestion of an infected blood meal. After the midgut transversal, ookinetes attach to the basal surface of the midgut where they round up into oocysts (Figure 1.4 (no. 11)) that undergo several rounds of replication by means of sporogony (Figure 1.4 (C)) releasing thousands of infective sporozoites (Figure 1.4 (no. 12)) into the hemolymph to eventually invade the salivary glands. During the next blood meal, these sporozoites are injected with the saliva into a vertebrate host including humans (Figure 1.4 (no. 1)) (Bennink et al., 2016; CDC, 2020; Paniker, 2017).

The time required for the development of the parasite within the vector is known as extrinsic incubation period (EIP) or sporogonic cycle. Depending on the parasite species and other environmental factors like temperature, the EIP ranges from 10 to 14 days. In order to be able to transmit the parasite, a female mosquito must survive longer than the EIP of the parasite (Ohm et al., 2018).

1.4 Determinants of malaria transmission

1.4.1 Longevity and vectorial capacity

As mentioned above less than a third of the *Anopheles* species are able to transmit malaria and even within the vector species the degree of importance varies greatly (Sinka et al., 2010; Sinka et al., 2012). One of the measurements to quantify the ability of a mosquito to transmit malaria is the

vectorial capacity (VC). The mosquito's longevity is the most critical factor to determine VC (Johnson et al., 2020). Indeed, it is essential for completion of the parasite life cycle that the mosquito is able to survive the initial invasion and lives long enough for successful transmission of sporozoites to the vertebrate host. As the sporogonic cycle of *Plasmodium* lasts between 10 to 14 days depending on the species, the mosquito life expectancy must be larger than this (Ohm et al., 2018). It was predicted that even the slightest decrease in longevity will have a most dramatic effect on disease transmission (Johnson et al., 2020).

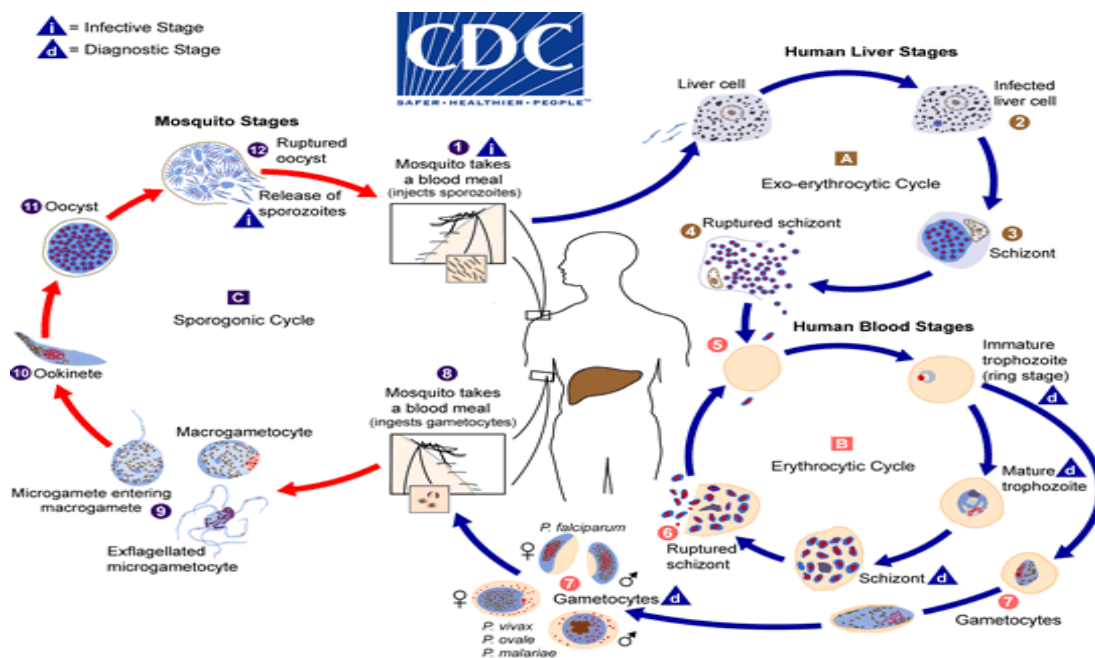


Figure 1.4. The malaria parasite life cycle: CDC Malaria. available at <https://www.cdc.gov/malaria/about/biology/>.

Direct measurement of the life span of mosquitoes is hardly possible in nature. But indirect estimates of daily survivorship have been made for several *Anopheles* species using catch, release and recapture methods. In West African Sudan Savanna village, for instance, the estimated daily mortality rate for the *An. gambiae* complex mosquito ranged from 12% to 20% (Costantini et al., 1996). Assuming this is constant throughout the adult life time, only a small fraction of mosquitoes

(<10%) would survive longer than the EIP. Furthermore, given the zoophilic behavior of *An. arabiensis*, the first blood meal(s) might well be on cattle and thus will not lead to ingestion of *Plasmodium*. In fact, it is thought that the large differences in VC between malaria vector mosquito species are mainly attributable to the life span of the mosquitoes.

1.4.2 Factors that affect longevity and vectorial capacity

The longevity and VC of adult mosquitoes can be affected by several factors of the larval breeding environment. These factors include the physicochemical characteristics of breeding water (Mwangangi et al., 2007; Oyewole et al., 2009) and quality (Linenberg et al., 2016) and abundance of the larval diet (Araujo and Gil, 2012; Moller-Jacobs et al., 2014). The larval breeding environment affects the survival and development of larvae, the pupation rate and the number of emerged adults (Moller-Jacobs et al., 2014; Oyewole et al., 2009). Importantly, it also influences adult biological traits including the size, reproductive success and survival (longevity) (Araujo and Gil, 2012; Moller-Jacobs et al., 2014; Shapiro et al., 2016). The longevity of female adult mosquitoes stemming from larvae kept in a high nutritive environment has been longer than those from a poor nutritive environment (Moller-Jacobs et al., 2014). A significant reduction in VC has also been observed in *An. stephensi* mosquitoes stemming from larvae reared in a low food environment (Moller-Jacobs et al., 2014) whereas greater VC has been observed in *An. darlingi* (Araujo and Gil, 2012) and *An. stephensi* (Shapiro et al., 2016) stemming from larvae maintained in a high food habitat. In addition, other biological traits of mosquitoes like adult wing size and overall size are also a function of the characteristics of the larval breeding environment (Moller-Jacobs et al., 2014). It is known that both overall size and wing size directly affects the mosquito's longevity positively (Moller-Jacobs et al., 2014).

1.5 Malaria control and prevention measures

Malaria control and elimination strategies are being scaled up and strengthened in current efforts to reduce and interrupt disease transmission in countries in order to attain the World Health Assembly, Roll Back Malaria, and Millennium Development universal access and coverage

targets. From 2000 to 2015, a total of 49 countries reported fewer than 10,000 malaria indigenous cases, compared with 46 countries in 2007 and 40 countries in 2010 (WHO, 2016). This progress is largely attributable to the scaling-up of vector control interventions, such as LLINs and IRS (Bhatt et al., 2015), as well as improved diagnostics and effective treatment using artemisinin-based combination therapies (ACT), and intermittent preventive treatment in pregnant women (IPTP) (WHO, 2020).

1.5.1 Vector Control

Vector control is the major malaria control and elimination strategy. This approach addresses both mature and immature developmental stages of malaria transmitting mosquitoes.

1.5.1.1 ITNs/ LLINs / IRS

Insecticide treated nets, LLINs and IRS are the key malaria intervention tools. These tools reduce the vectorial capacity of mosquitoes through reducing the survival rate of the mosquito to a point at which transmission is interrupted. Insecticide treated nets and ITNs interrupt malaria transmission through providing both a physical barrier and insecticidal effect. Indoor residual spraying is based on the application of long-acting chemical insecticides on the interior walls and roofs of houses to kill adult mosquitoes resting on these surfaces. It reduces malaria transmission through killing or reducing longevity of adult mosquitoes when they rest on insecticide sprayed surfaces inside houses or other structures (WHO, 2015).

The ITN supply and utilization is increasing globally. From 2004 to 2020 almost 2.3 billion ITNs have been supplied by the manufacturer worldwide, of which 2 billion (86%) were distributed to sub-Saharan African countries (WHO, 2021). In 2020, about 229 million ITNs have been distributed to malaria endemic countries and 65% of the households had at least 1 ITN compared to about 5% in 2000. The percentage of the population with access to an ITN within their household increased from 3% to 50%. The percentage of people sleeping under an ITN increased significantly between 2000 and 2020 from 2% to 43% for the whole population, from 3% to 49% for children under five and from 3% to 49% for pregnant women (WHO, 2021).

The world health organisation recommends four classes of insecticides: pyrethroids, organochlorines, carbamates and organophosphates (WHO, 2021). Pyrethroid has been the most commonly used insecticide to treat LLINs (ITNs). Recently, a newly developed net, the piperonyl butoxide (PBO) net, has a significant role in reducing the number of malaria infections in pyrethroid resistant areas, overcoming the resistance problem of pyrethroids (Gleave et al., 2021). Piperonyl butoxide is a synergist that has been incorporated into pyrethroid-LLINs to form pyrethroid-PBO nets. This chemical does not have an insecticidal effect but it rather blocks the effect of an enzyme inside the mosquito that prevents the effect of pyrethroids. This net has been more effective than standard pyrethroid-only nets both in killing mosquitoes and preventing blood feeding in areas where pyrethroid resistant mosquitoes are distributed (Gleave et al., 2021). In SSA an increased rate of scaling up of LLINs resulted in reductions in malaria transmission in many endemic countries (Bhatt et al., 2015).

Indoor residual spraying has been a basis in malaria control and resulted in a reduction in malaria incidence during the 20th century and contributed to malaria elimination in many countries (Mabaso et al., 2004; Shiff et al., 2002; Wakabi, 2007). Between 1940 and 1980, millions of lives were saved largely due to IRS (WHO, 2015). With the current efforts to scale up malaria control in Africa, IRS has been introduced in a number of countries with high levels of transmission (Kigozi et al., 2012; Kleinschmidt et al., 2006). In Ethiopia, vector control intervention has started since 1959 during the malaria eradication era. Dichlorodiphenyltrichloroethane (DDT) was used for IRS until 2008. Malathion has also been used as an alternative when mosquitoes were resistant to DDT. In 2009, DDT was replaced by pyrethroid due to the development of insecticide resistance by the vectors (Mekuriaw et al., 2020). Currently, carbamate is the common insecticide used for IRS (Abraham et al., 2017). However, these tools are designed for mosquitoes that are active at night and display strong endophagic (seeking blood meal indoors) and endophilic (resting indoors following a blood meal) behavior (WHO, 2015). For example, LLINs and ITNs treated with pyrethroids have been more effective at killing *An. gambiae* and *An. funestus* compared to *An. arabiensis*. Experimental hut trials of ITNs and LLINs conducted in northeastern Tanzania demonstrated a lower mortality rate of *An. arabiensis* (25–52%) compared to *An. gambiae* s.s. (63–88%) and *An. funestus* (53–78%) (Kitau et al., 2012).

Similarly, in southern Tanzania a 79% reduction in *An. gambiae* was reported compared to only 38% in *An. arabiensis* following high LLIN coverage (Russell et al., 2010). The above findings are supported by other studies in Africa (Russell et al., 2011; Sougoufara et al., 2016) where ITNs and LLINs have been more effective for the endophagic/endophilic *An. gambiae* and *An. funestus* mosquito compared to the exophagic/exophilic *An. arabiensis* mosquito. Therefore, new vector control approaches targeting outdoor biting malaria vectors, like *An. arabiensis*, are necessary to complement the existing malaria control methods. A recent review on the existing and novel malaria vector control approaches has also shown the need for new vector control methods targeting outdoor biting and resting behavior (Sougoufara et al., 2020).

1.5.1.2 Attractive toxic sugar baits (ATSBs) methods

These methods are a new class of vector control measures to control mosquitoes through "attract and kill" strategies. Attractive toxic sugar baits attract and kill mosquitoes when mosquitoes feed on sugar solution containing a mosquitocidal agent in both the indoor (Qualls et al., 2015; Stewart et al., 2013) and outdoor environment (Traore et al., 2020). These approaches use fruit or flower smell as an attractant, sugar solution as a feeding stimulant and oral insecticides to kill the mosquitoes (Qualls et al., 2015; Stewart et al., 2013; Tenywa et al., 2017) .

Attractive toxic sugar baits have a significant effect on the reduction of *Anopheles* mosquitoes as observed in different field trial studies (Tenywa et al., 2017; Traore et al., 2020) although it is not 100% effective. Moreover, indoor ATSB enhance the control of pyrethroid-resistant *An. gambiae* in combination with LLINs. The addition of ATSB to LLINs increased the mortality rates of wild pyrethroid-resistant *An. gambiae* from 19% (LLIN alone) to 28% together with boric acid ATSB and to 39% together with chlorfenapyr ATSB (Furnival-Adams et al., 2020).

1.5.1.3 Sterile male technique

Population suppression can be mediated by releasing male mosquitoes that have been made sterile. The technique involves mass rearing, radiation mediated sterilization and release of a large number of male insects carrying a dominant lethal gene (RIDL) (Klassen et al., 2021). Upon mating with the female wild population and thus out-competing non-sterile wild males, the inseminated females

will not be able to produce offspring. This technique is a species-specific and environmentally friendly method for insect population suppression. This strategy is self-limiting because repeated releases are necessary to keep the lethal gene acting in the target population (Wilke and Marrelli, 2012).

1.5.1.4 Other vector control tools

Treatment of livestock: This approach is based on the treatment of animals using Ivermectin and is appropriate for zoophagic mosquitoes. The drug is used to treat endoparasites both in human and animal, and also ectoparasites in animals (Chaccour et al., 2018; Naz et al., 2013). Ivermectin is a new vector control approach to reduce malaria transmission. It kills the mosquito when she feeds on treated hosts. The drug is ingested with the bloodmeal and binds to the glutamate-gated chloride channel of the nerve system and muscle of the mosquito and paralyzes it. The treatment can be given in injectable veterinary formulation and oral formulation (Ivermectin Roadmappers, 2020). Effects has been observed in *An. arabiensis* fed on cattle treated with Ivermectin up to forty weeks (Chaccour et al., 2018).

Individual bite protection methods: This method includes insect repellents and protective clothing that can provide protection to mosquito-vectored diseases in combination with established control programs. There are many simple mosquito biting personal protective measures, both mechanical and chemical. The mechanical measures include wearing long sleeved shirts, long trousers and covered shoes, especially at dusk and dawn when mosquitoes are active. The chemical measures include repellents applied to the exposed skin and chemicals applied to clothes and mosquito coils (Maia et al., 2018).

1.5.2 Treatment

1.5.2.1 Artemisinin combination therapies (ACTs)

Early diagnosis and treatment with highly effective anti-malaria drug regimens remains crucial to control malaria. Artemisinin-based combination treatments (ACTs) are the best first- and second-line treatments for uncomplicated *P. falciparum* malaria infections as well as chloroquine resistant

P. vivax infections across malaria endemic countries. Artemisinin-based combination treatments combine an artemisinin derivative with a partner drug. The role of the artemisinin compound is to reduce the number of parasites during the first three days of treatment, while the role of the partner drug is to eliminate the remaining parasites (cure) (WHO, 2018). Access to effective treatment with WHO-recommended ACTs or chloroquine (where still efficacious) for *P. falciparum* and ACTs plus primaquine for *P. vivax* is essential in controlling malaria (WHO, 2018). ACTs have a good tolerability and a high crude drug efficacy against *P. falciparum* (Shibeshi et al., 2021) both in endemic (Ippolito et al., 2021) and non-endemic countries (Pousibet-Puerto et al., 2016). In several studies in different malaria endemic areas, death and complicated malaria have not been observed among cases treated with ACTs. A review (Adam et al., 2018) demonstrated a considerably high (98%) treatment success with malaria patients treated with artemether +lumefantrine compared to patients treated with artesunate + sulfadoxine–pyrimethamine.

1.5.2.2 Intermittent preventive treatment in pregnancy

In 2020, there were 33.8 million pregnancies in the WHO high malaria transmission African area, of which 11.6 million were exposed to malaria (WHO, 2021). Malaria infection during pregnancy has significant risks for both the mother and fetus/infant (Bardaji et al., 2011; Moya-Alvarez et al., 2014). Intermittent preventive treatment of malaria in pregnancy is a full therapeutic course of an antimalarial given to pregnant women at routine antenatal care visits, regardless whether the woman is infected with malaria or not (WHO, 2020). Intermittent preventive treatment of malaria in pregnancy reduces maternal malaria incidence and other complications of malaria infections including maternal and fetal anemia, placental parasitemia, low birth weight and neonatal mortality (Bardaj et al., 2011; Menendez et al., 2007; WHO, 2020;). The world health organisation recommends IPTP using the antimalarial drug sulfadoxine-pyrimethamine (SP) to protect women in Africa living in moderate and high malaria transmission areas. In 2018, among 36 African countries that reported on IPTP coverage levels, an estimated 31% of eligible pregnant women received the recommended doses of IPTP, compared with 22% in 2017 and 2% in 2010 (WHO, 2019).

1.5.3 Malaria vaccines

There are three types of malaria vaccine candidates that have been intensively investigated targeting the different malaria parasite developmental stages. These vaccines are categorized into three groups: pre-erythrocytic, blood-stage and transmission-blocking vaccines. To date, only RTS,S/AS01 is tested at a phase III clinical trial level. The vaccine is a pre-erythrocytic vaccine and based on the circumsporozoite protein (CSP) of sporozoites. The trial has been conducted in 7 sub-Saharan African countries including Burkina Faso, Malawi, Ghana, Kenya, Tanzania, Gabon and Mozambique. In the trial, 15,459 subjects (8,922 infants and 6,537 young children) have been enrolled. For the children that received 3 to 4 doses of the vaccine followed by a booster at 20 months of age, the vaccine confers partial protection against malaria among children aged 5 to 17 months. The results of the trial show a reduction of 39% in the incidence of malaria cases and 29% in the incidence of severe malaria (Murray and Chambers, 2015). However, the vaccine efficacy against clinical malaria was under the standard of 75% reduction set by the WHO (Murray and Chambers, 2015). The protection also declines rapidly after vaccination. This vaccine is now being rolled out in a pilot implementation program in three countries: Malawi, Ghana and Kenya (WHO, 2020a). Based on the results of this pilot program the RTS,S vaccine might be recommended by WHO for children in sub-Saharan African countries and regions with high and moderate *P. falciparum* malaria transmission (WHO, 2021).

1.5.4 Novel vector control methods to block *Plasmodium* transmission

1.5.4.1 Genetic vector control methods

Genetic vector control methods are promising alternative methods to achieve malaria control and elimination. Mosquito transgenesis and paratransgenesis are two novel genetic malaria control strategies in which the vectors are made incompetent to sustain *Plasmodium* development and thus transmission (Wang and Jacobs-Lorena, 2013).

1.5.4.1.1 Transgenesis: engineering the mosquito to block transmission

Mosquito transgenesis involves direct genetic engineering of the mosquito for delivery of anti-*Plasmodium* effector molecules. It can be done by releasing genetically modified mosquitoes that

express a killing or disabling agent for the malaria parasites (Knols et al., 2007; Zieler et al., 2001). When these mosquitoes are released into the area of interest the transgene is introduced into the target population which results in the interruption of malaria transmission. This is a self-sustaining approach which needs only one or some additional releases for the exogenous gene to increase its frequency and to be fixed in the target population (Jasinskiene et al., 2007) using gene drive technology (see Chapter 5). *An. stephensi*, for instance, is genetically modified to express a small peptide, known as salivary gland and midgut peptide 1 (SM1), resulting in blocking around 80% of the oocyst development (Ito et al., 2002). Similar effects have been observed in other mosquito species.

1.5.4.1.2 Paratransgenesis: engineering microbiota of the mosquito

Paratransgenesis reduces the VC using genetically modified symbiotic organisms. In this approach symbiotic organisms are genetically transformed to express anti- pathogenic effector molecules (Wang and Jacobs-Lorena, 2013). Next, the transformed organisms are reintroduced into the mosquito, where they express effector molecules that block pathogen development and transmission. Engineered microorganisms including bacteria (Riehe et al., 2007) and viruses (Ren et al., 2008) have been used to express refractory genes in the mosquito (Wang et al., 2012). For example, *Escherichia coli* in *An. stephensi* mosquitoes hampered oocyst formation (Yoshida et al., 2001) and inhibited *P. berghei* development in the same vector host (Riehle et al., 2007).

Wolbachia, a vertically transmitted endosymbiotic bacteria known to infect arthropods, has also caused resistance to pathogens through a mechanism called cytoplasmic incompatibility (CI) (Hurst et al., 2000). Cytoplasmic incompatibility results in the generation of unviable offspring when a non-infected female mates with a *Wolbachia*-infected male while eggs of infected females are viable. This allows the spread of *Wolbachia* through the population by giving infected females a reproductive advantage. It is common in the *Anopheles* vector of Africa (Gomes and Barillas-Mury, 2018) including *An. coluzzii*, *An. gambiae* s.s., *An. arabiensis*, *An. moucheti* species (Jeffries et al., 2018).

1.5.4.2 Transmission-blocking vaccines (TBVs)

Transmission blocking vaccines target the sexual stage of the malaria parasite in the midgut (Laurens, 2018) and thus block the transmission to the human host by preventing mosquito infection. There are two groups of TBV antigens known as pre-fertilization and post-fertilization antigens. The pre-fertilization antigens are expressed on the surface of gametocytes and gametes and include Pfs48/45, Pfs47 and Pfs230. The post-fertilization antigens are expressed on the surface of gametocytes, zygotes, and ookinetes and include Pfs25 and Pfs28. These antigens are all targets for candidate vaccines. PfHAP2 is another recently known target of interest expressed on the male and female gametocytes (Nunes et al., 2014).

Transmission-blocking vaccines block transmission through a mechanism of antibody mediated neutralization of the function of the receptor genes (surface antigens). This can be accomplished by either 1) complement (TEP) mediated lysis of gametes or ookinetes, 2) agglutination of gametes or 3) phagocytosis. When the *Anopheles* vector feeds on the vaccinated and infected human host, the antibody together with the parasites is ingested. In the mosquito midgut these antibodies block the fertilization either by directly binding to the surface antigens on gametocytes/gametes (Pf230, Pf48/45) or by blocking the attachment of ookinetes (Pf25) on the gut wall. To date, there are three transmission blocking monoclonal antibodies: 45.1 (α -Pfs48/45), 2A2 (α -Pfs230), and 4B7 (α -Pfs25). Monoclonal antibodies 45.1 and 2A2 are the most potent and 4B7 currently forms the most advanced TBV.

1.6 Challenges of the current malaria control tools

1.6.1 Insecticide resistance

The effectiveness of insecticide-based vector control is hampered by the emergence of insecticide resistance of the malaria vector. Resistance has occurred in the major malaria transmitting vectors (Munywoki et al., 2021; Nardini et al., 2017; Riveron et al., 2015). Resistance has been observed for the 4 commonly used insecticide classes including pyrethroids, organochlorines, carbamates and organophosphates (WHO, 2020). According to a recent WHO report, insecticide resistance has been detected in 78 malaria endemic countries to at least 1 of the 4 insecticide classes, and in

19 countries resistance for the four classes has been found in at least one site and one local vector (WHO, 2021).

Furthermore, in some endemic areas, insecticide resistance in *An. arabiensis* is associated with agricultural usage of organophosphate, carbamates (Abuemaali et al., 2013) and pyrethroids (Orondo et al., 2021). Simultaneous application of similar chemicals for pesticide and insecticide purposes results in insecticide resistance in malaria vectors (Reid and McKenzie, 2016). Agricultural pesticides exert strong selection pressure which contributes to vector resistance (Reid and McKenzie, 2016; Yadouleton et al., 2009).

In Ethiopia wide spread insecticide resistance has been reported for the major malaria vectors (Mekuriaw et al., 2020). This might be due to the long use of insecticides by the national malaria control program for IRS since the 1950s and intensive use of IRS and LLINs since 2005 (Abate and Hadis, 2011; Yewhalaw et al., 2012). Wide spread insecticide resistance has also been reported in *An. arabiensis* populations in different parts of the country (Abate and Hadis, 2011; Yewhalaw et al., 2012). DDT resistance was first detected in 1986, and from 2005 to 2015 the trend of resistance widely increased in most parts of the country (Mekuriaw et al., 2020). *Anopheles arabiensis* has developed resistance to all classes of insecticides: organochloride (Abate and Hadis, 2011; Balkew et al., 2012), pyrethroids (Asale et al., 2014; Fettene et al., 2013; Massebo et al., 2013), carbamates (Asale et al., 2014; Massebo et al., 2013) and organophosphate (Massebo et al., 2013). In Ethiopia different types of insecticide resistance mechanisms have been reported. Knock down resistance is associated with a mutation in the voltage gated sodium channel. Knock down resistance involves substitution of leucine to phenylalanine (L1014F) or substitution of leucine to serine (L1014S). Knock down resistance was reported for DDT and deltamethrin (Alemayehu et al., 2017). Metabolic resistance, in which insects produce enzymes that detoxify the insecticides, is also reported. This mechanism is due to a modification in the cuticle or digestive tract lining that prevents absorption. Cuticular and P450 detoxification have been reported for deltamethrin, DDT (Simma et al., 2019) and pyrethroids (Simma et al., 2018).

1.6.2 Behavioral resistance

Behavioral resistance of malaria vectors is one of the main challenges for the malaria control and elimination agenda, and is the cause of residual malaria transmission. Behavioral resistance is any modification to mosquito behavior that facilitates an avoidance of insecticides following the intensive application of insecticide-based malaria control. Some mosquito vectors, e.g., *An. arabiensis*, adjust (shift) their behavior and exhibit behavioral plasticity to avoid contact with insecticides (Killeen et al., 2016). The most common behavioral changes include: (1) early exit to avoid contact with treated surface, mosquitoes are being active earlier in the evening before people have gone to sleep and run after feeding, (2) feeding on humans when they are active and unprotected outdoors, (3) increase feeding on animals to minimize contact with insecticide treated humans or houses and (4) feeding and resting outdoor away from insecticide treated nets, wall and roof (Killeen, 2014; Killeen et al., 2016).

Such resilient behavior of malaria vectors contributes to persistent transmission of the disease even when using a scale-up of appropriate vector control tools (Killeen et al., 2016; Loha et al., 2019). In areas where most humans use bed nets, two thirds of blood feeding and half of malaria transmission events of *An. arabiensis* has been estimated to have occurred outdoors (Killeen et al., 2016). This study has also shown that more than 98% of outdoor transmission by resilient *An. arabiensis* has been preceded by unsuccessful indoor biting of a human. This unsuccessful indoor biting is attenuated by the availability of alternative hosts or the ability to attack humans outdoors. Across Africa, an estimated 10.6 million additional malaria cases related with outdoor transmission have been predicted (Sherrard-Smith et al., 2019). Together with the above factors, these exophagic and exophilic vectors are not well addressed by the currently widely used vector control tools, LLINs and IRS (Bayoh et al., 2010; Kitau et al., 2012; Sougoufara et al., 2020). In Ethiopia, *An. arabiensis* exhibits blood feeding with a high preference for livestock (Eba et al., 2021; Massebo et al., 2013; Massebo et al., 2015) and engages mainly outdoor although they contribute to both indoor and outdoor malaria transmission (Degefa et al., 2021). Residual malaria transmission has been reported (Abraham et al., 2017; Zemene et al., 2018), indicating *An. arabiensis* is one of the major challenges for malaria control in Ethiopia. Moreover, the above

findings have an implication on the development of complementary vector tools to address vectors that contribute to residual malaria transmission.

1.6.3 *Plasmodium* resistance to effective drugs

Improved access to effective malaria treatment is a key contributing factor to the significant reduction in the malaria burden in the last few decades. However, the emergence of resistance to antimalarial medicines is a threat to the global efforts to control and eliminate malaria. Artemisinin resistance in *P. falciparum* has emerged and the local spread of artemisinin resistant parasites has been observed in Africa including Rwanda, Uganda and elsewhere in the Horn of Africa (WHO, 2021). A recent study in Uganda by Balikagala et al. (2021) shows that 14 of 240 patients are infected with artemisinin resistant *P. falciparum* parasites.

1.6.4 Asymptomatic malaria

Asymptomatic malaria is a silent threat and challenge for an effective malaria control and elimination program (Hassanpour et al., 2017). Asymptomatic individuals serve as a reservoir host and contribute to parasite transmission (Lidblade et al., 2013; Lin et al., 2014). Community based surveys have demonstrated that a significant number of asymptomatic malaria carriers reside in the community. For instance, in asymptomatic children under 5 years old 33% carried the gametocyte stage of *P. falciparum* in Kenya (Bousema et al., 2004). A significantly higher number of asymptomatic malaria carriers has been found among the Amazonian population in Brazil for both *P. vivax* and *P. falciparum*. Asymptomatic malaria carriers occur 4 to 5 times more frequently than symptomatic ones (Alves et al., 2005).

1.7 Mosquito immunity against the *Plasmodium* parasite

One of the key factors that determine the VC is the mosquito's immune response. There is a remarkable loss of malaria parasites in the midgut of *Anopheles* mosquitoes during the parasite sporogony. In each developmental stage of this cycle, there is a significant reduction in the number of parasites (Sinden, 1999; Sinden and Billingsley, 2001; Whitten et al., 2006). Studies have shown that a female *Anopheles* mosquito normally ingests thousands of gametocytes in a blood

meal. The volume of the blood meal ingested by the female mosquito is 1-2 micro liters (Sinden, 1999). Of these thousands of gametocytes only 50-100 develop into ookinetes and about five survive to form oocysts (Gouagna et al., 1998; Sinden, 1999; Sinden and Billingsley, 2001). This loss of the parasites is due to the exposure of the parasite to different mosquito and human anti-parasite factors including cytokines, white blood cells and mosquito's immune defence, gut microbiota and digestive enzymes (Whitten et al., 2006).

1.7.1 Innate immune system

The innate immune system of mosquitoes is the main immune defense mechanism against parasites and other microbes including bacteria, viruses and fungi (Cirimotich, 2010; Meister 2004). There are three major immune signaling pathways in the defence against *Plasmodium* parasites: Toll, Immune deficiency (IMd), and Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathways. The Toll pathway is elicited by gram-positive bacteria and has also been implicated in the defence against the rodent malaria parasite (Cirimotich et al., 2010; Frolet et al., 2006). The IMd pathway is elicited by both gram-negative and gram-positive bacteria and has also been implicated in modulating human *Plasmodium* parasite (Cirimotich et al., 2010; Meister et al., 2005).

The IMd pathway is responsible for the basal level immune response against *P. falciparum*. It is activated when *PGRP-LC* recognizes the cell wall component peptidoglycan of the bacteria. This pathway is regulated by NF-kappaB (*NF-kB*) relishing family transcription factors Rel2 (Dong et al., 2009; Frolet et al., 2006). Rel2 exists in two forms, Rel2-F and Rel2-S. Rel2 F is a full-length form that includes the carboxyl-terminal ankyrin, ANK, and death domains, that is inactive until immune activation occurs. Rel2-S is a short form lacking the inhibitory Ankyrin domain that is constitutively active to regulate basal immune genes expression (Meister et al., 2005).

The activation of the IMd pathway results in the cleavage of the carboxyl-terminal ankyrin end of Rel2-F and the translocation of active Rel2-S into the nucleus where it regulates the transcription of immune genes, Anti-Microbial Peptides (AMPs) including *Defensins*, *Cecropins*, *Attacin* and *Gambicin*. These AMPs act against gram-negative and gram-positive bacteria and the *Plasmodium*

parasite *Gambicin* is upregulated in *Plasmodium* infection (Figure 1.5) (Luna et al., 2006; Meister et al., 2005).

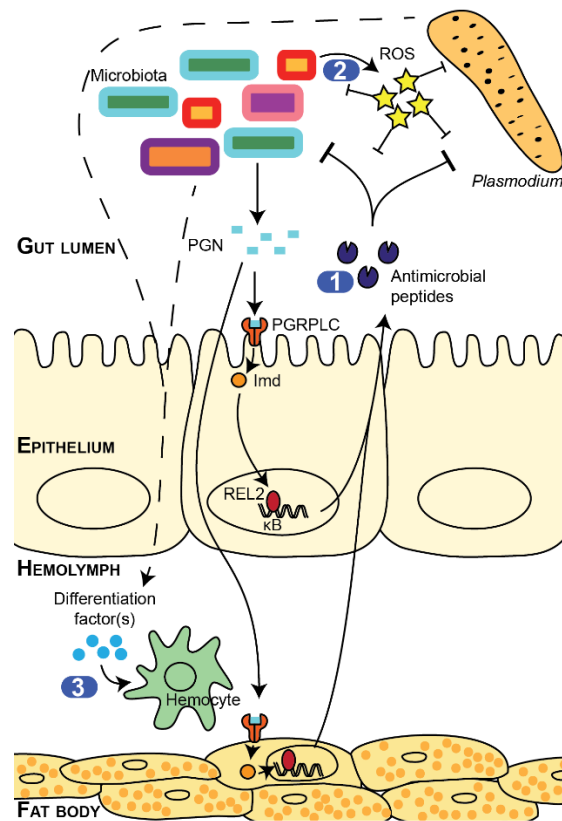


Figure 1.5. Mechanisms of colonization resistance conferred by *Anopheles* microbiota against *Plasmodium* infection: (1) direct effect via synthesis of ROS by the *Enterobacter* EspZ strain, (2) indirect effect via induction of NF- κ B antibacterial responses that have antiparasitic effects, which is likely to be the most general mechanism and (3) induction of hemocyte differentiation by unknown soluble hemolymph factors during *Plasmodium* infection, which has a priming effect against a subsequent *Plasmodium* infection (Gendrin and Christophides, 2012).

1.7.2 Role of midgut microbiota in the Imd pathway

The midgut of the mosquito harbors mainly gram-negative bacteria belonging to the *Enterobacteriaceae*. They are essential for the activation of the basal level immunity (Frolet et al., 2006) and preventing the development of the *Plasmodium* parasite (Dong et al, 2009; Meister et al., 2009). The midgut microbiota exert anti-*Plasmodium* effects either directly through synthesis

of ROS (reactive oxygen species) (Molina-Cruz et al., 2008) or indirectly through activation of the IMD pathway (Molina-Cruz et al., 2008; Romoli and Gendrin, 2018).

Studies have shown that following the ingestion of a bloodmeal the number of mosquito mid gut bacteria increases (Meister et al., 2009) with a concomitant increase in the release of bacterial immune elicitors such as peptidoglycans which further triggers an immune response in the mosquitoes. The IMD pathway is then activated through recognition of the bacteria by receptors (*PGRPLC* and *PGRPLA*) which leads to the production of antimicrobial peptides and other effectors against both bacteria and the parasite, and reduces the number of proliferated bacteria to the basal level and maintain the gut homeostasis (Dong et al., 2009; Gendrin and Christophides, 2013).

When the immune system is hampered through silencing these immune receptor genes, the mosquito's lifespan shortens. When bacterial receptors of this pathway (*PGRPLC* and *PGRPLA*) are silenced, gut homeostasis is disrupted and mosquitoes die prematurely either due to opportunistic infections or altered physiology (Meister et al., 2009). A recent study in a laboratory system has confirmed that immune suppression in *An. gambiae* by depletion of immune related genes or proteins including, FN3Ds (including *FN3D1*, *FN3D2*, *FN3D3*) and the gustatory receptor *GPRGr9* significantly compromised the mosquito gut homeostasis (Stathopoulos et al., 2014). In the present study, we investigated the effect of silencing some of the immune related genes involved in gut homeostasis on longevity on field *An. arabiensis* populations using RNAi interference techniques.

1.7.3 Immune genes involved in gut homeostasis

About 242 genes belonging to 18 gene families are implicated to have a role in the innate immune function in *An. gambiae* (Christophides et al., 2002). There are different immune genes that are involved in recognition, modulation, signal transduction and effectors. Recognition of a pathogen associated molecular pattern by PGRPs activate immune signaling path way and proteolytic cleavage death domain results in the production of effectors. PGRPs are among the best studied Pattern Recognition Receptor (PRR) genes in insects. In addition, the FN3D family have a significant role to maintain the gut homeostasis. RNAi mediated gene silencing studies

demonstrated that three type III fibronectins domain proteins (*FN3Ds* including *FN3D1*, *FN3D2* and *FN3D3*) and gustatory receptor *GPRGr9* modulate the gut microbiota homeostasis in *An. gambiae* (Stapholus et al., 2014). Antimicrobial peptides are the hall mark of insect humoral immune response effector immune genes. Four families of AMPs have been identified in *Anopheles* including *Defensins*, *Cecropins*, *Attacin* and *Gambicin*. Defensin 1 and Cecropin 1 are most active against gram-positive bacteria and yeast. In addition, the mosquito *Defensins* are active against fungi. Gambicin 1 has a broad antibacterial (both gram-negative and gram-positive) effect. It is also upregulated against *Plasmodium* parasites. In the present study immune related genes involved in recognition were screened and their effect assessed on longevity of mosquitoes.

1.7.3.1 Peptidoglycan recognition proteins

Peptidoglycan recognition proteins in *Anopheles* mosquitoes belong to the Pattern Recognition Receptor family and are responsible for the activation of the insect immune system including the melanization cascade, phagocytosis and signal transduction pathways for production of antibacterial effectors (Christophides et al., 2002). *Anopheles gambiae* encodes seven *PGRPs* isoforms (*PGRPS1*, *PGRPS2*, *PGRPS3*, *PGRPLA*, *PGRPLB*, *PGRPLC* and *PGRPLD*) of which *PGRPLC* and *PGRPLA* are responsible for the activation of the IMd pathway. (Christophides et al., 2002).

A further study by Meister et al. (2009) showed the role of *PGRPLC* in mosquito infections with bacteria and malaria parasites. Silencing *PGRPLC* had a large impact on mosquito survival related with the dramatic proliferation of the midgut microbiota after ingestion of a bloodmeal. *PGRPLC* encompasses three domains, i.e., *PGRPLC1*, *PGRPLC2* and *PGRPLC3*. Microarray analysis demonstrated that the *Anopheles PGRPLC3* is upregulated only by bacteria (Christophides et al., 2002). A further study on the contribution of each of the three isoforms showed that *PGRPLC3* is the most important of the three main isoforms in the response against bacterial infections particularly with *Escherichia coli* or *Staphylococcus aureus* (Meister et al., 2009). Moreover, it modulates *Plasmodium* infection too.

1.7.3.2 Type III fibronectins (*FN3D1*, *FN3D2* and *FN3D3*)

FN3Ds are family of the PRRs. They have an antibacterial effect and are involved in modulating the bacterial load and composition of the mosquito (Christophides et al., 2002). Stathopoulos et al. (2014) investigated the genetic basis of a mosquito infection with a gram-negative gut bacterium commonly found in laboratory reared *An. gambiae* populations through the combination of a genome wide association study and SNP genotyping arrays. They identified three type III fibronectins (*FN3D1*, *FN3D2* and *FN3D3*) that modulate homeostasis of the gut microbiota mainly against *Enterobacteriaceae*. A RNAi mediated gene silencing study showed the role of the *FN3D* genes in shaping the outcome of *An. gambiae* and *Serratia marcescens* infections and in modulating the proliferation of midgut bacteria. Even though the mechanism of action of the type III fibronectins is not yet fully understood, it can be deduced from homology with other molecules that the FN3Ds have antibacterial effect and are involved in modulating the bacterial load and composition of the mosquito. For instance, FN3D2 is an ortholog of *Drosophila* Dscam4, shown to bind bacteria and influence the efficiency of phagocytosis. It has been observed that silencing these genes increased the *Serratia* load and altered the gut microbiota composition in favor of *Enterobacteriaceae*.

1.7.3.3 Gustatory receptors

Gustatory receptors (for example *GPRGr9*) genes have been involved in modulating feeding behavior by acting as nutrient sensors and antibacterial response through recognition of bacteria-derived metabolites. Behavioral responses, following *S. marcescens* infection, can modulate the bacterial load (Stathopoulos et al., 2014). The above authors showed the role of these immune genes to regulate the gut homeostasis in *An. gambiae* mosquitoes in laboratory colonies.

In the previous study, it has been confirmed that immune suppression in *An. gambiae* by depletion of immune related genes or proteins including *PGRPLC*, *FN3Ds* (including *FN3D1*, *FN3D2*, *FN3D3*) and the gustatory receptor *GPRGr9* significantly compromised and disrupted the mosquito gut homeostasis (Meister et al., 2009; Stathopoulos et al., 2014). In the present study, we investigated the effect of silencing some of the immune related genes on longevity on field *An. arabiensis* populations by RNAi mediated gene interference technique (Stathopoulos et al., 2014).

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Chapter 2 Objectives

Malaria is a major threat to public health and economic development in Africa. Malaria has a significant impact on the health of infants, young children, and pregnant women worldwide. In the last few decades, malaria incidence has decreased substantially mainly due to efficient vector control tools, but this positive evolution has stalled the last few years and complete eradication and elimination does not seem to be attainable in the near future.

The reasons why the malaria elimination programs book less success are many and diverse. On the one hand, there are reports on the emergence of drug resistance against artemisinin-based combination therapies, the recommended first-line treatment of *P. falciparum* malaria in all countries with endemic disease (WHO, 2021). Rapid case diagnostics and treatment might thus be less efficient in reducing the pool of infectious persons.

On the other hand, malaria vector control tools also have lost part of their efficacy. Insecticide resistance of the vector is quickly evolving. Behavioral resistance against insecticides has also developed in some vector species. Furthermore, a shift in malaria vectors has been observed in Africa where the endophagic/endophilic species such as *An. gambiae* and *An. funestus* are being replaced by the exophagic/exophilic species *An. arabiensis* (Kitau et al., 2012; Mwangani et al., 2013; Russell et al., 2010). This vector is less susceptible to ITN and IRS implementation.

Thus, development of new tools (drugs, insecticides and vaccines) and strategies is urgently needed in order to combat malaria and the above malaria eradication challenges. The aim of the present study was to lay the foundations for the development of new tools to disrupt mosquito gut-homeostasis to reduce the life span of vector mosquitoes to that extent that the transmission cycle of malaria can be broken.

A first objective of this PhD thesis consists of the screening of different candidate genes involved in the regulation of gut-bacterial homeostasis of the *An. arabiensis* mosquito and assess their importance for survival. Candidate genes are silenced in the mosquito and the effect on survival is assessed. This is based on a study with a naturally occurring *An. arabiensis* population of southwest Ethiopia. This topic is presented in Chapter 3.

A second objective is to generalise the results found in Chapter 3 to different *An. arabiensis* populations bred in different circumstances. It is essential to investigate whether the gene silencing effect on survival is consistent over different practical situations before embarking on the development of a new anti-mosquito tool targeting gut-homeostasis. This topic is presented in Chapter 4.

A third objective is to critically assess different potential tools for disrupting gut-homeostasis in *An.arabiensis* populations but also other *Anopheles* populations or even other arthropod disease vectors such as sandflies. These topics are touched upon in the discussion section.

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Chapter 3 The effect of silencing immunity related genes on longevity in a naturally occurring *Anopheles arabiensis* mosquito population from southwest Ethiopia

Adapted from: Debalke S, Habtewold T, Duchateau L, Christophides GK. The effect of silencing immunity related genes on longevity in a naturally occurring *Anopheles arabiensis* mosquito population from southwest Ethiopia. Parasit Vectors. 2019;12:174.

3.1 Abstract

Background: Vector control remains the most important tool to prevent malaria transmission. However, it is now severely constrained by the appearance of physiological and behavioral insecticide resistance. Therefore, the development of new vector control tools is warranted. Such tools could include immunization of blood hosts of vector mosquitoes with mosquito proteins involved in midgut homeostasis (anti-mosquito vaccines) or genetic engineering of mosquitoes that can drive population-wide knockout of genes producing such proteins to reduce mosquito lifespan and malaria transmission probability.

Methods: To achieve this, candidate genes related to midgut homeostasis regulation need to be assessed for their effect on mosquito survival. Here, different such candidate genes were silenced through dsRNA injection in the naturally occurring *Anopheles arabiensis* mosquitoes and the effect on mosquito survival was evaluated.

Results: Significantly higher mortality rates were observed in the mosquitoes silenced for *FN3D1*, *FN3D3* and *GPRGr9* genes as compared to the control group injected with dsRNA against a non-related bacterial gene (*LacZ*). This observed difference in mortality rate between the candidate genes and the control disappeared when gene-silenced mosquitoes were treated with antibiotic mixtures, suggesting that gut microbiota play a key role in the observed reduction of mosquito survival.

Conclusions: We demonstrated that interference with the expression of the *FN3D1*, *FN3D3* or *GPRGr9* genes causes a significant reduction of the longevity of *An. arabiensis* mosquito in the wild.

Keywords: *Anopheles arabiensis*, Longevity, Gene silencing, Microbiota, Gut homeostasis

3.2 Background

Sub-Saharan Africa hosts some of the most efficient malaria vectors, *An. gambiae*, *An. arabiensis* and *An. funestus*, and carries the heaviest malaria burden worldwide. A substantial reduction in malaria related cases and deaths have been recorded in the past decade (WHO, 2018). This progress is largely attributable to the scaling-up of vector control interventions, such as long-lasting insecticide nets (LLINs) and indoor residual spraying (IRS), as well as improved diagnostics and effective treatment using artemisinin-based combination therapies (MacDonald, 1957).

LLINs and IRS impact malaria transmission largely by reducing the daily survival rate of mosquitoes that are mostly active at night and display strong endophagic (seeking blood meals indoors) and endophilic (rest indoors following a blood meal) behavior (Bayoh et al., 2010; Gratz, 1985). These tools have been more efficient for the endophagic/endophilic *An. gambiae* and *An. funestus* mosquitoes compared to the exophagic/exophilic *An. arabiensis* mosquito (Kitau et al., 2012; Eckhof et al., 2017; Russell et al., 2011; Ferguson et al., 2010; Sokol et al., 2015). In addition, field studies have reported evidence of behavioral adaptation of *An. arabiensis* to the LLINs and/ or ITNs as mosquitoes feed earlier and more frequently outdoors, and rest more frequently outdoors (Russell et al., 2011; Mwangangi et al., 2013; Yohannes and Boelee, 2012).

Due at least in part to this difference in vector control efficacy, a marked shift in vector composition has been observed. In east African countries where these vectors coexist, *An. arabiensis* is gradually replacing *An. gambiae* and *An. funestus*, e.g., in Kenya (Derua et al., 2015; Mutuku et al., 2011; Zhou et al., 2011) and Tanzania (Russell et al., 2011; Trape et al., 2011). Although *An. arabiensis* is known to be a less efficient vector compared to *An. gambiae* and *An. funestus*, the inherent resilience of the mosquito to LLINs and IRS has been linked to reports of resurgence or stagnation in rates of malaria cases and deaths in African countries (Zhou et al., 2011). Therefore, there is a need to develop new complementary vector control technologies targeting vectors that are resilient to the current vector control tools.

Malaria transmission blocking vaccines and mosquito population replacement via genetic modification have recently become attractive technologies to complement the existing vector control interventions (Eckhof et al., 2017; Alphey et al., 2002; Deredec et al., 2011; Burt, 2003; James, 2005; Gantz et al., 2015). Another novel approach is to reduce the mosquito lifespan by introducing a lethal gene or a pathogen in the mosquito population (Ricci et al., 2012; Pumpuni et al., 1996). Along the same lines, the mosquito lifespan could be reduced by immunizing primary mosquito blood hosts (i.e., humans and domestic bovids) with mosquito proteins involved in the midgut homeostasis. For this approach, candidate genes need to be found, which is the topic of this paper. Different *An. arabiensis* genes related to midgut homeostasis were screened for their potential to reduce mosquito longevity. Our work was prompted by data showing that shortly after a blood meal the number of microbiota in the mosquito midgut increases drastically, up to 1000 times (Pumpuni et al., 1996; Kumar et al., 2010; de O Gaio et al., 2011), which in normal circumstances triggers immune reactions that soon reduce the microbiota number to the basal level (Gendrin and Christophides, 2013; Meister et al., 2009; Cirimotich et al., 2011; Rodrigues et al., 2010; Dong et al., 2009). We hypothesized that by compromising the immune system the mosquito would no longer be able to control the microbiota in the gut, which would lead to a shorter lifespan. Previous studies have demonstrated in a laboratory colony of *An. gambiae*, that when genes encoding putative bacterial receptors such as *PGR-PLC*, type III fibronectin domain proteins (*FN3Ds* including *FN3D1*, *FN3D2*, *FN3D3*) and the gustatory receptor *GPRGr9* were silenced, gut homeostasis was disrupted (Meister et al., 2009; Stathopoulos et al., 2014). Here, we investigated the effect of silencing some of these genes by RNA interference on longevity in naturally occurring *An. arabiensis* populations.

3.3 Methods

3.3.1 Mosquitoes

Adult *An. arabiensis* mosquitoes were reared from larvae and pupae collected from natural breeding sites around Jimma, southwest Ethiopia (07°40'00"N, 36°50'00"E). Larvae and/or pupae were collected using a 350 mL mosquito dipper following the standard larvae collection procedure (WHO, 1975; Gerberg, 1970). The collected larvae and pupae were transported to an on-site mud-

house where the pupae were separated from the larvae and transferred into a 10 mL beaker with water and kept in a mosquito cage with a dimension of 24.5×24.5×24.5 cm³ (Bugdorm-41515; Watkins & Doncaster, Leominster, UK) until emergence. The remaining larvae were transferred to a plastic tray containing water obtained from their natural habitat and fed on yeast and tropical fish food. The water was changed every 2 days and pupae were collected daily and transferred to the adult cage. Emerged adult mosquitoes were maintained on 10% sugar solution. Adult females of zero to two day old were transported to the experimental insectary at Jimma University for double stranded RNA (dsRNA) injection.

3.3.2 Gene silencing

Total RNA was extracted from ten field-collected and laboratory reared *An. arabiensis* mosquitoes using TRIzol (Invitrogen, Inchinnan, UK) and cleaned with Turbo DNase I (Ambion, Huntington, UK). Complementary DNA (cDNA) was synthesized by reverse transcribing 1 µg of the total RNA using a Prime-Script™ 1st-strand cDNA Synthesis Kit (TaKaRa, Kusatsu, Shanghai). Fragments of the five target genes were amplified by PCR using specific gene primers tailed with the short T7 promoter sequence TAA TAC GAC TCA CTA TAG G and the cDNA as a template. The targeted genes were *FN3D1*, *FN3D2*, *FN3D3*, *GPRGR9* and *PGRPLC3*. The PCR fragment for the *LacZ* gene that served as a control was synthesized using a plasmid template containing the *LacZ* gene (for full primer sequences see Table 3.1). DsRNA was synthesized from purified PCR products using the TranscriptAid T7 High Yield Transcription Kit (Termo Fisher Scientific, Waltham, USA). The dsRNA was then purified using a RNeasy Mini Kit (Qiagen, Manchester, UK), following the manufacturer's protocol. The concentration of dsRNA was determined spectrophotometrically by a Nanodrop 1000 spectrometer (Termo Fisher Scientific, Wilmington, USA) at 260 nm and adjusted to 3 µg/µl using ultra-pure water. Gel electrophoresis (1% TBE agarose) was performed on a sub-sample of the PCR products to confirm that products of the expected size were detected for each gene. Zero- to two-days-old *An. arabiensis* mosquitoes were injected with 69 nl of dsRNA specific to a target gene or the *LacZ* control gene following a published RNA interference technique (Habtewold et al., 2016). Gene silencing efficiency was measured for each of the 5 silenced genes using qPCR. Quantification of transcript abundance was performed on cDNA synthesized from total RNA extracted from

mosquitoes injected with dsRNA 3 days earlier and maintained on 10% sugar solution. Fast SYBR® Green Master Mix Real-Time PCR Master Mix (Applied Biosystems, Warrington, UK) was used in the PCR reaction and amplification was detected by a 7500 Fast Real-Time PCR system (Applied Biosystems). Each target gene was quantified in duplicate. The AgS7 gene was used as an internal control. Primer sequences are given in Table 3.1.

Primer Name	Reaction	Sequence
FN3D1 F	T7, dsRNA preparation	<u>taatacgactcactataggggatggacgtggatcagcc</u>
FN3D1 R	T7, dsRNA preparation	<u>taatacgactcactataggggtggatcgctcctcatcactgt</u>
FN3D2 F	T7, dsRNA preparation	<u>taatacgactcactatagggacggccgttttaaagtgtca</u>
FN3D2 R	T7, dsRNA preparation	<u>taatacgactcactatagggcccagcattgtgtacaga</u>
FN3D3 F	T7, dsRNA preparation	<u>taatacgactcactataggggaatcatttccttcgcattcc</u>
FN3D3 R	T7, dsRNA preparation	<u>taatacgactcactatagggacattgtcctgtaccacacca</u>
Gr9 F	T7, dsRNA preparation	<u>taatacgactcactatagggagcaccggcatgcgacatc</u>
Gr9 R	T7, dsRNA preparation	<u>taatacgactcactatagggagctcagctcgtttggcgca</u>
LC3 F	T7, dsRNA preparation	<u>taatacgactcactatagggacgaaatgcatgtatcagg</u>
LC3 R	T7, dsRNA preparation	<u>taatacgactcactatagggctcgctcggttgtggtgtcgttc</u>
LacZ F	T7, dsRNA preparation	<u>taatacgactcactatagggagaatccgacgggttgttact</u>
LacZ R	T7, dsRNA preparation	<u>taatacgactcactatagggcaccacgctcatcgataattt</u>
FN3D1 F	qRT-PCR	ggccgccggtttcatccaca
FN3D1 R	qRT-PCR	ytgcggtccaggtggtgga
FN3D2 F	qRT-PCR	ggtgcgctgacggtgacgg
FN3D2 R	qRT-PCR	tgcgccggaagccggaaat
FN3D3 F	qRT-PCR	cgtgacggccaacgtgacga
FN3D3 R	qRT-PCR	ggtcggaacccaccgactg
Gr9 F	qRT-PCR	cgcttgttctgctgattgt
Gr9 R	qRT-PCR	aacggcacagaatgtttgc
LC3 F	qRT-PCR	gcatggcaaggaacctac
LC3 R	qRT-PCR	tcgtcggttgtggtgtcggttc
AgS7F	qRT-PCR	gtgcgcagttggagaaga
AgS7R	qRT-PCR	atcggtttgggcagaatgc

Table 3.1 Primer sequences used for dsRNA preparation or qRT-PCR. F, forward; R, reverse (Stathopoulos et al., 2014).

3.3.3 Monitoring of mosquito survival

The dsRNA injected mosquitoes were put into their respectively labelled cups. For each gene between 20 and 30 mosquitoes were injected per replicate. Cups with the mosquitoes were placed inside a $2 \times 1 \times 0.75$ m³ (length $\times$ width $\times$ height) microclimate regulatory box constructed from chipboard. It has a window of 25 cm² on each side and the top cover, and was covered with metal mesh to allow airflow (Figure 3.1). The box was placed in a typical rural house. To prevent ant attacks, the box was placed on a 50 cm raised stand that was dipped halfway into water. It was lined with sawdust, 30 cm deep, which was sprinkled daily with water and kept closed.



Figure 3.1 Microclimate regulatory box to contain mosquitoes.

The box maintained a humidity of 60–70% RH and temperature of 25–28 °C throughout the whole study period, hence providing a microclimatic condition that resembled the natural resting habitat of *An. arabiensis* mosquitoes. The mosquitoes were offered a 10% sugar solution daily and a blood meal every 4 days by direct-feeding on a goat. Survival was monitored daily for 25 days, starting 24 h post-injection. Six independent replicates of mosquito injection were performed per gene.

3.3.4 Midgut microbiota analysis

A separate experiment was performed to determine microbiota load in both blood-fed and sugar-fed mosquitoes injected with the specified dsRNA. For each gene, between 20 and 30 mosquitoes were injected and transferred to their corresponding labelled cups. The mosquitoes were then kept in a microclimate regulatory box. On day 4 post-injection, the mosquitoes were either fed on blood or kept on a sugar meal. Twenty-four hours post feeding, 5 blood-fed and 5 sugar-fed mosquitoes were sampled and their midguts were dissected. Individual midguts were homogenized in 100 µl of 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The number of bacteria was quantified using flow cytometry on the 5 pooled midgut samples per replicate (Habtewold et al., 2016). A total of 3 replicates of dsRNA injections were carried out per gene.

3.3.5 Antibiotic treatment

We tested the effect of reducing/eliminating the midgut microbiota load on the survival of the gene knocked down mosquitoes. For this purpose, dsRNA-injected mosquitoes were placed in 6 different cups, each cup containing 20–30 mosquitoes. The cups were kept in the microclimate regulatory box. On the day of dsRNA injection, the mosquitoes were given a cotton ball soaked in an antibiotic cocktail of streptomycin and norfloxacin, both at a dose rate of 10 µg/mL in a 10% sugar solution. On day 4 post-injection, the mosquitoes were blood-fed on a goat. The cotton balls were re-soaked with the antibiotic cocktail every fourth day for a period of 12 days (on days 4, 8 and 12). The antibiotics/sugar feed was carried out in the morning for about 1 h, and then cotton ball was removed from the cups for the next 5 h to starve the mosquitoes. The starved mosquitoes were offered a blood meal and the cotton ball was replaced on the cups after the feed. Mosquito survival was monitored daily for 25 days starting from 24 h post-injection. For this experiment 5 replicates of dsRNA injection were performed per gene.

3.3.6 Data analysis

Data analysis was performed using the statistical software package R v.3.3.2. For the gene silencing efficiency test, the relative expression of mRNA was calculated. The standard curve method was used for real-time qPCR quantification analysis. For each test sample, the PCR cycle number at which the fluorescent intensity of the reaction curve intersects the threshold line, i.e., level of detection or the point at which a reaction reaches a fluorescent intensity above background levels, known as threshold cycle values (Ct-values), was determined for the target and reference gene. Additionally, a standard curve was generated for both the target gene and the reference gene in each assay run using serial dilution of same template. Then, for each sample, the Ct-value of the target or reference gene was standardized using corresponding a standard curve and then the expression level of the target gene was normalized to the reference gene (AgS7 gene). The relative expression of mRNA of a target gene was compared between mosquitoes for which the target gene was silenced, and mosquitoes injected with control *dsLacZ* by a paired t-test using the replicate as a block factor. Gene silencing efficiency was expressed as the ratio of the relative expression of the target gene in the *dsLacZ* injected mosquitoes and the target gene silenced mosquitoes. Survival of the target gene and *dsLacZ* RNA-injected mosquitoes was depicted by Kaplan-Meier survival curves. The effect of silencing the different target genes on survival was modelled by the Cox proportional hazards frailty model, with replicate used as frailty term (Duchateau and Janssen, 2008). The hazard ratio of a target gene over the control *dsLacZ* injection was used as summary statistic, together with the median time to death for the different gene silenced mosquitoes. To investigate the effect of the antibiotics cocktail, the same Cox proportional hazards model was fitted using the hazard ratio again as summary statistic. The bacterial counts were first log-transformed and then compared by a mixed model with replicate as random effect, and treatment, feed and the two-way interaction as fixed effects factor. The F-test was used to compare the silencing of the different target genes and the injection of control *dsLacZ*. The ratios of bacterial counts in the control *dsLacZ* injections and the target gene silencing were used as summary statistics.

3.4 Results

3.4.1 Effect of gene silencing on survival

A significant reduction in expression level of mRNA compared to the dsLacZ-injected mosquitoes was achieved for the targeted candidate genes *FN3D1* ($t = -8.36$, $df=2$, $P=0.007$), *FN3D2* ($t = -7.09$, $df=2$, $P=0.010$), *FN3D3* ($t = -3.82$, $df=2$, $P=0.031$) and *GPRGr9* ($t = -6.60$, $df=2$, $P=0.011$) but not for *PGRPLC3* ($t = -1.42$, $df=2$, $P=0.145$). The data are presented in Figure 3.2.

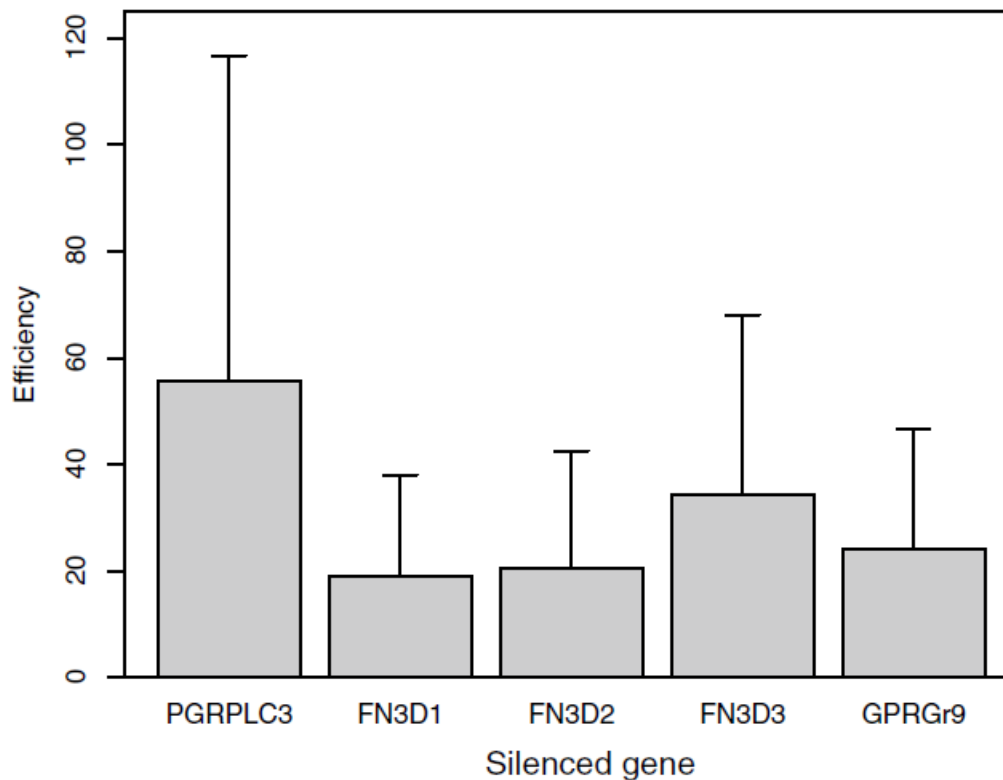


Figure 3.2 The average reduction (vertical line is standard error) in expression level of mRNA for the specific knocked-down gene mosquitoes as compared to LacZ knocked-down mosquitoes ($n = 3$).

Mosquito survival following silencing of the target genes compared to the control *dsLacZ* injected mosquitoes is depicted as a function of time for the each of the above genes in Figure 3.3. Significantly higher mortality rates were observed for the *FN3D1*, *FN3D3* and *GPRGr9* knocked-

down mosquitoes as compared to the control group, but not for the FN3D2 and *GRPLC3* knocked down mosquitoes (Table 3.2). A marked reduction in average time to death was observed in mosquito groups where target genes were silenced compared to dsLacZ-injected controls, e.g., for *GRPGr9* silenced mosquitoes the median time to death was almost halved from 20 (*LacZ*) to 11 days (Table 3.3).

3.4.2 Effect of gene silencing on the midgut bacterial count

The global analysis demonstrated that there was no overall significant difference between the target gene silenced and the dsLacZ-injected mosquitoes with respect to bacterial count ($F(5,24)=1.148$, $P=0.363$). However, there was an overall effect of the feed source ($F(5,24)=20.287$, $P<0.001$), i.e. blood-feeding versus sugar-feeding, whereas the interaction between the factors was not significant ($F(5,24)=1.902$, $P=0.131$). The ratio of bacteria count in blood-fed mosquitoes as compared to sugar-fed mosquitoes was 2.13 (95% CI: 1.49–3.06).

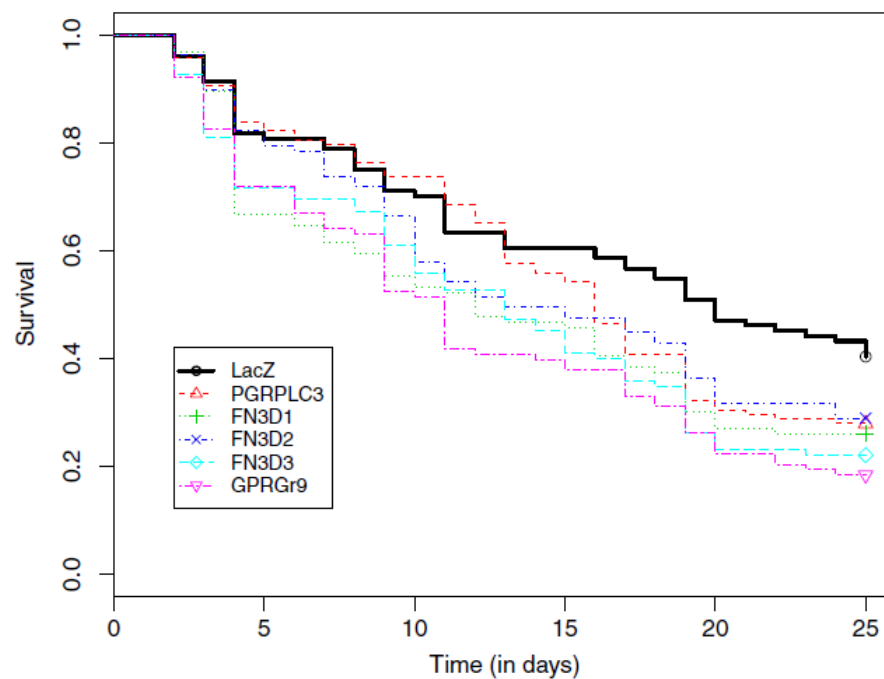


Figure 3.3 Kaplan-Meier curves depicting the survival rate as a function of time for gene-silenced mosquitoes. In the experiment, five test and one control genes were considered. The Kaplan-Meier curve for each gene is based on six replicates each consisting of 20–30 mosquitoes.

Gene	Hazard ratio (95% CI; <i>P</i> -value)	
	Without antibiotics	With antibiotics
<i>LacZ</i>	1.00	1.00
<i>FN3D1</i>	1.64 (1.17–2.30; <i>P</i> = 0.004)	1.02 (0.75–1.37; <i>P</i> = 0.91)
<i>FN3D2</i>	1.40 (1.00–1.95; <i>P</i> = 0.050)	1.17 (0.86–1.58; <i>P</i> = 0.32)
<i>FN3D3</i>	1.79 (1.28–2.50; <i>P</i> < 0.001)	0.90 (0.67–1.21; <i>P</i> = 0.50)
<i>GPRGr9</i>	2.00 (1.45–2.76; <i>P</i> < 0.001)	0.90 (0.67–1.21; <i>P</i> = 0.47)
<i>PGRPLC3</i>	1.35 (0.97–1.87; <i>P</i> = 0.072)	0.89 (0.66–1.21; <i>P</i> = 0.46)

Table 3.2 Effect of gene silencing on mosquito survival. The second column presents the hazard ratio (HR) of dying between a gene knockdown and dsLacZ control. The third column presents the hazard ratio of dying between a gene knockdown and dsLacZ control when mosquitoes are treated in parallel with antibiotics.

When considering the blood-fed mosquitoes alone, no statistically significant differences in the bacterial count of the target gene silenced and the dsLacZ-injected mosquitoes were observed. The ratio of midgut bacterial count of target gene silenced to the dsLacZ-injected mosquitoes is presented in Table 3; the ratios for all target gene silenced mosquitoes compared to the control groups (LacZ injected mosquitoes) were above 1, with the highest value for FN3D1 equal to 2.66 (95% CI: 0.94– 7.57) but not significantly different from 1 (*P*=0.085). Treatment of mosquitoes with an antibiotics cocktail eliminated the gene silencing effect on survival for all 5 target genes. No statistically significant differences were noted in terms of mortality between the target genes silenced and the dsLacZ-injected mosquitoes (Table 3.2). We also observed that for best performing genes, FN3D1 and FN3D3, dsRNA treatment had no effect on mosquito fecundity (number of eggs laid/female) or fertility (egg hatchability) of the mosquitoes (Table 3.5).

Gene	Median time to death (days) (95% CI)
<i>LacZ</i>	20 (16 – +∞)
<i>FN3D1</i>	12 (9–17)
<i>FN3D2</i>	13 (10–19)
<i>FN3D3</i>	13 (10–17)
<i>GPRGr9</i>	11 (9–15)
<i>PGRPLC3</i>	16 (13–19)

Table 3.3 Median time to death in *An. arabiensis* mosquitoes when silenced with genes through injection of gene-specific dsRNA.

Gene	Bacterial count ratio (95% CI, <i>P</i> -value)
<i>LacZ</i>	1.00
<i>FN3D1</i>	2.66 (0.94–7.57; <i>P</i> = 0.085)
<i>FN3D2</i>	1.15 (0.40–3.27; <i>P</i> = 0.793)
<i>FN3D3</i>	1.36 (0.48–3.85; <i>P</i> = 0.570)
<i>GPRGr9</i>	2.30 (0.81–6.54; <i>P</i> = 0.136)
<i>PGRPLC3</i>	1.08 (0.38–3.07; <i>P</i> = 0.886)

Table 3.4 Bacterial count in *An. arabiensis* mosquitoes silenced with genes involved in midgut homeostasis using by microinjection of gene-specific dsRNA.

Gene (kids)	Oviposition (%)	Mean egg/Female	Egg hatchability (%)
Lacz (14)	64.3	49 (67)	100%
FN3D1 (15)	60.0	42 (62)	100%
FN3D3 (10)	50.0	58 (68)	80 %

Table 3.5 Reproductive fitness of gene silenced mosquitoes. Numbers inside bracket represent number of mosquitoes or eggs tested.

3.5 Discussion

The scale-up of vector control interventions in conjunction with early patient diagnosis and therapy has resulted in a substantial reduction in malaria-related cases and deaths since 2000. However, the newest data suggest that this progress is progressively coming to a halt or, in fact, being reversed in some countries, indicating that the current malaria intervention tools and strategies may have reached their maximum capacity (WHO, 2019). This highlights the urgency of developing new tools to complement LLINs to achieve the malaria elimination agenda in Africa, especially against the exophagic and exophilic *An. arabiensis* mosquito. A viable option against this opportunistic feeder species is to challenge different blood hosts with whole protein or antigenic peptide generated from mosquito itself (i.e., mass vaccination). The resulting host antibody is ingested by the mosquito during blood-feeding on an immunized host. This anti-mosquito antibody can be designed to target mosquito molecules involved in midgut homeostasis to reduce the longevity of the mosquito to such an extent that insufficient time remains to transmit the *Plasmodium* parasite.

In order for a mosquito to transmit a pathogen, it must acquire a blood meal from an infected person, support pathogen replication, dissemination to the salivary glands and take a subsequent blood meal from a susceptible host; this period is commonly called extrinsic incubation period (EIP). The EIP ranges from 10 to 14 days (2 to 6 gonotrophic cycles in areas of high malaria

transmission) and a female mosquito must survive longer than the EIP to transmit the parasite (Ohm et al., 2018), hence a very small fraction of mosquitoes (<10%) can survive long enough to transmit the pathogen. This underlines the fact that mosquito longevity is a critical factor affecting malaria transmission and provides a basis for guiding vector control strategies (Bruce-Chwatt, 1980; Bara et al., 2015). A small reduction in the mean mosquito survival period would have a significant impact on the disease transmission (Charlwood et al., 1997; Scholte et al., 2005; Dye, 1986; Garrett-Jones and Shidrawi, 1969).

In the present study, we assessed five midgut-expressed genes *FN3D1*, *FN3D2*, *FN3D3*, *GPRGr9* and *PGRPLC* for their potential as lifespan-limiting targets in a wild *An. arabiensis* population. Previous studies with *An. gambiae* under laboratory settings have demonstrated that the above genes regulate midgut microbiota (Meister et al., 2009; Stathopoulos et al., 2014). Unlike these studies, our work focused on the effect of those genes on the survival of field-caught *An. arabiensis*. Our experimental mosquitoes were captured as larvae or pupae and maintained in an environment simulating the mosquito natural resting habitat. They were also allowed to blood-feed on a goat every fourth day, mimicking their natural habits. We have demonstrated that in these seminatural conditions, the reduction of *FN3D1*, *FN3D3* or *GPRGr9* expression significantly reduces the longevity of the *An. arabiensis* mosquitoes to an average of 12, 13 and 11 days, respectively, compared to a 20-day average longevity of control mosquitoes.

The observed effect on the mosquito survival is probably linked to the disruption of the mosquito midgut homeostasis as the observed effect of gene silencing (particularly of *FN3D1*, *FN3D3* and *GPRGr9*) on their survival was no longer seen when mosquitoes were treated with an antibiotic cocktail to eliminate their gut microbiota. Our hypothesis that the reduced survival is due to the inability of mosquitoes to control their gut microbiota is also supported by other studies (Asgari et al., 2017; Gendrin et al., 2015). Furthermore, it has been previously demonstrated that FN3Ds and GPRGr9 have a specific effect on microbiota of the family Enterobacteriaceae (Stathopoulos et al., 2014).

Our results demonstrate that interfering with the expression and/or function of these genes reduces the mosquito lifespan which in turn will significantly reduce malaria transmission. A permanent

gene inactivation/ knock-out can be achieved in various ways. A new and most promising tool is the recently developed CRISPR/ Cas9-based genome editing methodology and gene-drive systems for *Anopheles* mosquitoes (Gantz et al., 2015; Zhang et al., 2017; Dong et al., 2018). Recently a CRISPR/Cas9-induced somatic gene disruption technique has been established in *An. gambiae* (Dong et al., 2018; Hammond et al., 2016). Such a knock-out line can be crossed with a germline-Cas9 strain as described in to generate a germ-line gene knockout line which might be released to introgress the life-shortening trait into the wild malaria vector population (Macias et al. 2017). A second approach could be through the immunization of the blood-providing host, whether human or domestic animals, with molecules derived from target vector proteins that play a role in midgut homeostasis. The purified form of such molecules (i.e., antigens) can be inoculated into the vertebrate host to induce host immune reaction, and ultimately produce specific antibodies. Mosquitoes feeding on the immunized hosts would then ingest antibodies that neutralize the function of the protein leading to disrupted midgut homeostasis and shortened lifespan. It has been previously demonstrated that *An. gambiae*-derived anti-midgut monoclonal antibodies significantly reduce vector survivorship (Lal et al., 2001). This approach is particularly attractive for zoophilic mosquitoes such as *An. arabiensis*. Mosquitoes that have taken an infectious blood meal will typically take three to four additional blood meals before the completion of a sporogonic period as they normally blood-feed every 2–3 days. This could ensure repeated ingestion of antimosquito antibodies with consequential disruption of gut bacterial homeostasis to ultimately induce reduced lifespan. This technology can also impact secondary malaria vectors including *An. rivolorum*, *An. pharoensis*, *An. coustani*, and *An. ziemanni* and *An.squamosus* that are responsible for about 5% of total malaria transmission in Africa and are often highly zoophilic (Afrane et al., 2016).

As the anti-mosquito vaccine technologies are expected to only reduce the long-term survival of vectors of the mosquitoes, female mosquitoes will complete some of their gonotrophic cycle. Thus, the technologies are expected to have minimum selection pressure inducing resistance (Read et al., 2009).

This approach has been successfully used for an antitick vaccine targeting the midgut antigens Bm86 and an anti-tick and anti-mosquito vaccine targeting the subolesin/akirin (SUB/AKR)

antigens (Jonsson et al., 2000; da Costa et al., 2014; de la Fuente et al., 2011; de la Fuente et al., 2013). The functional model for the SUB/AKR vaccine involves the nuclear factor-kappa B (NF- κ B) of vector insects to inhibit the Immunodeficiency (IMd) pathway, which is important for regulation of the gut microbiota (Naranjo et al., 2013; Wan et al., 2009). A major challenge with such protein antigens is that they can result in autoimmunity due to molecular mimicry as such molecules can possess ‘mimotopes’ that are peptides mimicking the antigenic conformation structures that are recognized by the paratope antibody, leading to autoimmunity (Huang et al., 2011).

In the present case, amino acid sequence analysis of the *An. arabiensis* *FN3D1* and *FN3D3* genes has shown the presence of 25–37% sequence identity with proteins of some human genes such as the protein tyrosine phosphatase receptor type F gene. This level of homology is often referred as the ‘twilight zone’ where the structural similarity between the target mosquito genes and the human genes cannot be ruled out, suggesting a potential risk of induction of autoimmune diseases in individuals upon immunization with the mosquito genes (Rost, 1999). For instance, an analysis involving over a million sequences with known structures showed that at the top cut-off of the twilight zone, about 90% of protein pairs were structurally homologous, but the level of homology reduced drastically (to <10%) when the sequence similarity between protein pairs was below 25% (Huang et al., 2011). On the other hand, both mosquito genes have no significant sequence similarity in the domestic Bovidae, hence induction of an autoimmune reaction in the vaccinated animals is negligible and the protein molecules can be used to develop vaccines to immunize the animals with minimum risk.

3.6 Conclusions

Eliminating the expression of the midgut proteins *FN3D1*, *FN3D3* or *GPRGr9* significantly reduces the lifespan of naturally occurring *An. arabiensis* mosquitoes reared in field conditions. The effect is probably caused by disruption of the mosquito midgut homeostasis through interference with the midgut microbiota, eventually hampering the mosquito immuno-metabolic functions. Therefore, these proteins can be good targets of mosquito life-shortening interventions, such as antimosquito vaccines or mosquito genetic modification, resulting in mosquitoes that can

survive long enough to complete a gonotrophic cycle but not long enough to transmit malaria parasites to a new host. Thus, the technologies are expected to have minimum selection pressure inducing resistance.

Acknowledgments

We acknowledge the financial support from the Institutional University Cooperation IUC-JU program to LD, in the framework of the Flemish Interuniversity Council (VLIR-UOS).

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4

Chapter 4 Stability of the effect of silencing fibronectin type III domain-protein 1 (*FN3D1*) gene on *Anopheles arabiensis* reared under different breeding site conditions

Adapted from:

Debalke S, Habtewold T, Christophides GK, Duchateau L. Stability of the effect of silencing fibronectin type III domain-protein 1 (*FN3D1*) gene on *Anopheles arabiensis* reared under different breeding site conditions. Parasit Vectors. 2020; 13:202.

4.1 Abstract

Background: Malaria vector mosquitoes acquire midgut microbiota primarily from their habitat. The homeostasis of these microbial communities plays an essential role in the mosquito longevity, the most essential factor in the mosquito vectorial capacity. Our recent study revealed that silencing genes involved in regulation of the midgut homeostasis including *FN3D1*, *FN3D3* and *GPRGr9* reduced the survival of female adult *Anopheles arabiensis* mosquitoes. In the present study, we investigate the stability of the gene silencing efficiency of mosquitoes reared in three different breeding conditions representing distinct larval habitat types: town brick pits in Jimma, flood pools in the rural land of Asendabo and roadside pools in Wolkite.

Methods: First-instar larvae of *An. arabiensis* mosquitoes were reared separately using water collected from the three breeding sites. The resulting adult females were micro-injected with dsRNA targeting the *FN3D1* gene and their survival was monitored. Control mosquitoes were injected with dsRNA *Lacz*. In addition, the load of midgut microbiota of these mosquitoes was determined using flow cytometry.

Results: Survival of naïve adult female mosquitoes differed between the three sites. Mosquitoes reared using water collected from brick pits and flood pools survived longer than mosquitoes reared using water collected from roadside. However, the *FN3D1* gene silencing effect on survival did not differ between the three sites.

Conclusions: The present study revealed that the efficacy of *FN3D1* gene silencing is not affected by variation in the larval habitat. Thus, silencing this gene has potential for application throughout sub-Saharan Africa.

Keywords: *Anopheles arabiensis*, Larval breeding sites, Gene silencing stability, Survival, *FN3D1* gene

4.2 Background

Malaria still thrives in SSA. The region holds over 90% of the 219 million malaria cases with an estimated 435,000 malaria deaths in 2017 (WHO, 2018). This disproportionate share is due to the principal malaria vectors in the region that exhibit a higher vectorial capacity, i.e., *An. gambiae* (s.s), *An. coluzzii*, *An. arabiensis* and *An. funestus*. Key parameters for vectorial capacity include preference to feed on humans, susceptibility to *Plasmodium* infection and longevity of the mosquito. It can take two weeks and beyond for the *Plasmodium* parasites to complete the stage development after which they can be transmitted to another human host. On the other hand, in tropical regions, the average lifespan of *Anopheles* mosquitoes is 14 to 19 days (Gillies et al., 1965; Service and Towson, 2002), resulting in only a few long-living vectors that can transmit malaria. Therefore, a promising strategy to eliminate malaria transmission is to reduce the mosquito lifespan, as even a small reduction would have a large impact on transmission (Smith and McKenzie, 2004).

Adult mosquito survival is influenced by the environment during the preceding immature stage. Environmental factors consist of the physicochemical characteristics of larval habitats (Mwangangia et al., 2007; Oyewole et al., 2009), and the quality and availability of nutrients (Linenberg et al., 2016; Araújo et al., 2012). Previous studies have demonstrated that larval fitness has a major effect on the adult survival of different malaria mosquitoes (Araújo et al., 2012; Moller-Jacobs et al., 2014; Okech et al., 2007; Shapiro et al., 2016). For instance, female adult mosquitoes maintained in a high nutritive larval environment lived longer than in a poor nutritive environment (Moller-Jacobs et al., 2014; Joy et al., 2010). A nutritionally restrictive larval environment yields mosquitoes with reduced size with significantly shorter survivorship (Moller-Jacobs et al., 2014; Okech et al., 2007). Microorganisms and organic materials in the larval pool are the major constituents of the larval diet (Timmermann and Briegel, 1996). Previous studies have demonstrated that bacteria in the breeding water are most critical for the quality of the larval diet and their absence leads to increased larval mortality (Merritt et al., 1992; Wotton et al., 1997; Toure et al., 2003; Coon et al., 2014).

An increasingly important factor that affects the life traits of adult mosquitoes is the pollution of the larval habitats. Rapid and unplanned expansion of urbanization in SSA increases pollution of the surface waters with domestic or industrial discharges of untreated effluents (Fayiga et al., 2018; Fossog et al., 2012). *Anopheles* larvae have shown a high resilience to the high toxicity of some of these wastes in the urban and suburban sites, but the impact of such pollution on the life traits of the resulting adult mosquito is poorly understood. However, there is speculation that exposure of larvae to toxins dissolved in the water might contribute to the rising prevalence of vector resistance to pyrethroids in the cities across the region (Santolamazza et al., 2008; Ranson et al., 2009). It was also suggested that larvae developing in polluted water can lead to a significant fitness cost (Riaz et al., 2009).

Based on the above facts, we assessed the survival of adult female *An. arabiensis* mosquitoes reared in water collected from three different breeding sites. In our previous study we observed that silencing the midgut gene fibronectin type III domain-protein 1 (*FN3D1*) involved in the midgut homeostasis reduced the longevity of *An. arabiensis* mosquitoes (Debalke et al., 2019). Therefore, in the present study we further assessed the stability of the *FN3D1* gene silencing effect on *An. arabiensis* mosquitoes reared in water collected from three different breeding sites.

4.3 Methods

4.3.1 Study sites and sample collection

Water samples for mosquito rearing were collected from three malaria endemic sites including Wolkite, Jimma and Asendabo, south-west Ethiopia (7°40'0.01"N, 36°49'59.99"E) (Figure 4.1). From each site, a single pool confirmed to support *An. arabiensis* larvae was selected to collect rearing water using a clean jug and plastic container/jerry cans. Simultaneously water samples for bacterial counts were collected using sterile, screw cup bottles following standard operational procedures (Association American Public Health, 1992). The water collected for rearing purposes was filtered using a fine mesh linen cloth in order to remove debris and mosquito eggs and larvae. The filtered water was then transferred directly to clean larvae rearing pan or boiled for 10 min and cooled before transferring to the rearing pans.

4.3.2 Analysis of water from selected sites

pH, conductivity and dissolved oxygen was measured on site using a Portable Multi meter (HQ40D, HACH). Salinity was measured using a salinity meter (TRACER PocketTester) and turbidity with a turbidity meter (Wag-WT 3020, Wagtech International, Gateshead, UK). Total dissolved solids (TDS) and total suspended solids (TSS) were measured by the gravimetric method. In brief a volume of 100 mL of well-mixed water samples was filtered using a glass-fiber-filter with applied vacuum. The filtered samples were then washed with three successive 10 mL volumes of distilled water which permitted complete drainage between washings. The suction was continued for about 3 min after filtration was completed. The filtrate (with washing) was then transferred to a weighed evaporating dish and evaporated to dryness on a steam bath. If the filtrate volume exceeded dish capacity, successive portions were added to the same dish after evaporation. Finally, the samples were dried for at least 1 h in an oven at 180 ± 2 °C for TDS and 103–105 °C for TSS, allowed to cool in a desiccator to balance temperature, and weighed (Association American Public Health, 1992). All the parameters were measured based on the supplier's guidelines and the instruments were calibrated before the samples analyzed. A total of two replicates was taken for each site.

The total bacterial count was assessed for the water samples using membrane filter techniques. For each sample ten-fold serial dilution was prepared with a total volume of 100 mL and allowed to pass through a funnel covered with a membrane filter (with a size of 47mm diameter by 0.45µm porosity) in order to trap the bacteria available in the water. Then the membrane with the trapped bacteria was placed in a Petri dish containing a pad saturated with sterilized M-lauryl sulfate broth (Sigma-Aldrich, Bangalore, India). The M-lauryl sulfate broth media was prepared according to the manufacturer's guidelines. The passage of nutrients through the filter during incubation facilitates the growth of organisms in the form of colonies on the upper surface of the membrane. Then, the inoculates were incubated at a temperature of 37 °C for 24 h where after the total number of the colonies was counted using a magnifying lens (Association American Public Health, 1992).

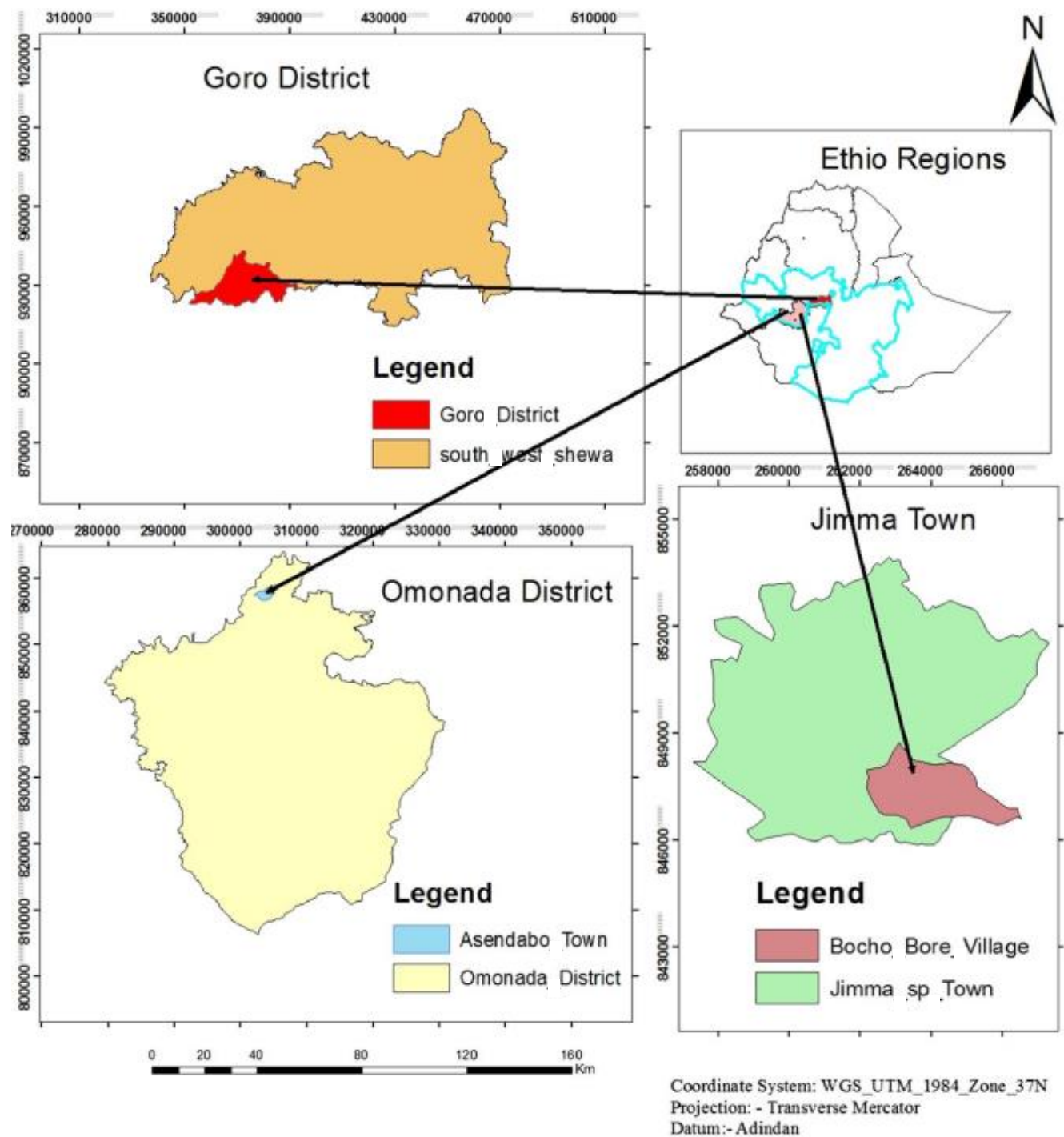


Figure 4.1 Map showing larval breeding sites where rearing water samples were collected.

4.3.3 Analysis of water from selected sites

Anopheles arabiensis obtained from a laboratory strain of the Adama Malaria Research Centre, Ethiopia, was used in this study. Recently hatched first-instar larvae were reared in the water collected from the three breeding sites. Two-hundred larvae from the same batch of eggs were dispensed into the rearing trays and supplied with an equal amount of a mixture of yeast and fish food. The rearing water was changed every two days and development of larvae was monitored daily. Next, the enclosed pupae were counted and transferred into 10 mL glass beakers independently and kept in adult cages (W15 × D15 × H15 cm, BugDourm, Watkins & Doncaster, Leominster, UK) until the emergence of the last pupae. The emerged adults were maintained on 10% sugar at 27 °C, 70% humidity with 12:12 h light:dark photocycle (Gerberg, 1970). The experiment was done in triplicate.

Adult wing size was measured to evaluate differences in the adult female body size. For this experiment, 10 individual mosquitoes were removed from each adult cage to measure their wing size as follows: a single wing was clipped from each mosquito using scalpel blade and mounted onto a glass microscope slide. Then the wing size was measured using a micrometer eyepiece with stereomicroscope. Measurements were taken from the tip of the wing (excluding fringe) to the distal end of the alula (Nanci, 1990).

4.3.4 Gene silencing and survival assay

From each breeding site, 15–24 adult female mosquitoes were CO₂ anesthetized and treated with 69 µl of the *FN3D1* dsRNA gene and another group with non-mosquito ds*LacZ* gene. Double stranded RNA (DsRNA) for *FN3D1* and the control *LacZ* gene was prepared from complementary DNA (cDNA) as described in our previous study (Debalke et al., 2019). The DNA was synthesized from 1 µg of the tRNA using Prime-Script™ 1st-strand cDNA Synthesis Kit (TaKaRa, Saint-Germain-en-Laye, France) which was extracted from 10 whole female *An. arabiensis* mosquitoes using TRIzol reagent (Invitrogen, Carlsbad, USA). The following primer sequences tailed with the T7 promoter were used to synthesize the two dsRNA: *FN3D1* (forward: GAT GGA CGT GGA TCA GCC; reverse: TGG ATC GTC CTC ATC ACT GT) and *LacZ* (forward: AGA ATC CGA CGG GTT GTT ACT; reverse: CAC CAC GCT CAT CGA TAA TTT).

On day four, after the mosquitoes were starved overnight, they were fed on blood. At 24 h post-blood-meal, 5 mosquitoes were sampled for each breeding site independently for both unboiled and boiled water and their midguts were dissected. The midgut samples were then homogenized in 100 µl 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The number of bacteria was then counted using flow cytometry from five pooled midgut samples per replicate (Habtewold et al., 2016). A total of 3 replicates of microbiota analysis were performed. The survival of mosquitoes was monitored starting from 24 h post-injection for 20 days. All mosquitoes were supplied with 10% sugar solution and monitored daily, and they obtained blood meal at 4-day intervals post injection. Three replicates each consisting of 15–24 mosquitoes were performed.

4.3.5 Data analysis

Data analysis was performed using the statistical software package R version 3.3.2. Survival of the *FN3D1* gene- and *LacZ* gene-silenced mosquitoes and the naïve mosquitoes with boiled and unboiled water were depicted by Kaplan Meier survival curves as a function of site. The effect of gene silencing on survival was modelled by the Cox proportional hazards frailty model (Duchateau and Janssen, 2008), with replicate as frailty term and including as covariates the gene (the *FN3D1* target and *LacZ* control gene), the site (Jimma, Asendabo and Wolkite) and the two-way interaction. The effect of site and boiling in the naïve mosquitoes was analyzed in the same way. The hazard ratio was used as a summary statistic, together with the median time to death. The bacterial counts were first log-transformed and then compared by a mixed model with replicate as random effect and the *F*-test was used to compare the silencing of the *FN3D1* target gene and the control *LacZ* gene on the one hand, and the boiling on the other hand. The ratio of the bacterial counts in the control *LacZ* gene and the *FN3D1* target gene was used as a summary statistic. The t-test was used to compare the number of emerged adults and pupae and the adult wing length between the three breeding sites. All tests were performed at a significance level of 5%.

4.4 Results

4.4.1 Analysis of water samples

Pictures of the three breeding sites are shown in Figure 4.2 and their physicochemical characteristics are provided in Table 4.1. The water samples from Jimma and Asendabo had close to the neutral pH, while water samples from Wolkite were alkaline with the highest salinity. Nevertheless, the pH for all the sites was within a range that was previously reported suitable for the natural breeding habitats of *An. arabiensis* (Hamza and Rayah, 2016). The level of dissolved oxygen was considerably higher for Wolkite and Asendabo compared to Jimma. The Asendabo had also the highest total bacterial count compared to Jimma or Wolkite.



Figure 4.2 Breeding sites with larval rearing water samples were collected representing brick-pit pool at Jimma town (a), flooded farmland at Asendabo (b) and roadside pool at Wolkite (c).

Parameter	Wolkite	Jimma	Asendabo
pH	8.2 (7.9–8.5)	7.3 (7.1–7.5)	7.15 (7.0–7.4)
Salinity (ppm)	225 (180–320)	85 (75–125)	160 (100–175)
DO (mg/l)	7.2 (2.3–7.6)	4.5 (1.8–8.8)	6.3 (5.1–9.0)
Turbidity	27.3 (4.9–80.2)	51.7 (2.2–87.1)	64.3 (47.2–146.0)
TDS (mg/l)	507.5 (256.0–665.5)	237.0 (185.5–317.0)	167.0 (163.0–256.2)
TSS (mg/l)	157.5 (72.0–358.0)	275.5 (156.0–293.0)	170.0 (151.0–323.0)
Chlorophyll a	11.6 (11.3–12.0)	11.9 (11.7–12.3)	12.2 (12.0–12.4)
Bacterial count	317 (233–400)	233 (192–291)	546 (252–660)

Table 4.1 Physicochemical characteristics and bacterial count of water samples from the three sites. The data are presented as median (range). Abbreviations: DO, dissolved oxygen; TDS, total dissolved solids; TSS, total suspended solids.

4.4.2 Larval development

Our results demonstrated that, generally, the larvae reared with water from Asendabo performed better when compared to the rest of the sites (Table 4.2). For instance, the pupation rate for Asendabo (85.8%) was significantly higher compared to Wolkite (68.5%) ($t = 5.69$, $df = 4$, $P = 0.005$) but not to Jimma (78%) ($t = 5.69$, $df = 4$, $P = 0.062$). No significant differences of percentage of pupae developed in adult mosquitoes could be found between Asendabo, Wolkite and Jimma. The mean adult wing length was significantly larger for Asendabo (3.2 mm) compared to Jimma (3.0 mm) ($t = 2.58$, $df = 87$, $P = 0.012$) and Wolkite (2.7 mm) ($t = 8.23$, $df = 87$, $P < 0.001$). Together, these results suggest that the above parameters may be positively impacted by the level of dissolved oxygen and bacterial count in the breeding water. Accordingly, the water from Asendabo is considered most suitable for larval development.

4.4.3 Survival rate of adult mosquitoes

Survival of the mosquitoes is depicted as a function of time in Figure 4.3 for the different settings, i.e., different sites and water boiled or not. There was no significant interaction between the site and boiling effect, i.e., the effect of boiling the water has a similar effect for the three sites. A significant effect of boiling the water ($\chi^2 = 17.2$, $df = 1$, $P < 0.001$) and an almost significant difference between the sites ($\chi^2 = 5.64$, $df = 2$, $P = 0.059$) was found. The hazard ratio of the mosquitoes grown in boiled water compared to unboiled water was 2.25 (95% CI: 1.42–3.56). The hazard ratio of Jimma compared to Wolkite was 0.53 (95% CI: 0.32–0.88) whereas the hazard ratio of Asendabo compared to Wolkite was 0.51 (95% CI: 0.31–0.84). The adult gut bacterial count ratios in mosquitoes grown in the unboiled *versus* the boiled water for the three sites are presented in Figure 4.4.

Breeding site	Pupa (%)	Adult (%)	Mean wing size (mm)
Wolkite	68.8	93.7	2.7
Jimma	78.2	93.6	3.0
Asendabo	85.8	95.9	3.2

Table 4.2 The rates of pupation and adult emergence ($n = 3$ batches of 200 eggs), and the mean adult wing size ($n = 30$) in mosquitoes reared in the water collected from the three sites

4.4.4 Effect of the *FN3D1* gene silencing

Survival of the *FN3D1*- and *LacZ*-silenced mosquitoes is depicted as a function of time in Figure 4.5 for the different sites. There was no significant interaction between the gene silencing effect and the site, i.e. the effect of silencing was the same for the three sites. Both gene silencing ($\chi^2 = 25.7$, $df = 1$, $P < 0.001$) and site ($\chi^2 = 8.15$, $df = 2$, $P = 0.017$) had a significant effect on survival. The hazard ratio of the *FN3D1*-silenced mosquitoes compared to the *LacZ*-silenced mosquitoes was 1.96 (95% CI: 1.58–2.43). The hazard ratio of Jimma compared to Wolkite was 0.69 (95% CI: 0.54–0.89) whereas the hazard ratio of Asendabo compared to Wolkite was 0.86 (95% CI: 0.67–1.10).

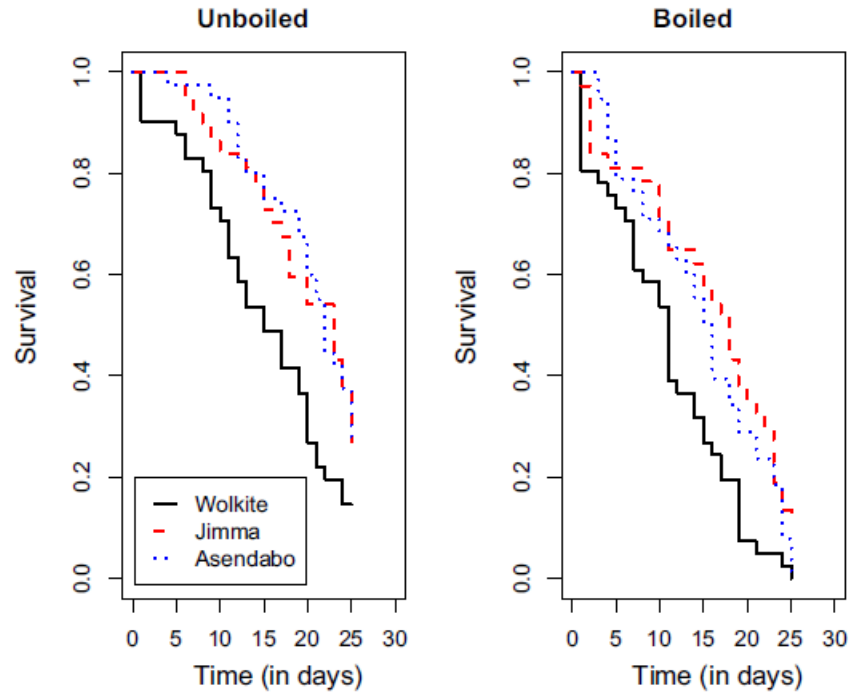


Figure 4.3 Survival as a function of time for the naive mosquitoes for the three sites (Wolkite, Jimma and Asendabo) and water boiled or unboiled.

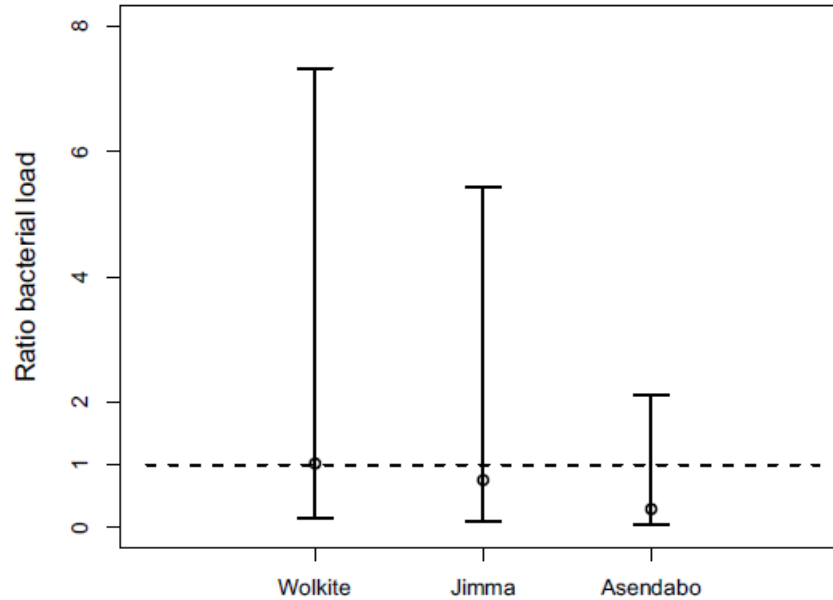


Figure 4.4 Ratio of adult gut bacterial load (95% confidence interval) of mosquitoes in boiled versus unboiled water in the three sites.

Significant effects for the microbiota were found for the gene silencing ($F(1, 14) = 7.59, P = 0.016$) and for the interaction between gene silencing and site ($F(2, 14) = 4.23, P = 0.038$). Therefore, we study the gene effect at each location separately. A significantly higher bacterial load in the *FN3D1*-silenced mosquitoes as compared to the *LacZ*-silenced mosquitoes was only found for Asendabo, with a ratio equal to 2.50 which differs significantly from 1 ($F(1, 14) = 14.21, P = 0.002$) (Figure 4.6).

4.5 Discussion

In the present study, we demonstrated that the larval breeding habitats in the three sites vary distinctly with regard to their physico-chemical characteristics and microbial abundance. Previous studies have demonstrated that the physico-chemical characteristics (Christiansen-Jucht et al., 2014; Wallace and Merritt, 1999; Emide et al., 2017) of the larval breeding habitat determine the larval and pupal density, the size and number of emerged adults and the survival of both larvae and adult mosquitoes. Thus, the mosquitoes in the study sites could also differ in their fitness (e.g. size, longevity, fecundity) and capacity to support and transmit the malaria parasite (Araújo et al., 2012; Okech et al., 2007). Previous studies have shown that different species of mosquitoes maintained and reared in low food environments had a reduced longevity, smaller body size and lower vectorial capacity (Araújo et al., 2012; Moller-Jacobs et al., 2014; Wallace and Merritt, 1999; Chouaia et al., 2012; Mitraka et al., 2013). Most mosquito life traits (fitness) are affected by environmental factors, and specifically the breeding habitat (Araújo et al., 2012; Moller-Jacobs et al., 2014; Okech et al., 2007; Pfaehler et al., 2006; Tun-Lin et al., 2000).

The present observation of an increased total number of emerged pupae for the larvae that were reared in water collected from Asendabo is an indicator for a higher suitability of this larval habitat. For this site, two parameters that stand out include a high bacterial abundance and increased oxygenation. Previous studies have established that bacteria in the breeding habitat constitute the main food source for larvae enhancing larval growth and the productivity and survival of the adult mosquitoes (Araújo et al., 2012; Moller-Jacobs et al., 2014; Coon et al., 2014). Studies have also demonstrated that higher dissolved oxygen favors the development of *Anopheles* mosquitoes (Dida et al., 2018; Dejenie et al., 2011).

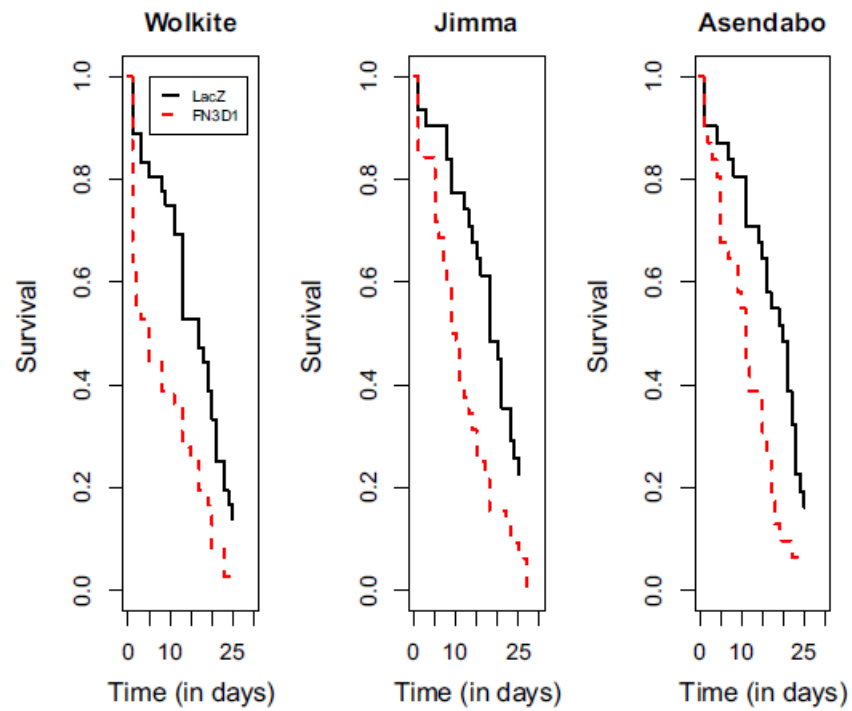


Figure 4.5 Survival as a function of time for the control LacZ and target FN3D1 gene silenced mosquitoes at the three sites.

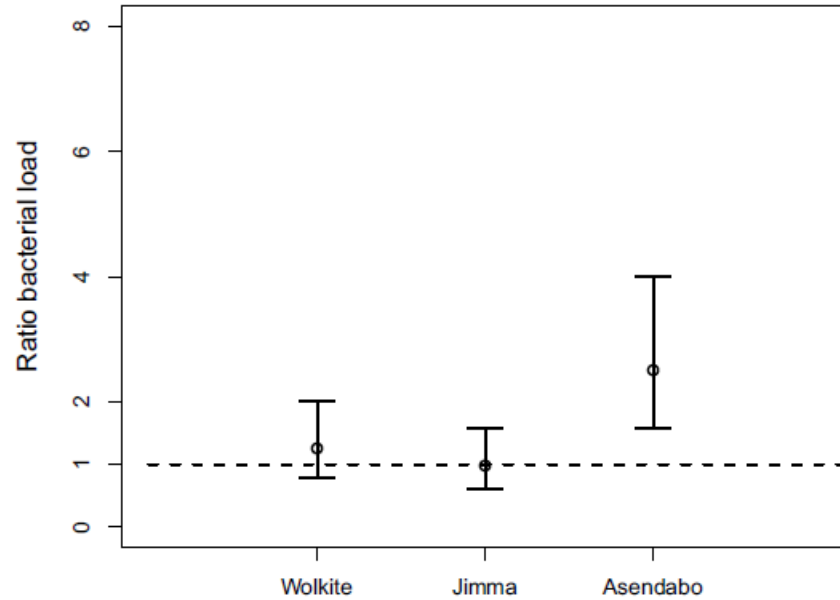


Figure 4.6 Ratio of adult gut bacterial load (95% confidence interval) in FN3D1-silenced versus LacZ-silenced mosquitoes in the three sites.

In this study, wing size of the adult female mosquitoes was measured because the wing size is also an indicator for the suitability of the larval breeding environment. Our data revealed there is a marked variation in the wing size between the mosquitoes originating from different breeding sites with the Asendabo site yielding mosquitoes with the largest wing compared to Jimma and Wolkite. A similar variation in the wing size among the mosquitoes grown in different larval habitats was reported earlier for *An. stephensi* (Moller-Jacobs et al., 2014) and *An. darlingi* (Araújo et al., 2012; Lounibos et al., 1995). It was concluded that such variation in the wing size is closely linked to the nutrient availability in the habitat ((Araújo et al., 2012; Moller-Jacobs et al., 2014).

Wing size is directly correlated with survival of adult female mosquitoes, i.e. the mosquitoes with longer wing showed a higher longevity than short winged mosquitoes (Moller-Jacobs et al., 2014; Joy et al., 2010; Ameneshewa and Service, 1996; Lehmann et al., 2006). Comparison of the survival of the mosquitoes reared with water from the three sites in the present work was supported by the above observation. For instance, the mosquitoes reared in the water from the Asendabo site that had the longest wing displayed the longest survival.

Previously we have demonstrated that the depletion of some midgut gene proteins including the *FN3D1*, *FN3D3* and *GPRGr9* genes markedly shorten the longevity of female *An. arabiensis* (Debalke et al., 2019). In the present study, we assessed whether the variation between breeding habitats may affect the gene silencing effect. Our survival data revealed that the effect of gene silencing was not affected by variation in the larval breeding site, i.e., for all the study sites the *FN3D1*-treated mosquitoes had a similarly reduced survival rate compared to the control *LacZ* group. Thus, the variation in breeding sites does not affect the gene silencing effect on reducing the longevity of *An. arabiensis* mosquitoes. Gene silencing induces mosquito mortality by disrupting the midgut homeostasis (Debalke et al., 2019), which is evidenced also in the present study where a higher bacterial load was observed in the *FN3D1*-silenced mosquitoes as compared to the *LacZ* silenced mosquitoes.

4.6 Conclusions

The longevity of the *An. arabiensis* mosquitoes varied between the three larval breeding sites depending on biotic as well as abiotic characteristics of the larval breeding water. However, there was no evidence that these differences compromise the gene silencing effect of the *FN3D1* gene on the mosquito survival. Therefore, interventions based on the silencing of such genes, and thus the reduction of mosquito longevity, offer a universal strategy to block malaria transmission.

Acknowledgements

We acknowledge the financial support from the Institutional University Cooperation IUC-JU project to LD, in the framework of the Flemish Interuniversity Council (VLIR-UOS). The project was additionally supported by the Wellcome Trust Investigator Award to GKC, and at late stages, by the Bill and Melinda Gates Foundation grant OPP1158151 to GKC.

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Chapter 5 General discussion

In this chapter, the possible impact of the results of our research is discussed. Our research is focused on the reduction of longevity of the *Anopheles* mosquito. The vectorial capacity concept, introduced in Section 5.1, is a useful tool to understand how such a vector control tool impacts on malaria transmission. A discussion follows on how the promising results on particular genes and their effect on longevity could be used in vaccine development in Section 5.2 or in genetic engineering of mosquitoes in Section 5.3. The potential of our findings in *An. arabiensis* for other *Anopheles* species is discussed in Section 5.4 and for some other arthropod vectors in Section 5.5. The chapter is concluded with future perspectives in Section 5.6.

5.1 Reducing and eliminating *Anopheles* vector populations

Vector control tools have had the largest impact on the reduction of malaria incidence in SSA in the last few decades (Bhatt et al., 2015). Unfortunately, this positive evolution has stalled in the last few years due to the fact that some previously successful vector control tools do not have the same efficacy anymore (Hancock et al., 2018). Therefore, existing vector control tools need to be adapted and improved and new tools need to be developed.

The best way to understand how different vector control tools have an impact on malaria incidence is through the concept vectorial capacity (VC), also called the daily reproductive number. Vectorial capacity corresponds to the average number of potentially, i.e., from a *Plasmodium*-infected mosquito, infectious bites per day per subject and can be determined as

$$VC = \frac{m a^2 p^n}{-\ln p}$$

with m the number of mosquitoes in the neighborhood of an infected subject, a the proportion of those m mosquitoes that will actually bite, p the daily survival probability and n the number of days required for *Plasmodium* to develop in the mosquito and appear in the salivary glands, which is called the extrinsic incubation period (EIP). Vectorial capacity can be interpreted in the following way. The number of mosquitoes in the neighborhood of an infected subject is given by m , of which a proportion a will actually bite, leading to ma mosquitoes that will be infected with *Plasmodium*. First, the mosquitoes will have to survive the EIP consisting of n days, and

probability to still be alive after EIP is thus p^n . Thus, $ma p^n$ mosquitoes will survive the EIP and can then start transmitting *Plasmodium*. The average lifespan of these infectious mosquitoes is given by $1/(-\ln p)$, and thus the total available infectious bites corresponds to $(ma p^n)/(-\ln p)$, but only a proportion a will be received by a subject, leading to the VC formula. Obviously, VC is immediately linked to malaria dynamics and incidence (Johnson et al., 2020).

A number of factors that have an impact on VC are listed in Table 5.1. Reduction of mosquito density, the fecundity of mosquitoes, environmental factors in the larval breeding environment and stress from larval competition all have an impact on m . The biting frequency and rate have an effect on a . Longevity has an impact on p . The length of the EIP impacts on n . Finally, mosquito susceptibility to the *Plasmodium* parasite is not implicitly present in the model, but can be absorbed in a , by reducing the proportion with the fraction of mosquitoes that will not be infected despite a blood meal on an infected subject. In this work, we mainly concentrate on the reduction of longevity.

Already harsh conditions prevail in the environment of the malaria vector. After an infectious blood meal, the mosquito has to survive at least 10 to 14 days (the EIP) before it can start to transmit the two most virulent malaria parasites, *P. falciparum* and *P. vivax* (Ohm et al., 2018). Only a small fraction of the mosquitoes, typically smaller than 10%, survives long enough to start to transmit (Figure 5.1). It even gets worse for zoophilic malaria vectors such as *An. arabiensis*. Those mosquitoes could first feed on a non-human host which can never result in *Plasmodium* infection, and therefore the mosquito would have to live even longer to be able to transmit *Plasmodium*. On the other hand, assuming that the mosquito first feeds on human and ingests *Plasmodium*, there remains ample time for a zooprohylactic approach as the mosquito will have to blood feed on several occasions during the EIP (Figure 5.2).

5.2 Reducing mosquito longevity by host vaccination

The present work demonstrated that silencing of the midgut genes, and more specifically FN3D1, through RNAi techniques significantly shortens the lifespan of *An. arabiensis* mosquitoes (Debalke et al., 2019).

Factors	Mechanisms
Mosquito density	• Reduce abundance of adult mosquitoes using, e.g., LLINs, IRS (WHO, 2015), and Ivermectine (Chaccour et al., 2013) • <i>Wolbachia</i> (Cytoplasmic Incompatibility) (Hughes et al., 2011) • Larvicidals ((Fillinger and Lindsay, 2011)
Fecundity	• <i>Wolbachia</i> (Cytoplasmic Incompatibility) (Hughes et al., 2011) • Ivermectin: (Chaccour et al., 2013)
Biting frequency/rate	• Reduce contact: physical barrier, e.g., LLINs (WHO, 2015) • Improving housing condition (Mburu et al., 2018) • Repellent (Karunamoorthi et al., 2008)
Longevity	• Interrupting the IMD pathway (Debalke et al., 2019) • Insecticidal effect (WHO, 2015) -LLINs -IRS • Ivermectin (Chaccour et al., 2013)
Susceptibility to parasite	• Inducing the immune system (Dong et al., 2009) • Genetic modification of organisms to express anti-pathogenic effector molecules (Favia et al., 2008) • Direct inhibition of parasite development (Van Tol and Dimopoulos, 2016)
Extrinsic incubation period	• Environmental factors & genetic diversity (Ohm et al., 2018)
Environmental factors in larval breeding environment	• Physicochemical characteristics of water (Oyewole et al., 2009) • Availability and abundance of food (Okech et al., 2007)
Stress from larval competition	• High number of competitors and predators (Muriu et al., 2013) • Impact on their biological trait: growth, fecundity, longevity

Table 5.1 Factors that impact on vectorial capacity (VC) and possible mechanisms

This gene is involved in maintaining the homeostasis of midgut microbiota (Stathopoulos et al., 2014). The blood meal triggers microbiota proliferation in the mosquito midgut following the feed. After every blood meal, the number of bacteria in the mosquito gut increases 100-1000 times in 24-30 hrs (de O Gaio et al., 2011; Kumar et al., 2010; Meister et al., 2009). By 72 hours after the blood meal, the mosquito must restore to pre-blood feeding levels to limit damage to the gut epithelium and to re-establish homeostasis in order to prevent a systemic infection which eventually kills the mosquito (Gendrin et al., 2015). Manipulation of the homeostasis of mosquito gut microbiota can be the basis for the development of novel malaria vector control methods (Rodgers et al., 2017). The FN3D1 gene (Debalke et al., 2019) could be a good candidate to interfere with the immune system and thus the manipulation of the homeostasis of mosquito gut microbiota. Such strategies could include anti-mosquito vaccines, discussed in this section, and anti-mosquito gene-drive using genetic engineering technology, discussed in the next section.

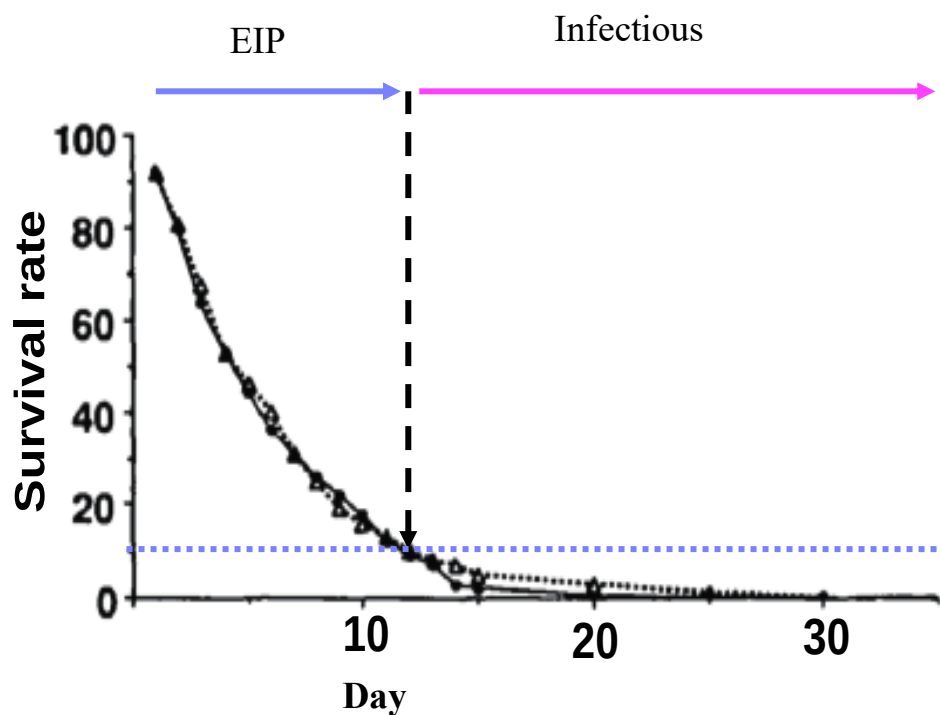


Figure 5.1 Daily mosquito survival with respect to EIP of *Plasmodium* and age at which the mosquito can become infectious (adapted from Geoffery et al., 1990).

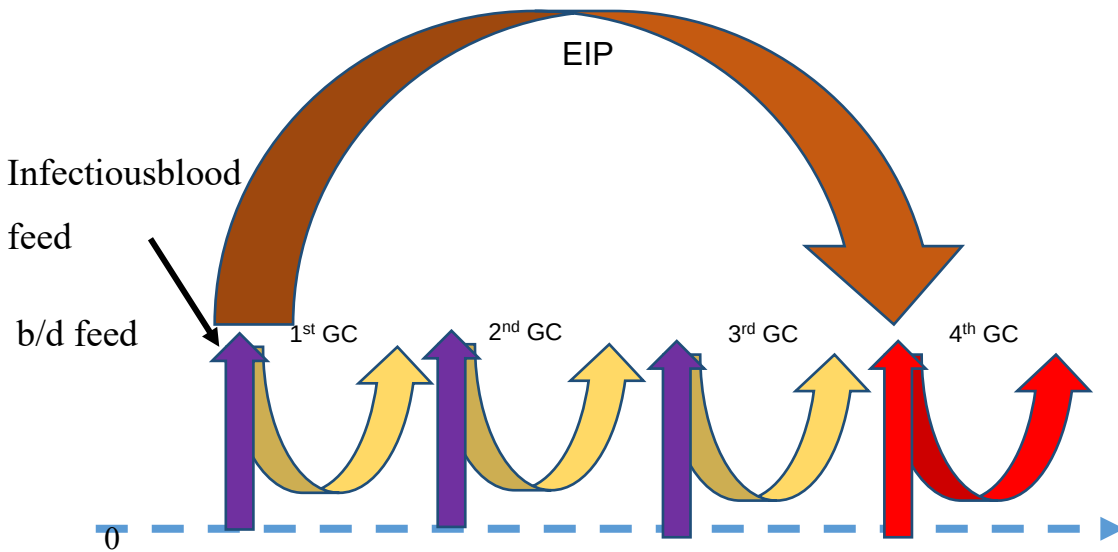


Figure 5.2 Chronological age of mosquito in relation to consecutive blood feed (purple arrow), gonotrophic cycle (GC) (yellow arrow) and EIP (orange arrow).

The aim of the anti-mosquito vaccine based on the manipulation of the immune system is to interrupt the malaria transmission cycle by shortening the mosquito lifespan after a blood meal on a vaccinated host. When the mosquitoes feed on the immunized host they ingest antibodies that neutralize the function of the immune system protein leading to a disruption of the midgut homeostasis leading to a reduction of longevity. Mosquitoes that have taken an infectious blood meal will typically take three to four additional blood meals before the completion of a sporogonic period as they normally blood-feed every 2–3 days (Johnson et al., 2020) (Figure 5.2). This ensures repeated ingestion of anti-mosquito antibodies with consequent and repeated disruption of gut bacterial homeostasis.

This strategy exploits the fact that the important malaria vector *An. arabiensis* takes blood meals both on humans and livestock. *Anopheles arabiensis* is a promiscuous blood feeder with a high preference for livestock (Habtewold et al., 2004). Studies in Ethiopia (Asale et al., 2017; Eba et al., 2021; Massebo et al., 2013, 2015), Eritrea (Okbaldet et al., 2006), Kenya (Degefa et al., 2017)

and Tanzania (Mahande et al., 2007) show that two-thirds of *An. arabiensis* mosquitoes take their blood meal on cattle. In a large part of Africa, humans and livestock live in close association which makes zooprophyllaxis a possible strategy for vector control (Donnelly et al., 2015; Mahande et al., 2007). Livestock vaccination against blood-sucking ticks has already been developed, hence it is possible to bring together the two vaccination strategies into an integrated solution for animal and human health.

Potential vaccine targets are therefore the *FN3D1* genes as they are essential in the functioning of the immune system. Such vaccines could also be integrated with the delivery systems that already exist in many low-income countries for other diseases .

An analysis using antibody epitope prediction tools (<http://tools.immuneepitope.org/bcell/>) confirmed that *FN3D1* genes are promising immunogenic epitopes. Delivery of anti-mosquito vaccine via cattle will have the following advantages: (1) Easy integration with the existing annual veterinarian vaccination system and (2) tackling outdoors/residual transmission, hence complementing the existing control strategies.

A potential problem with an anti-mosquito vaccine strategy is the high cost of the development of vaccines. The limited prospects of financial reward of this technology may make it unattractive for the private sector to invest in such a technology. Secondly, there might exist a lack of willingness of the cattle holders to present animals regularly and pay for the vaccine as no direct impact of the vaccine on the performance of immunized animals is observed. This will reduce the vaccine coverage and compromise the overall impact of this intervention. Therefore, this strategy needs to be implemented at the public level and not left to the private sector.

5.3 Reducing mosquito longevity by genetic manipulation of mosquitoes

In the context of the current work, particular genes that reduce the longevity when silenced were found. Mosquitoes can be engineered to contain genes to counter the effect of essential genes for the mosquito, e.g., for its immunity system. Such genes are termed effector genes, and are discussed below in the first section. These engineered mosquitoes can next be introduced in the mosquito population to spread these effector genes. However, the effector gene will probably not

survive in the population as it does not have a survival or reproduction benefit. Therefore, another system must be engineered in the mosquito, the gene drive, in order to increase the frequency of the effector gene in the wild mosquito population quickly. This is the topic of the second part of this section.

5.3.1 Effector component

Using the RNAi technique, we demonstrated that whenever certain immunity-related genes are silenced, it leads to the disruption of the homeostasis of midgut microbiota which will eventually shorten the mosquito's life. We can utilise CRISPR-based techniques to generate mosquito strains that can express molecules that could silence the target gene to disrupt mosquito midgut homeostasis. This can be achieved using two different approaches, either by neutralizing the effect of the proteins of the immunity-related genes or by destruction the mRNA that would lead to the production of such proteins.

5.3.2 Single-chain antibodies neutralizing the FN3D1 gene protein

A single-chain variable fragment (scFv) is a fusion protein of the variable regions of the heavy (vh) and light chains (vl) of immunoglobulins, connected with a short linker peptide of 10 to about 25 amino acids. The single-chain antibody molecule retains the original specificity of the parental immunoglobulin (Bates et al., 2019). The bigger the size and complexity of monoclonal antibodies (mAb), the more they are unsuitable as effector molecules and difficult for the use in genetic engineering. Single-chain antibodies retain the binding specificity and are much smaller and more suitable. Besides their specificity and efficacy, their relatively smaller size makes single-handed antibodies suitable for integration in the host genome by using genetic engineering techniques with minimum stress and less fitness cost for the host organism (Kang et al., 2020; Raag et al., 1995).

Previously, scFvs produced from a single transcription unit targeting the *P. gallinaceum* circumsporozoite protein (CSP) effectively inhibited sporozoite invasion of salivary glands of transgenic *Aedes aegypti* mosquitoes (Jasinskiene et al., 2007). More recently, engineered *An. stephensi* mosquitoes expressing the scFvs m1C3, m4B7 (derived from monoclonal antibodies that bind the *P. falciparum* ookinete proteins Chitinase 1 and Pfs25,

respectively) or m2A10 (binding the CSP, the predominant surface protein of sporozoites) have significantly blocked *P. falciparum* development (Isaacs et al., 2011). Also, in *Anopheles* mosquitoes a gene was transgenically integrated to express an anti-sporozoite single-chain antibody fused to the antiparasitic protein Scorpion resulting in almost complete suppression of the sporozoite form of the parasite (Dong et al., 2020). In the present context, scFvs based on FN3D1 gene mAb can be engineered into the genome of *An. arabiensis* mosquitoes to generate transgenic strains expressing the single chain antibodies in a specific tissue (here, midgut) and at a specific time-point (here, post bloodmeal). FN3D1 directed scFvs can be fused to specific host genes to achieve site and physiological-time specific expression of transgenes. Several promoter elements driving tissue-specific expression in relevant tissues have been characterised in malaria vectors. These include the regulatory elements of the zinc carboxypeptidase A1 (CP), peritrophin1 (Aper1) and the vitellogenin (Vg) genes that have been reported to drive transgene expression (Abraham et al., 2005; Nirmala et al., 2006; Nolan et al., 2011; Volohonsky et al., 2015).

The advantage of this engineering approach is that it allows to fuse a series of multiple scFvs of different genes in the pathway of midgut microbiota homeostasis which could provide an additive effect and slow development of resistance against the transgene.

5.3.3 RNA-targeting CRISPR–Cas effector Cas13 to silence *FN3D1* gene mRNA

Standard gene silencing using dsRNA delivered to organism is capable to induce RNA interference and can efficiently knockdown RNAs. This principle is furthered by recent discoveries and development of RNA-guided RNA-targeting CRISPR–Cas effector Cas13 (Abudayyeh et al., 2016; Shmakov et al., 2015). Cas13 RNA nucleases are the new members in the CRISPR nuclease family which specifically target endogenous mRNAs. Most Cas13 proteins are single effector proteins with two Higher Eukaryotes and Prokaryotes Nucleotide-binding (HEPN) domains (Abudayyeh, et al 2016). Once loaded with a target- specific crRNA, a Cas13 protein will locate target RNA and execute nuclease activity to degrade the target. To date, four subtypes of the Cas13 family are identified: Cas13a, Cas13b, Cas13c and Cas13d. The smaller size of Cas13 compared to Cas9 makes the former more suitable for transgenic integration into the host genome with a minimum gene modification effort.

Unlike the scFvs, the Cas13 system targets mRNA. Specific guide-RNA (known as crRNA) of 60 to 66 nucleotides is used for optimum target specificity. As CRISPR/Cas13 mediates RNA degradation, it has a promise to replace the RNA interference (RNAi) technique (Huyhn et al., 2020). Like Cas9, the Cas13 system uses guide RNA to identify its substrate, however, the latter targets RNA rather than DNA. In the presence of a target-specific crRNA, a Cas13 protein will locate target RNA and execute nuclease activity to degrade the target.

In the context of the present work, Cas13d mediated knock-down of *FN3D1* transcript could be achieved to block the translation of *FN3D1* mRNA into protein and hamper mosquito regulation of gut homeostasis. Transgene constructs can be designed in midgut-specific manner to restrict mRNA silencing to the gut and at physiological time when midgut proliferation takes place so that the mosquito is prevented from regulating the homeostasis. The construct could be fused to midgut specific host genes known to restrict mRNA knock-down to the midgut.

The Cas13 system has been tested in mosquito species recently. The Cas13 mediated CRISPR technique was developed for targeted mRNA silencing in mosquitoes by Kulkarni et al. (2020) using a Cas13a plasmid which was delivered by direct intrathoracic injection into adult mosquitoes. For example, in *Anopheles gambiae*, the vitellogenin gene was effectively silenced by Cas13a/Vg-crRNA resulting in a significant reduction in egg production. The Cas13a/crRNA system was also applied in *Aedes aegypti* to successfully silence the COPI genes to induce mortality and fragile midguts. Co-silencing genes simultaneously is achievable when a cocktail of target crRNAs is given without obvious fitness cost (Kulkarni et al., 2020).

5.3.4 Gene drive component

Gene-drive systems function in the germline to change heterozygous to homozygous cells and achieve the super-Mendelian inheritance necessary for introducing the effector gene in the naturally occurring mosquito population. This system features a DNA-cutting enzyme (called Cas9) and a guide RNA (or gRNA) that targets cuts at specific sites in the genome. In the newly developed split gene drive system, the cas9 enzyme guided by gRNA in the effector component induces a double-strand break at the same position on the wild-type allele, which is then repaired via homology-directed repair (HDR) using the intact chromosome carrying the effector element

and the gene drive component as a template. The effector element, in the present context, is the anti-*FN3D1* single chain antibody.

Recently an integral gene drive engineering technique was evaluated involving gRNAs expressed within a synthetic intron placed inside the transgene coding sequence for the CRISPR-Cas9 system (Hoermann et al., 2021). In the presence of a source of Cas9, the effector single-chain antibody gene is homed into a wild-type chromosome, triggering a gene drive effect that can increase the frequency of the modification in the mosquito population.

The desired traits can be forced to spread in wild populations using the gene drive mechanism. Gene drive is currently being developed to eradicate malaria by eliminating *Anopheles gambiae*, one of the main mosquito vectors for the transmission of malaria in Africa (Kyrrou et al., 2018). Other examples of the use of gene drive are to protect endangered species by eliminating rats from New Zealand islands (Teem et al., 2020), to eliminate a target population (Hoermann et al., 2021; Kyrrou et al., 2018) or to replace a population, e.g., by spreading an anti-malarial trait within a vector population (Adolfi et al., 2020; Gantz et al., 2015; Pham et al., 2019). The laboratory experiments demonstrate that these strategies could be deployed to eliminate malaria transmission in the field. The first field trial is expected to involve gene drive mosquitoes in Uganda, Mali or Burkina Faso in 3-5 years (precursor trials of GM mosquitoes occurred in Burkina Faso this year).

Like other mosquito control interventions, efficacy will greatly depend on minimising the development of resistance to the gene drive mechanism. Resistance against the cas9 drive system might develop in the mosquitoes. One cause could be the presence of a pre-existing allele variation in the wild mosquito population where the gRNA can not recognise the DNA cut site and/or *FN3D1* gene mRNA destruction site. Such population might grower faster in the presence of selection pressure and impade the gene drive. This risk can be minimised by targeting sequences that are highly conserved, which implies that changes cannot easily be tolerated. Also, multiplex scFv engineering can slow the takeover of the resistance as the probability of individual mosquitoes carrying multiple resistant alleles is slim. Second, gene drive resistance could arise when the repair of the cleaved chromosome is mediated by non-homologous end joining. One approach to reduce the development of resistance to a gene drive is to target regions that are

structurally or functionally constrained and therefore less likely to tolerate insertions or deletions ('indels') and substitutions that could lead to resistance.

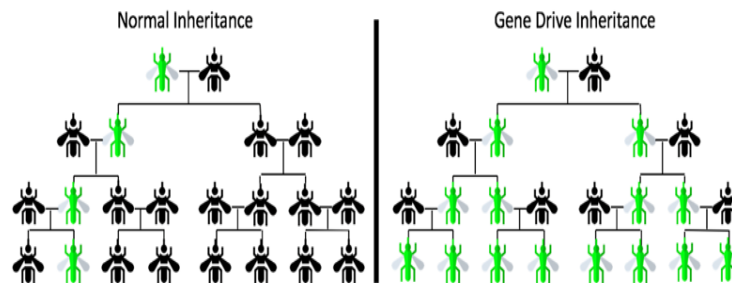


Figure 5.3. Gene drive model showing super mendelian transmission of transgene in the wild population. The left panel shows mendelian inheritance. An engineered mosquito with the effector on one of the two genes (green mosquito) is introduced and mating with the naturally occurring population (black mosquitoes). Only one quarter of the offspring will have the effector gene on one of its chromosomes. Eventually, the effector gene will disappear as it does not have any survival or reproduction benefit. The right panel shows the gene drive inheritance. Although the mosquitoes are also heterozygote with respect to the effector gene, the gene drive system makes that during the production of gametes most of them contain the effector gene (and also the gene drive system), which makes that the effector gene can spread through the naturally occurring population (Source, VigiLab, Imperial College London).

5.4 Reducing mosquito longevity for *An. arabiensis* and other *Anopheles* species

Anopheles arabiensis dominates malaria transmission in Africa and is often the sole vector of *Plasmodium* across the Sahelian belt and sub-deserts, the Great Horn region and the southern cone of Africa (Drake et al., 2014; Moffett et al., 2007). Importantly, these regions are along the geographical margins of perennial malaria and are therefore considered to be the likely “second line elimination regions” to follow their southernmost and northernmost malarious neighbors. The importance of *An. arabiensis* as a primary human malaria vector is continually increasing, even in the inland Afrotropical regions, e.g., the mosquito species was responsible for 66% of annual malaria transmission in Senegal, West Africa (Fontenille et al., 1997). In rural African countries

including Ethiopia, cattle are the most abundant form of livestock, owned by many households (Fereja et al., 2017). They are penned at night in corrals located within the homestead or cohabitate with humans, particularly in Ethiopia and Eritrea. In fact, strategic placement of livestock as lures is suggested to deflect nocturnal *An. arabiensis* bites and is termed malaria zooprophylaxis (Franco et al., 2014; Habtewold et al., 2004; Hasyim et al., 2018). From West to East Africa, this mosquito is increasingly zoophilic (BØgh et al., 2001).

Accumulating evidence shows that *An. arabiensis* is replacing *An. gambiae* and *An. funestus* in areas where these vectors co-exist. Meteorological records indicate that SSA, especially East Coast regions, becomes increasingly drier creating climatic conditions conducive for *An. arabiensis* and prohibitive for *An. gambiae* and *An. funestus* (Hinne et al., 2021). This process is thought to be accelerated by the expanded use of LLINs and IRS, the mainstream malaria vector control measures presently in SSA. Long lasting insecticide nets and IRS are designed to target female mosquitoes seeking a blood meal indoors from humans sleeping under the treated nets. Therefore, vectors that blood feed (exophagic) and/or rest outdoors (exophilic) are either excluded or not adequately targeted. Accordingly, LLINs and IRS remove the highly endophilic *An. gambiae* and *An. funestus* thus relieving larval competition from *An. arabiensis*. For instance, the expanded and sustained use of LLINs in Kenya and Tanzania where *An. arabiensis* co-existed with *An. gambiae* and *An. funestus* is thought to have led to an increased importance of *An. arabiensis* as a malaria vector whereas the role of other vector species has gradually diminished (Kreppel et al., 2020). Indeed, this non-uniform effect of current control tools on vector populations is one of the major setbacks to the present malaria eradication efforts.

Vaccination approaches can be applied for other *Anopheles* mosquitoes including the secondary malaria vectors *An. rivolorum*, *An. pharoensis*, *An. coustani*, *An. ziemanni* and *An. squamosus*. They are often highly zoophilic, exophagic and exophilic vectors. These vectors contribute to residual malaria transmission and challenge the malaria control and eradication agenda (Afrane et al., 2016; Cross et al., 2022)

In some African regions primary *Anopheles* vector species are replaced by the secondary species. For instance, in Zambia a temporal consistent spatial distribution and anopheline community

composition with a dominant number of secondary vector species compared with primary ones has been identified from larvae and a smaller survey of adult mosquitoes sampled in 2019 and 2017/18 (Cross et al., 2022). However, these vectors are not well addressed by the currently available approaches LLINs and IRS.

The vaccine strategy presented here is part of a zooprophylactic approach, i.e., bovines are vaccinated, and is therefore based on the premises that the particular mosquito species feeds also on bovines. This is not the case for vectors like *An. gambiae s.l.* for which this vaccination strategy cannot be used. In such cases, human vaccination could be used, but this comes with quite a few ethical problems as the vaccination does not have an immediate protective effect for the recipient. Such problems were also encountered with the so-called transmission blocking vaccines.

5.5 Beyond Anopheles: the sand fly case

To explore the similarities and potential in other important vectors, the sand fly is discussed as an example in this section.

5.5.1 Leishmaniasis, its etiology and vector

Along similar lines as a mosquito killing vaccine, killing vaccines could possibly be developed for other hematophagous vectors such as the tsetse fly and the sand fly. This is based on the fact that some insects share similar characteristics in terms of their host preference, blood feeding habit, immune system and interaction with and dynamics of their gut microbiota. In the context of the present work we consider sand fly as a potential case. The sand fly is a vector that transmits *Leishmania* and other viral and bacterial diseases. *Leishmania* is an intracellular protozoan parasite belonging to the genus *Leishmania* which causes the disease called leishmaniasis (Maroli et al., 2013).

The *Leishmania* parasite infects different parts of the body including the skin, the mucosa layer of the mouth and nose and internal organs including liver, spleen and bone marrow. It causes different disease types including cutaneous leishmaniasis (localized and disseminated) (Reithinger et al., 2007), muco-cutaneous leishmaniasis (tegumentary leishmaniasis) and visceral leishmaniasis

(kala-azar) (Maroli et al., 2013) (Table 5.2). Cutaneous leishmaniasis (CL) is a less severe form of the disease and is usually self-healing. Muco-cutaneous leishmaniasis (MCL) is characterized by mucosal deforming lesions on the mouth, nose and throat. Visceral leishmaniasis (VL) is the most severe form of the disease and results in a 100% death rate if left untreated (Hide, 2007). Leishmaniasis is the second most important disease next to malaria. Visceral leishmaniasis is endemic in East Africa including Ethiopia, Kenya, Somalia, Sudan and Uganda, where many people die due to epidemic outbreaks. Ninety percent of VL occurs in India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil (Alvar et al., 2012).

In African countries, *L. donovani* is the parasite that causes the visceral leishmaniasis while *L. major* and *L. tropica* are responsible for local cutaneous leishmaniasis (LCL) and diffuse cutaneous leishmaniasis (DCL) respectively. *Leishmania aethiopica* is restricted to the highlands of Ethiopia and Kenya (Cunze et al., 2019). In Ethiopia, *L. aethiopica* causes both DCL and LCL while *L. donovani* causes VL (Alemayehu and Alemayehu, 2017).

Vector borne diseases occur often where malaria is present (Golding et al., 2015), thus they are suited for integrated vector control targeting both diseases. Integrated vector control of multiple disease is a cost-effective approach (Golding et al., 2015). This could be extended, in our approach, to integrated vector control through double vaccination.

Leishmania is transmitted mainly by the infected female sand fly (Hide, 2007). *Phlebotomus* and *Lutzomyia* are known vectors of *Leishmania* in the old and new world respectively (Alemayehu and Alemayehu, 2017; Cox, 1996; Maroli et al., 2013). In the Old World there are about 42 suspected species and 20 are proven to be involved in disease transmission (Maroli et al., 2013). In most cases, a particular vector species is responsible for the transmission of only one *Leishmania* parasite species. However, in some regions, one vector species transmits more than one *Leishmania* parasite species. This is the case in Ethiopia for *Phlebotomus sergenti* which transmits both *L. tropica* and *L. aethiopica* (Gebre-Michael et al., 2004).

5.5.2 Host preference

The host preference and feeding behavior of vectors are essential parameters to understand the transmission of the parasite and develop control strategies for the diseases they transmit. Like *An. arabiensis*, *P. orientalis*, the main vector in East Africa (Elnaiem et al., 2011), is a highly opportunistic feeder (human and animal) with a main preference for large domestic animals that are commonly found around the village (Yared et al., 2019). Mixed blood meals from different blood sources (primarily human and cattle) have been reported and constitute an indication for their opportunistic feeding behavior (Lemma et al., 2014; Yared et al., 2019). Zoophagic characteristics have also been observed (Gebre-Michael et al., 2010; Yared et al., 2019). Other supporting findings constitute the presence of antibodies against *L. donovani* in the blood of domestic animals in northwestern Ethiopia (Kenubih et al., 2015) and other parts of African countries, e.g., Sudan (Mukhtar et al., 2000).

Recently, in Ethiopia a new species, *Adlerius* has been reported (Pareyn et al., 2020). The African sand fly vector species and their geographical distribution are presented in Table 5.3. In Ethiopia, VL occurs in the lowlands concurrent with malaria (EPHI, 2015; Gebre-Michael et al., 2010) although it has been found as well in the highlands of northwestern Ethiopia (Herrero et al., 2009; Pareyn et al., 2019).

5.5.3 The sporogonic life cycle and blood feeding habit

Leishmania has two main morphological forms in the life cycle, the intracellular amastigotes and promastigotes in the mammalian and in the vector host respectively. Promastigotes can be found in different forms. Female sand flies feed on different blood sources from mammalian hosts for their egg production. During the blood meal, multiplicative procyclic promastigotes of the parasite are ingested. The procyclic promastigotes differentiate into non-dividing nectomonad promastigotes, which migrate to the anterior midgut where they differentiate into leptomonad promastigotes forms. Next, they differentiate into non-differentiating mammalian infective metacyclic promastigotes (Bates and Rogers, 2004). In the laboratory the colonization of anterior parts of the midgut and the stomodeal valve of *P. orientalis* has been observed at day 5 post blood meal with small infection rate and at day ten with a high infection rate at a temperature of 26°C

Leishmania species	Disease type	Geographical Distribution	Reservoir host	Vector species
Old World				
<i>L. tropica</i>	Diffuse	Europe, Asia, N. Africa	Dogs	<i>Phlebotomus</i> spps.
<i>L. aethiopica</i>	Diffuse/Localized	Ethiopia/Kenya	Hyraxes	<i>P. pedifer</i> , <i>P. longipes</i>
<i>L. major</i>	Localized	Asia/subSaharan Africa/Sahel belt, N. Africa, Sudan	Rodent	<i>P. papatassi</i> , <i>P. duboscqi</i>
<i>L. infantum</i>	Localized/Visceral	Mediterranean	Dogs/Foxes, cats/Jackals	<i>P. perniciosus</i> , <i>P. ariasi</i>
<i>L. donovani</i>	Visceral	Africa, Asia		<i>P. orientalis</i> , <i>P. martini</i> , <i>P. hiebotomus argentipes</i>
New World				
<i>L. mexicana</i>	CL	Central America	Rodents	<i>Lutzomyia olmeca</i>
<i>L. amazonensis</i>	CL	Brazil	Rodents	<i>L. flaviscutellata</i>
<i>L. pifanoi</i>	CL	Venezuela	Rodents	<i>Lutzomyia</i> spp.
<i>L. venezulensis</i>	CL	Venezuela		<i>Lutzomyia</i> spp.
<i>L. braziliensis</i>	MCL	Brazil	Rodents	<i>Psychodopygus</i>
<i>L. guyanensis</i>	C	S. America		<i>Lutzomyia</i> spp.
<i>L. panamensis</i>	C	Panama	Sloths	<i>Lutzomyia</i> spp.
<i>L. peruviana</i>	C	S. America	Dogs	<i>L. verrucarurn</i> , <i>L. pvmenis</i>
<i>L. chagasi</i>	V	S. America	Foxes	<i>Lutzomyia</i> spp.

Table 5.2 The main species of Leishmania that causes disease in human (Alemayehu and Alemayehu, 2017; Cox, 1996). *P. orientalis* and *P. martini* are the principal transmitters of *L. donovani* (El-naïem et al., 2011). In Ethiopia *P. orientalis* is the main vector of *L. donovani* (Gebre-Michael et al., 2004; Yared et al., 2019). *P. pedifer* and *P. longipes* are the two main vectors for *L. aethiopica* (Krayter et al., 2015).

(Seblova et al., 2013). Oviposition in the sand fly occurs 3 days after blood feeding and lasts 6 days. High mortality of females occurs after the first gonotrophic cycle and only few remain for the second oviposition (Srinivasan et al., 1993). The duration of the gonotrophic cycle is affected by the season, it takes 8-11 days during winter and 6-9 days in summer. Therefore, disruption of homeostasis can reduce the survival of these older females to block their ability to transmit the parasite.

5.5.4 Microbiota and gut homeostasis

Sand flies, as other hematophagous insects, maintain the homeostasis of gut after a blood feed. The insect microbiota has several roles including the maturation and triggering of the immune system of the vectors (Cirimotich et al., 2011; Telleria et al., 2012). Microbiota diversity has been shared by sand fly (Telleria et al., 2012) and mosquito (Boissière et al., 2012; Cirimotich et al., 2011; Ngo et al., 2015). The microbiota diversity in the gut of different sand fly species, both from laboratory and field specimens, has been reviewed recently. The result has shown that there are some common bacteria between laboratory and field sand fly vectors. The diversity of microbiota is affected by different factors including vector breeding habitat and the adult food source (Saab et al., 2020). For instance, *Phlebotomine* sand flies lay their eggs in the soil, animal burrows or tree trunk niches in the presence of a diversity of microorganisms. The developing larvae feed on these microbes together with other substrates. This is an important difference with *Anopheles* mosquitoes that breed in water where the larvae ingest bacteria and other foods available in the water. Volf et al. (2002) demonstrated the reduction in the diversity of bacteria after the blood meal, and the reverse is true for abundance with a dramatic increase of the number of bacteria. When the number of bacteria increases, the release of antimicrobial peptides (AMPs) is triggered.

The role of antimicrobial peptides (AMPs) including attacin, cecropin, four defensins (Def1, Def2, Def3, Def4) on the gut bacteria and the parasite has been studied in female *L. logipalpis*. The bacterial and parasite numbers increased 48 hours after a blood meal that was seeded with parasites. Following the blood meal, a high expression of attacin and Def2 has been observed at 72 hours. Concomitantly, the parasite and the bacterial load decreased at 72 hours post infection,

Vectors	Geographical distribution
<i>P. pedifer</i> , <i>P. longipes</i>	Ethiopia and Kenya
<i>P. papatassi</i> , <i>P. duboscqi</i> , <i>P. salehi</i>	sub Saharan Africa & Sahel belt, N. Africa, Sudan
<i>P. perniciosus</i> <i>P. ariasi</i>	Africa
<i>P. orinntalis</i>	Africa
<i>P. argentipes</i>	East Africa
<i>P. martini</i>	Ethiopia*, Kenya*, Somalia, Uganda
<i>P. sergenti</i>	North Africa
<i>P. celiae</i>	Ethiopia*, Kenya
<i>P. martini</i>	Ethiopia*, Kenya*, Somalia, Uganda
<i>P. orientalis</i>	Chad, Ethiopia*, Kenya, Sudan*
<i>P. vansomeranae</i>	Kenya
<i>P. ariasi</i>	Algeria, Morocco
<i>P. langeroni</i>	Egypt*, Tunisia*
<i>P. longicuspis</i>	Algeria, Morocco, Tunisia
<i>P. perfiliewi</i>	Algeria, Morocco
<i>P. perniciosus</i>	Algeria*, Morocco, Tunisia
<i>P. aculeatus</i>	Kenya
<i>P. arabicus</i>	Ethiopia
<i>P. chabaudi</i>	Morocco, Tunisia
<i>P. guggisbergi</i>	Kenya*
<i>P. rossi</i>	Namibia*
<i>P. saevus</i>	Ethiopia*
<i>P. sergenti</i>	Algeria*, Ethiopia* , Libya, Morocco*, Tunisia*
<i>P. duboscqi</i>	Burkina Faso, Chad, Ethiopia*, Gambia, Ghana, Guinea, Guinea-Bissau, Kenya*, Mali*,Mauritania, Niger, Nigeria, Senegal*, Sudan
<i>P. papatasi</i>	Algeria*, Egypt*, Libya, Morocco*, Palestine, Sudan, Tunisia*
<i>Adlerius</i> species	

Table 5.3 The African sand fly vector species and their geographical distribution. *Proven.

indicating the role of the AMPs expression both on the pathogen and the bacteria/gut homeostasis. On the other hand, silencing the expression of attacin favored the growth of bacteria (Telleria et al., 2021). Through this immune response the vectors maintain their gut homeostasis. In both the *Phlebotomine* sand fly (Telleria et al., 2012) and the *Anopheles* mosquito the gut microbiota homeostasis is essential to transmit the pathogen and to achieve vector longevity (Meister et al., 2009). In *An. arabiensis* mosquitoes, silencing the gut immune gene *FN3D1* significantly shortened the longevity of mosquitoes (Debalke et al., 2019). The ortholog of *FN3D1* gene has been discovered in *Phlebotomine* (PPAI001686) and *Glossina* (GBRI037331) with predicted similar function. Therefore, based on the above facts, integrated vaccine targeting vectors of both malaria and leishmaniasis could be a cost effective and considerable approach.

5.6 Future perspectives

This doctoral work demonstrated the effect of silencing specific immunity-related genes of the *Anopheles* mosquito on the disruption of gut homeostasis and its longevity. The reduction in longevity has a major effect on the vectorial capacity which might eventually lead to malaria incidence reduction and elimination. Especially the *FN3D1* gene had a substantial effect, which was demonstrated to be consistent.

There is still a long way to go from this fundamental observation to a viable vector control tool applicable in the field.

A first avenue could be vaccine development against the *FN3D1* gene. The antibodies generated in the livestock after immunisation need to block the effect of the *FN3D1* products.

A second avenue is the generation of transgenic *An. arabiensis* mosquitoes expressing Cas13 or single-chain antibody for silencing *FN3D1* and assessing its functionality. Obviously, the fitness of these transgenic mosquitoes must be assessed in a laboratory setting (in a semi-field system), and the best performing transgenic mosquito strain will be added to a gene drive system in order to ensure the spread in the population. Before starting this work, however, the acceptance of the introduction of transgenic mosquitoes and the gene drive approach must be evaluated in Ethiopia at the government level, but also through community discussion and surveys.

We further need to study the safety and environmental and ecological impact of the introduction of effector and gene driving transgenic mosquitoes. Especially the fact that this approach is based on weakening the immune system might also have an impact on *Plasmodium* infection and development in the mosquito. We need to establish whether silencing *FN3D1* has an effect on the susceptibility of the mosquito to *Plasmodium* to increase the mosquito VC.

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Summary

Malaria is a life-threatening infectious disease caused by the *Plasmodium* parasite. Especially in Sub-Saharan African countries malaria has high morbidity and mortality, particularly in children and pregnant women. About 3.4 billion people worldwide are at risk of contracting malaria and an estimated 627,000 people die of the disease each year with 90% of all malaria deaths occurring in the Sub-Saharan African region. In addition, malaria is a major constraint for economic development in Sub-Saharan Africa.

Malaria control interventions, particularly effective malaria drugs and vaccines, are crucial in malaria eradication programs, but the major tools in the fight against malaria remain the malaria vector control interventions.

Chapter 1 contains the general introduction discussing different topics. The epidemiology and burden of malaria is described. The next section concerns the *Anopheles* vector species that is responsible for malaria transmission. The sporogonic lifecycle of *Plasmodium* in the mosquito is first explained. The feeding and resting behavior and host preference of *An. Arabiensis* is described as this vector is one of the main challenges for the current malaria vector control tools as well as for the malaria control and eradication program. The chapter also introduces factors that determine the malaria transmission including longevity and VC of the mosquito. *Anopheles* mosquitoes do not necessarily transmit the *Plasmodium* parasite even if the female mosquito has been infected with malaria parasites. In order to be able to transmit malaria parasites a mosquito must live longer than the extrinsic incubation period of the parasite. The impact of environmental factors like quality and abundance of food in the larval breeding habitat, physical and chemical characteristics of breeding water on longevity and vectorial capacity of mosquito is also presented. The challenges posed on the current malaria control tools are also reviewed in this chapter with the main emphasis on the most commonly used current tools, i.e., long lasting insecticide treated net and indoor residual spraying. The innate immune system, especially the IMD pathway against the malaria parasite, is also discussed. The role of midgut microbiota in modulating the gut homeostasis is

discussed together with the immune genes (*FN3Ns*, *Gr9* and *PGRPLC3*) involved in gut homeostasis.

Chapter 2 presents the main objectives of the present study. The aim of this work is to lay the foundations for the development of a mosquito killing vaccine by depression of the immune system of the mosquito after blood feeding. Therefore, potential vaccine candidate genes involved in midgut epithelial immunity were screened by silencing these genes in the mosquito and evaluating its effect on survival of the mosquito. In a first study, a naturally occurring *An. arabiensis* population was used. In a second follow up study, the consistency of the silencing effect on mosquito survival was investigated in different *An. arabiensis* populations and under different circumstances.

Chapter 3 presents the result of the effect of silencing immunity related genes *FN3D1*, *FN3D2*, *FN3D3*, *GPRGr9* and *PGRPLC3* on longevity of naturally occurring *An. arabiensis* mosquito obtained from southwest Ethiopia. Significantly higher mortality rates were observed for *FN3D1* (hazard ratio (HR) =1.64, P=0.004), *FN3D3* (HR=1.79, P<0.001) and the *GPRGr9* silenced mosquitoes (HR=2.00, P<0.001) compared to a control group injected with dsRNA against a non-related bacterial gene LacZ. The bacterial load ratios for all target gene silenced mosquitoes compared to control mosquitoes was above 1, with the highest value for *FN3D1* equal to 2.66 (95%CI: [0.94;7.57]). When the mosquitoes have been treated with antibiotic mixtures (to reduce or eliminate the midgut bacteria) mortality rates in gene silenced mosquitoes reversed suggesting that gut microbiota have a major role in the observed reduction of mosquito survival.

Chapter 4 present the result of a follow up study performed on *An. arabiensis* mosquito which are reared in 3 different larval breeding site (Jimma, Asendabo, Wolkite) conditions to evaluate the stability of the gene silencing effect that has been observed in the previous study (Chapter 3). Environmental factor affects the longevity of mosquito. The physicochemical characteristics and bacterial load of water samples collected from different larval breeding habitat were presented. The level of dissolved oxygen was more suitable for larval development in water collected from Asendabo. Moreover, the bacterial load of water was also significantly higher in Asendabo compared to Jimma and Wolkite. The survival of *An. arabiensis* mosquitoes that have been reared

in different breeding site conditions has been assessed for both gene silenced and non-silenced (naive) groups. Naive mosquitoes reared using water collected from Jimma and Asendabo survived longer than mosquitoes reared using water collected from Wolkite. However, there was no significant variation between larval breeding sites (mosquitoes reared using water collected from different breeding sites) on the survival of *FN3D1* gene silenced mosquitoes indicating the gene silencing effect is stable.

In **Chapter 5**, we discuss the results of the studies and broaden the scope of these results towards different topics. First, the importance of vectorial capacity is discussed and the role that longevity of the mosquito plays in that equation. It is followed by a description of how the knowledge of the effect of the studied immunity genes on longevity could lead to vaccine development but also to engineered mosquitoes that could be released in the field and together with the gene drive mechanism could lead to depressing or eliminating *Anopheles* populations. Next, the potential of a mosquito killing vaccine is discussed generally for different *Anopheles* species and also other arthropod vector species such as sandflies. The chapter concludes with a section on future prospects and proposed research.

Samenvatting

Malaria is een levensbedreigende infectieuze ziekte die veroorzaakt wordt door de *Plasmodium* parasiet. Malaria heeft op de eerste plaats in Sub-Sahara Afrika een hoge morbiditeit en mortaliteit, vooral bij kinderen en zwangere vrouwen. Een totaal van 3,4 miljard mensen leven met het risico om malaria te krijgen en het aantal personen dat jaarlijks aan malaria sterft wereldwijd wordt geraamd op 627.000 personen waarvan 90% in Sub-Sahara Afrika. Malaria houdt ook in belangrijke mate de ontwikkeling tegen van Sub-Sahara Afrika.

Malaria controle interventies, en meer specifiek het gebruik van efficiënte malaria medicijnen en vaccins, hebben een cruciale rol in malaria uitroeiing programma's, maar de belangrijkste instrumenten in het gevecht tegen malaria blijven de malaria vector controle interventies.

Hoofdstuk 1 bevat de algemene introductie waarin verschillende topics worden bediscuteerd. De epidemiologie en de ziektelast van malaria worden beschreven. De volgende sectie handelt over de *Anopheles* vector die verantwoordelijk is voor malaria transmissie. Eerst wordt de sporogonische levenscyclus van *Plasmodium* in de mug uitgelegd. Het voedings- en rustgedrag en de gastpreferentie van *An. Arabiensis* worden beschreven aangezien deze vector één van de meest problematische is om te controleren met de huidige malaria vector controle instrumenten en om malaria controle en uitroeiing programma's tot een succes te maken. Het hoofdstuk introduceert factoren die een invloed hebben op malaria transmissie zoals levensduur en vectoriële competentie van de mug. *Anopheles* muggen dragen niet steeds de *Plasmodium* parasiet over zelfs als de vrouwelijke mug geïnfecteerd is met malaria parasieten. Om de *Plasmodium* parasiet over te dragen moet een mug minstens zo lang leven als de extrinsieke incubatie periode van de parasiet. De impact van omgevingsfactoren zoals kwaliteit en overvloed van voedsel in de larvale broedplaats habitat, fysieke en chemische karakteristieken van het water van de broedplaats op de levensduur en vectoriële capaciteit van de mug wordt ook voorgesteld. De uitdagingen die samenhangen met de malaria controle-instrumenten worden ook besproken in dit hoofdstuk met de nadruk op de meest gebruikte malaria controle instrumenten, i.e., “long lasting insecticide

treated nets' en "indoor residual spraying". Het aangeboren immuun systeem, vooral dan de IMD "pathway" tegen de malaria parasiet, wordt ook bediscuteerd. De rol van de microbiota in de middendarm van de mug in het moduleren van de darm homeostase wordt bediscuteerd tezamen met de immuniteit genen (*FN3Ns*, *Gr9* en *PGRPLC3*) die betrokken zijn bij de darm homeostase.

In **Hoofdstuk 2** worden de hoofdobjectieven van de huidige studie weergegeven. Het doel van dit werk is het leggen van de fundamenteën voor de ontwikkeling van een muggen dodend vaccin door onderdrukking van het immuunsysteem van de mug na een bloedmaaltijd. Potentiële vaccin kandidaat genen die betrokken zijn bij de middendarm epitheliale immuniteit werden gescreend door het uitschakelen van deze genen in de mug en het evalueren van het effect op overleving van de mug. In een eerste studie werd een natuurlijk voorkomende *An. arabiensis* populatie gebruikt. In een tweede opvolgstudie werd de consistentie van het effect van het uitschakelen van specifieke genen op de overleving van de mug bestudeerd in verschillende *An. arabiensis* populaties en onder verschillende omstandigheden.

In **Hoofdstuk 3** worden de resultaten voorgesteld van het effect van het uitzetten van immuniteit gerelateerde genen *FN3D1*, *FN3D2*, *FN3D3*, *GPRGr9* en *PGRPLC3* op de levensduur van natuurlijk voorkomende *An. arabiensis* muggen van Zuidwest Ethiopië. Er werd een significant hogere mortaliteit geobserveerd voor *FN3D1* (hazard ratio (HR) =1.64, P=0.004), *FN3D3* (HR=1.79, P<0.001) en de *GPRGr9* uitgeschakelde muggen (HR=2.00, P<0.001) in vergelijking met de controle groep die geïnjecteerd werd met dsRNA tegen een niet-gerelateerd bacterieel gen LacZ. De bacteriële lading ratio's voor alle doelgenen uitgeschakelde muggen in vergelijking met controle muggen was groter dan 1, met de hoogste waarde voor *FN3D1* gelijk aan 2.66 (95%CI: [0.94;7.57]). Wanneer de muggen behandeld werden met antibiotica mengsels (om de middendarm bacteriën te reduceren of te elimineren), daalden de mortaliteitscijfers tot op het niveau van de controle groep. Dit suggereert dat de darm microbiota een belangrijke rol hebben in het reduceren van de overleving van de mug.

In **Hoofdstuk 4** worden de resultaten besproken van de opvolgstudie uitgevoerd op *An. arabiensis* muggen populaties die afkomstig zijn van 3 verschillende larvale broedplaatsen (Jimma, Asendabo, Wolkite) met hun specifieke condities om de stabiliteit van het effect van het

uitschakelen van specifieke genen op overleving zoals geobserveerd in de vorige studie (Hoofdstuk 3) te evalueren. Omgevingsfactoren hebben een effect op de levensduur van de mug. De fysicochemische karakteristieken en bacteriële lading van waterstalen afkomstig van de verschillende larvale broedplaatsen werden voorgesteld. Het niveau van opgelost zuurstof was meer geschikt voor larvale ontwikkeling in water afkomstig van Asendabo. Bovendien was de bacteriële lading van het water ook significant hoger in Asendabo in vergelijking met Jimma en Wolkite. De overleving van *An.arabiensis* muggen afkomstig van de verschillende broedplaatsen met hun specifieke condities werd bestudeerd voor zowel muggen waarvan genen werden uitgeschakeld als controle muggen. Controle muggen die gekweekt werden in water van Jimma en Asendabo leefden langer dan muggen gekweekt met water van Wolkite. Er was evenwel geen significante variatie tussen de verschillende larvale broedplaatsen (muggen gekweekt met water afkomstig van de verschillende broedplaatsen) op de overleving van *FN3D1* gen uitgeschakelde muggen. Dit is een indicatie dat het effect van het uitschakelen van het *FN3D1* gen op de overleving van de mug stabiel is.

In **Hoofdstuk 5** worden de resultaten verder bediscuteerd en het gezichtsveld verruimd naar een aantal nieuwe thema's. Vooreerst wordt het belang van de vectoriële competentie aangeduid en de belangrijke rol die de levensduur van de mug in de vergelijking speelt. Daarop volgt een beschrijving hoe het effect van het uitschakelen van de geselecteerde immuniteit genen op levensduur zou kunnen gebruikt worden in het ontwikkelen van enerzijds een muggen dodend vaccin en anderzijds genetisch gemanipuleerde muggen die in het veld kunnen uitgezet worden en tezamen met het 'gene drive' mechanisme kunnen leiden tot het reduceren of elimineren van *Anopheles* populaties. Vervolgens wordt het potentieel van een muggen dodend vaccin besproken voor verschillende *Anopheles* species en eveneens voor andere arthropode vector species zoals de zandvlieg. Het hoofdstuk concludeert met een sectie aangaande de toekomstige perspectieven en voorgesteld onderzoek.

Curriculum Vitae

Serkadis Debalke Alene was born in Addis Ababa Ethiopia on 3 December 1979. After she completed her secondary school, she joined Jimma University medical laboratory school and obtained the diploma in medical laboratory technology in 2000. By the year 2005 she graduated in Bachelor of Science in medical laboratory technology from the same University. In 2009 she obtained the degree of Master of Science in Tropical and infectious diseases from Addis Ababa University, Ethiopia.

Serkadis was granted a PhD opportunity in 2015 via the VLIR-IUC program scholarship in a joint PhD program in Veterinary sciences and Biomedical sciences, organized by Ghent university and Antwerp University, Belgium.

Since 2000 Serkadis has been working in Jimma University medical laboratory school in different academic ranks. From 2014 up to date she is working for Jimma University medical laboratory school in an academic rank of assistant professor of infectious diseases.

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Acknowledgments

I would like to express my very sincere gratitude to my promoters: Prof. Dr. Ir. Luc Duchateau, Prof. Dr. Tibebu Habtewold, Prof. Dr. Guy Caljon, and also my advisor Prof. Dr. George K. Christopher. The actuality of this work wouldn't have been possible if it wasn't for each of my professors' continuous support, guidance, useful critique, and most of all their enthusiastic encouragement throughout my research and Ph.D. study journey.

Prof. Dr. Luc, I'm grateful for the consistent and friendly counsel you have put on from the very beginning of this project. Thank you for the compassionate support and generous helpfulness both in my scientific research and in other regards. Prof. Dr. George, thank you for generously sharing your time, knowledge, and resources including your laboratory. Your insights have deeply inspired and widened my perspective. And my sincere appreciation to Dr. Tibebu, your professional guidance, sharing your knowledge both in my lab and field activities, exacting attention, mentorship, and friendship have been huge assets to my work. Prof. Dr. Guy Caljon, you have been very supportive and willing to help me towards my career goals. Thank you for the extensive personal and professional support. It has been an honor to work under each of your guidance.

I would also like to extend my appreciation to Mr. Kora, thank you for the support you provided in facilitating my travel, supplementing for the successful completion of this work. Also my sincere appreciation to Mr. Kassahun Eba for assisting me on different occasions. And also big thanks to the Medical laboratory school of Jimma University for your encouragement. It's a pleasure to work with each of them. Thank you very much Mr. Zewdineh and Dr. Tilahun.

Yet, my completion of this project couldn't have been accomplished without the financial support and opportunity set forth by Ghent University Global Minds Funds, Antwerp University, VLIR_UOS (IUC-JU), and the NASCERE program. I am also thankful and appreciative to Andries, Klara, Annick, Medina, Buze, Belay, and Abiot for their support. My appreciation extends to the staff at Imperial College of the UK for the resources provided and for sharing with

me your knowledge. I am thankful to Gezahegn, Abdo, Mifta, Hussein, and Zahara for your contribution to the data collection.

Besides my professional environment, I'm extremely and deeply grateful for having a very caring, strong, determined mother, Wro. Achamyesh Duga. My mother, Emma, has always been there for me and my children; sacrificed herself, to support me in my journey and care for my kids in my absence without any complaint. I will never forget your support when I did a small experiment at home at a very young age. Your confidence in me has propelled me to achieve a place in the scientific field. Thank you, Mom.

My special thanks follow to my understanding and caring husband, Pr. Sisay. Sis, thank you for always standing by my side with committed support and prayer. My heartfelt thanks then go to my loving families; my sister Helina (Mimi), Dr. Nesha, Kana, my brothers Kidan, Tatek, Kalab, and Yibeltal, my uncles Dr. Taye, Gebey, and Melkamu. I'm so thankful for my family's persistent encouragement, love, prayer, support, and care ever since my childhood. And also grateful for my two sons: Dael Sisay (Joy) and Bennet Sisay (Sunny), both of you have been my abiding inspirations.

A huge thank you to Dr. Tibebu's wife, Astu, including their whole family, and my sister Rahel along with her husband and kids. I can't thank them enough for your generosity. Grateful for your gracious hearts and the comfort you delightfully served me with. George and Lily thank you very much for your care and love throughout my stay in Belgium.

My biggest appreciation to my family friends (Hundye, Seyu, Florence, Dr. Noh, Dr. Dink, Dr. Mahi, Kabi, Amani, Weyni, Mastewal, Yosef, Kumneger, Rahel, Hirut, and Sentayehu) who have always been my support throughout this journey with their time, generosity and consistent encouragement. I would also like to extend my appreciation to Pr. Delu, Mr. Ayshesh, Esku. Dr. Yeneneh, Aberu, Berhanu, Abegi, Geni, Bre, and many others for their sympathetic prayers and encouraging advice.

Last but most importantly, even though you won't be reading these, a huge thank you to my Father who planted the seed in me at a very young age; to go after and pursue the science field. Thankful for the confidence you dilated by believing in me.

Ultimately, I would like to praise and thank the almighty God, who has assured me with great opportunities, uncountable blessings, and for he has been faithful to be my strength through my weaknesses. Nothing would have been possible if it wasn't for God.