Curriculum Vitae

Haikel N. Bogale

haikelnb@gmail.com

EDUCATION

Ph.D. in Molecular Medicine May 2021 (expected)

Graduate Program in Life Sciences,

University Maryland, Baltimore

Dates Attended: July 2016-Present

Masters in Cellular, Molecular, and Biomedical Sciences 2016

Graduate Program in Life Sciences,

University Maryland, Baltimore

Dates Attended: August 2015-July 2016 (transferred to Ph.D.)

B.S. in Microbiology 2015

Department of Biochemistry and Molecular Biology,

The Pennsylvania State University

Dates Attended: August 2011-May 2015

Minor in Biochemistry and Molecular Biology 2015

Department of Biochemistry and Molecular Biology,

The Pennsylvania State University

Dates Attended: August 2011-May 2015

RESEARCH EXPERIENCE

Graduate Research Assistant 2017-Present

Institute for Genome Sciences,

University of Maryland School of Medicine, Serre Lab,

Research Advisor: David Serre

- Characterizing factors influencing parasitic infection of mosquito vectors

Undergraduate Research 2013-2015

Department of Biochemistry and Molecular Biology,

The Pennsylvania State University, Miyashiro Lab,

Research advisor: Dr. Tim Miyashiro

- Investigating the transcriptional regulator, NagC, in *Vibrio fischeri*

Ronald E. McNair Summer Research 2014

The Pennsylvania State University,

Research advisor: Dr. Tim Miyashiro

- Characterizing the regulation of *tfoX* in *V. fischeri*

Summer Research Opportunity Program (SROP), 2013

The Pennsylvania State University,

Research advisor: Dr. Tim Miyashiro

- Screening for differentially regulated *msh* genes in *V. fischeri*

PUBLICATIONS

1. **Bogale H.**, Pascini T., Kanatani S., Sa J., Wellems T., Sinnis P., Vega-Rodriguez J., Serre D. (2021) "Transcriptional heterogeneity and tightly regulated changes in gene expression during *Plasmodium berghei* sporozoite development". *PNAS.*

2. Cannon MV., **Bogale H.**, Bhalerao D., Yeroshefsky J., Dorothy J., Prendergast B., Fritz ML., Laurent B., Serre S. (2021) "High-Throughput Identification of Eukaryotic Parasites and Arboviruses in Mosquitoes". *In preparation.*
3. **Bogale H.**, Pascini T., Sa J., Wellems T., Vega-Rodriguez J., Serre D. (2021) "Transcriptional variations among salivary gland sporozoites of different *Plasmodium* species". *In preparation*.
4. **Bogale H.**, Cannon MV., Keita K., Camara D., Barry Y., Keita M., Coulibaly D., Kone AK., Doumbo OK., Thera MA., Plowe CV., Travassos M., Irish S., Serre D. (2020) "Relative contribution of various endogenous and exogenous factors on mosquito microbiota." *Parasites & Vectors.*
5. Cannon, MV., **Bogale**, **H**., Rutt, L., Humphrys, M., Korpe, P., Duggal P., Ravel, J., Serre, D. (2018) "A high-throughput sequencing assay to comprehensively detect and characterize unicellular eukaryotes and helminths from biological and environmental samples." *Microbiome*.
6. Sun, Y., Verma, SC., **Bogale**, **H**., Miyashiro, T. (2015) "NagC represses N-acetyl-glucosamine utilization genes in *Vibrio fischeri* within the light organ of Euprymna scolopes." *Frontiers in Microbiology.*

CONFERENCE & EXHIBITION PRESENTATIONS

Oral Presentations

Bogale H., Cannon MV., Keita K., Camara D., Barry Y., Keita M., Coulibaly D., Kone AK., Doumbo OK., Thera MA., Plowe CV., Travassos M., Irish S., Serre D. "Analysis of the genetic and non-genetic factors that influence the microbial composition of mosquitoes." 68th American Society of Tropical Medicine & Hygiene Annual Meeting, National Harbor, Maryland, 2019.

Bogale, **H**., Verma, SC., Sun, Y., Miyashiro, T. "Characterization of the Regulatory Network of *tfoX* in *Vibrio fischeri*." 2014 SROP/McNair Summer Symposium, The Pennsylvania State University, 2014.

Bogale, **H**., Verma, SC., Miyashiro, T. "Identification of *Vibrio fischeri* mutants that differentially regulate expression of the Mannose-Sensitive Hemagglutinin (MSHA) pilus genes." 2013 SROP/McNair Summer Symposium, The Pennsylvania State University, 2013.

Poster Presentations

Bogale H., Cannon MV., Keita K., Camara D., Barry Y., Keita M., Coulibaly D., Kone AK., Doumbo OK., Thera MA., Plowe CV., Travassos M., Irish S., Serre D. "A novel high-throughput molecular approach for vector-borne disease surveillance: Application to Malian and Guinean Mosquitoes." 67th American Society of Tropical Medicine & Hygiene Annual Meeting, New Orleans, Louisiana, 2018.

Bogale H., Cannon MV., Serre D. "High-throughput molecular assay for detecting and identifying eukaryotic parasites." 66th American Society of Tropical Medicine & Hygiene Annual Meeting, Baltimore, Maryland, 2017.

Bogale, **H**., Verma, SC., Sun, Y., Miyashiro, T. "Characterization of tfoX Regulation in *Vibrio fischeri* and its Impact on Colonization of the Squid Light Organ." American Society for Microbiology, Allegheny Branch meeting, Lycoming College, Williamsport PA, 2014.

Bogale, **H**., Verma, SC., Sun, Y., Miyashiro, T. "Characterization of tfoX Regulation in *Vibrio fischeri* and its Impact on Colonization of the Squid Light Organ." Spring Undergraduate Exhibition, The Pennsylvania State University, 2014.

Bogale, **H**., Verma, SC., Miyashiro, T. "Investigation of the regulatory network that controls expression of MSHA pilus genes." 17th Annual Environmental Chemistry and Microbiology Student Symposium, The Pennsylvania State University, 2014.

HONORS & AWARDS

Meyerhoff Graduate Fellowship 2016-Present
University of Maryland, Baltimore

Eberly College of Science Undergraduate Research Funds 2014-2015
The Pennsylvania State University

Ronald E. McNair Scholar 2014-2015
The Pennsylvania State University

Eberly College of Science Undergraduate Research Funds 2013-2014
The Pennsylvania State University

Bunton Waller Scholarship, 2011-2015
The Pennsylvania State University

Salizzoni Foundation Scholarship, 2011-2015
The Pennsylvania State University

University Trustee Scholarship, 2011-2013
The Pennsylvania State University

PROFESIONAL SOCIETY AFFILIATIONS

American Society of Tropical Medicine & Hygiene 2017-Present

American Society for Microbiology 2014-2015

MENTORING

Lab Mentor, Serre Lab, University of Maryland, 2019
Baltimore

- Supervised a college summer student on PCR-based experiments

UMB CURE Scholars Program, University of Maryland, Baltimore — 2017-2019

- Flex mentor to middle school science scholars

Learning Assistant, Introductory Microbiology Lab, The Pennsylvania State University — 2015

- Assisted the TA in teaching basic Microbiology lab techniques to undergraduate students

CAREER DEVELOPMENT

- TGen (Translational Genomics Research Institute) virtual meeting — 2019
- Genentech's Campus Engagement Week — 2020
- Meyerhoff Alumni Panel – Career Discussion — 2020

ACTIVITIES & SERVICE

- National Engineering Design Challenge Judge – Regional Maryland Engineering Science Achievement (MESA) Tournament for Baltimore County Public Schools — 2018
- Student Escort – Molecular Medicine Student Recruitment Committee — 2017-2019
- Activities Volunteer – Center for Talented Youth (CTY) Family Programs — 2017

Abstract

Title of Dissertation: Characterizing the influence of resident microbiota and mosquito factors on *Plasmodium* infections of *Anopheles* mosquitoes

Haikel N. Bogale, Doctor of Philosophy, 2021

Dissertation Directed by:

David Serre, PhD.,

Associate Professor,

Department of Microbiology & Immunology,

Institute for Genome Sciences

Pathogens transmitted by mosquitoes are responsible for illnesses that cause nearly 700 million cases every year. A significant proportion of this morbidity is due to malaria, a disease caused by *Plasmodium* parasites and spread through the bites of infected *Anopheles* mosquitoes. While we have seen a reduction in malaria mortality rates thanks to the development of antimalarial therapeutics and entomological controls, there is a potential of malaria resurgence associated with the emergence and spread of antimalarial and insecticide resistance, highlighting the need for additional malaria control strategies. Malaria transmission occurs only if one *Plasmodium* parasite develops through key stages in the mosquito, including passing through the midgut, which harbors a microbial community that can influence *Plasmodium* transmission. Despite the opportunities they present for novel interventions, the development of *Plasmodium* sporozoites and the factors

that shape the microbiota in mosquitoes are incompletely understood. Here, I sought to provide new insights into the microbial variations of wild-caught mosquitoes and the transcriptional regulatory programs of *Plasmodium* sporozoites. To this end, I simultaneously characterized the bacterial composition of 665 individual field-caught *Anopheles* mosquitoes in addition to their species, insecticide resistance genotype, blood-meal status, and infection status. My analyses revealed that mosquito collection site is the main driver of the microbial diversity, while other factors showed marginal or non-significant contribution. I also generated scRNA-seq data from 36,958 sporozoites of three *Plasmodium* species and collected from multiple anatomical sites of the mosquito and developmental stages of the parasite in an effort to better understand parasite developmental processes critical for malaria transmission. I identified transcriptional variations among salivary gland sporozoites of different *Plasmodium* species, patterns of gene regulation accompanying the journey of *Plasmodium berghei* sporozoites, and novel candidates potentially critical for mechanisms involved in sporozoite maturation. In addition, my analyses highlighted novel extensive transcriptional heterogeneity among sporozoites isolated from the same anatomical site, indicating asynchronous sporozoite development in the mosquito, that is regulated by intrinsic and environmental factors. Altogether, my findings improve our understanding of factors that influence malaria parasite transmission and lay the groundwork for identifying key transmission components, to inform development of novel intervention strategies.

Characterizing the Influence of Resident Microbiota and Mosquito Factors on *Plasmodium* Infections of *Anopheles* Mosquitoes

by
Haikel N. Bogale

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
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List of Abbreviations

ACT	Artemisinin-based combination therapy
ASV	amplicon sequence variant
DDT	dichloro-diphenyl-trichloroethane
DVS	dominant vector species
FDR	false discovery rate
GFP	green fluorescent protein
GMEP	Global Malaria Eradication Programme
HLC	Human Landing Catches
IBS	Johns Hopkins Institutional Biosafety Committee
Imd	Immune-deficiency
ITN	insecticide-treated bed net
JHSPH	Johns Hopkins School of Public Health
kdr	knockdown resistance
MRTC	Malaria Research and Training Center
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
OP	organophosphate
PBS	phosphate buffered saline
PCA	Principal component Analysis
PCoA	Principal coordinates analysis
PSC	Pyrethrum Spray Catches
RMgmDB	Rodent Malaria genetically modified Parasites database

rRNA	ribosomal RNA
scRNA-seq	single cell RNA-sequencing
SP	sulphadoxine-pyrimethamine
TBV	transmission blocking vaccines
WHO	World Health Organization

Chapter 1. Introduction

1. Malaria: a global infectious disease

1.1 The disease

Malaria is a disease caused by *Plasmodium* parasites and transmitted through infected *Anopheles* mosquito bites. The disease continues to have a significant global impact and the World Health Organization estimated that, in 2019, 229 million cases worldwide caused 409,000 deaths [1]. The mortality and morbidity caused by malaria is especially a burden on developing nations of Africa, Asia, and Central and South America [2]. More than 90% of the deaths associated with malaria occur in sub-Saharan Africa, with children younger than the age of five disproportionately afflicted [1, 2].

Many malaria endemic regions are economically limited and struggle with the challenges of providing finances for malaria prevention and treatment services [3, 4]. However, in the last decade, the increase of international aid in the form of funds from multiple agencies (e.g.,, US President's Malaria Initiative and Global Fund or the Gates foundation) have facilitated the expansion of malaria control measures, particularly in Africa [5, 6]. The expanded implementation of tools to control malaria, including antimalarial therapeutics and entomological control strategies, have significantly reduced malaria prevalence and mortality in endemic regions [7, 8]. Yet, the emergence and spread of *Plasmodium* parasites resistant to antimalarials and of insecticide-resistant *Anopheles* mosquitoes threaten to derail the efforts to eradicate malaria [9]. Hence, to sustain the decline of malaria mortality, and hopefully eventually eradicate the disease, there is a need for better

understanding malaria transmission in order to develop novel and complementary malaria control strategies.

1.2 The parasites

To date, over 150 *Plasmodium* species, unicellular organisms belonging to the phylum Apicomplexa, have been discovered [10]. Five *Plasmodium* species, *P. falciparum, P. vivax, P. malariae, P. ovale,* and more recently *P. knowlesi* [11, 12] were shown to frequently infect humans and cause malaria. *P. falciparum* is considered the most dangerous malaria parasite, accounting for the majority of cases and fatalities, while *P. vivax* is more geographically distributed, affecting human populations from four different continents [13].

In addition to infecting humans, *Plasmodium* parasites have been shown to infect primates (e.g., *P. cynomolgi* and *P. simium*), birds (e.g., *P. gallinaceum* and *P. relictum*), and rodents (e.g., *P. berghei* and *P. yoelii*) and studying these non-human malaria parasites has advanced our understanding of human malaria [14-16]. *P. berghei*, in particular, often serves as a model of mammalian malaria and provided valuable insights on parasite developmental biology and host-parasite interactions [14, 17, 18], and therapeutics (i.e., development and testing of vaccines and drugs) [19, 20]. The rodent malaria parasite, *P. berghei*, is recognized as an instrumental model parasite because of its similarity to human malaria parasites in overall parasite biology and genome organization [17, 21], availability of *in vivo* investigation of the complete life cycle, and *in vitro* culture with capabilities of mass production [22, 23], and amenability to genetic modification (e.g., reverse genetics) [24, 25].

Transmission of *Plasmodium* parasites, enabling malaria to persist over the course of human history, relies on a complex parasitic life cycle, consisting of several morphological stages occurring in the vector and mammalian host (Figure 1.1) [26]. In the mammalian host, *Plasmodium* parasites undergo multiple asexual multiplication steps, while sexual reproduction takes place in the mosquito.

Malaria transmission starts when a female *Anopheles* mosquito feeds on a *Plasmodium*-infected individual. *Plasmodium* parasites then develop inside the mosquito and when the mosquito takes a second blood-meal, the parasites are transmitted into a new host. However, transmission to new mammalian hosts requires the parasites to develop through different stages within the mosquito over 8 to 15 days. The parasite development starts in the mosquito with fertilization of a *Plasmodium* female gamete by a male microgamete, producing a zygote that differentiates into an ookinete in the mosquito midgut. Motile ookinetes then traverse the midgut epithelium and emerge on the basal surface to develop into round oocysts. Parasite development continues as each oocyst grows in size and produces through mitosis thousands of sporozoites. After oocyst rupture, the released sporozoites are carried by hemolymph towards the salivary glands. Some sporozoites then invade the salivary glands and remain there until they are released into a human host during a following blood meal [27, 28].

Upon injection into the human skin by a mosquito bite, sporozoites migrate from the dermis to the circulation before invading hepatocytes in the liver. In hepatocytes, sporozoites replicate and mature into schizonts which rupture and release merozoites in the blood. Merozoites then invade mammalian red blood cells to start the erythrocytic cycle in which merozoites multiply and mature into schizonts, schizonts rupture, and released merozoites

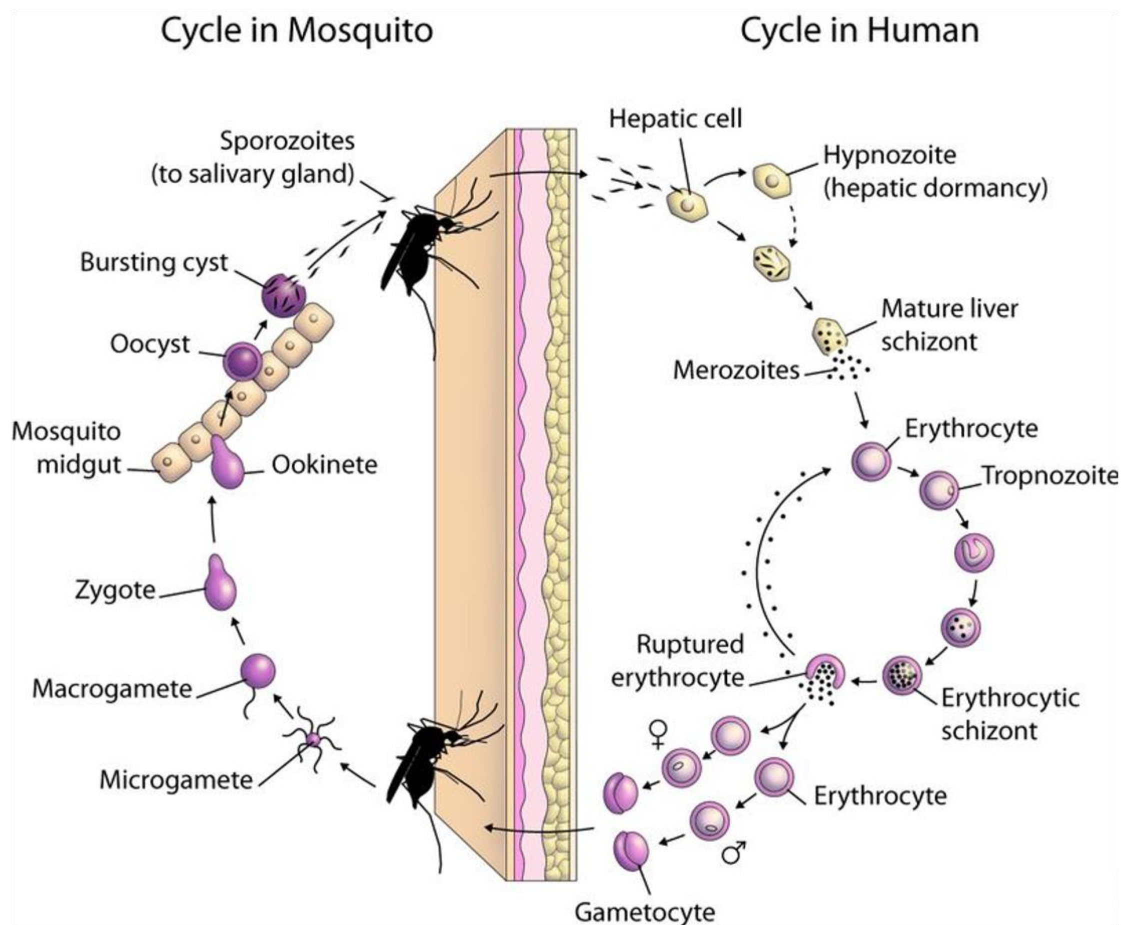


Figure 1.1. The life cycle of Plasmodium parasites in the mosquito vector and mammalian host.

Once injected into the mammalian host, sporozoites travel to the liver and invade hepatic cells. In hepatocytes, the parasites develop into merozoites (and some Plasmodium species can arrest their development in hepatic dormancy). Merozoites are released into the blood stream after the maturation of schizonts and rupture. Then merozoites invade erythrocytes, mature and multiply to produce new merozoites that go on to invade other red blood cells after discharge. Some parasites, however, break this cycle and develop into gametocytes, which are ingested by mosquito during blood feeding. Male and female gametes reproduced to form zygotes that develop into ookinetes. Ookinetes traverse the mosquito midgut and attach the basal side of the epithelium to form oocyst. Oocysts in turn mature and produce sporozoites which migrate to the mosquito salivary gland and are stored there until transmission into a mammalian host. (The schematic was adapted from [26]).

that invade new red blood cells. Unique to *P. vivax* and *P. ovale,* a dormant stage of the parasite termed hypnozoite, can result in delayed liver stage schizogony, in which merozoites are released into the blood stream weeks to years after the primary infectious mosquito bite [29]. Some parasites exit the erythrocytic cycle and differentiate into male and female gametocytes, the sexual stages of *Plasmodium*. The process of transmission is reinitiated when gametocytes are ingested by the mosquito and gametogenesis occurs inside the midgut.

The erythrocyte stages of *Plasmodium* cause the clinical symptoms of malarial disease [30, 31]. The erythrocytic proliferation of the parasite, through merozoite invasion of erythrocytes, multiplication, and erythrocyte rupture, occurs in a cyclic manner (i.e., 48-hour cycle for *P. falciparum*) that is associated with the symptoms of malaria that include: fevers, sweats, chills, fatigue, and headaches [30, 31]. The disease can also progress to severe malaria, which is characteristic of *P. falciparum* malaria, with dangerous and at times lethal complications such as cerebral malaria, severe anemia, and pulmonary edema [31, 32].

1.3 The vectors

Mammalian malaria transmission exclusively occurs through mosquito species of the *Anopheles* genus. The *Anopheles* mosquito life cycle consists of four morphologically distinct stages: egg, larvae, pupa and adult (Figure 1.2). Unlike their male counterparts, female mosquitoes blood feed and therefore transmit malaria parasites, as they require a blood meal to produce eggs. Throughout their entire life span, female *Anopheles* mosquitoes usually mate only once and lay eggs in a three-day period after each blood meal

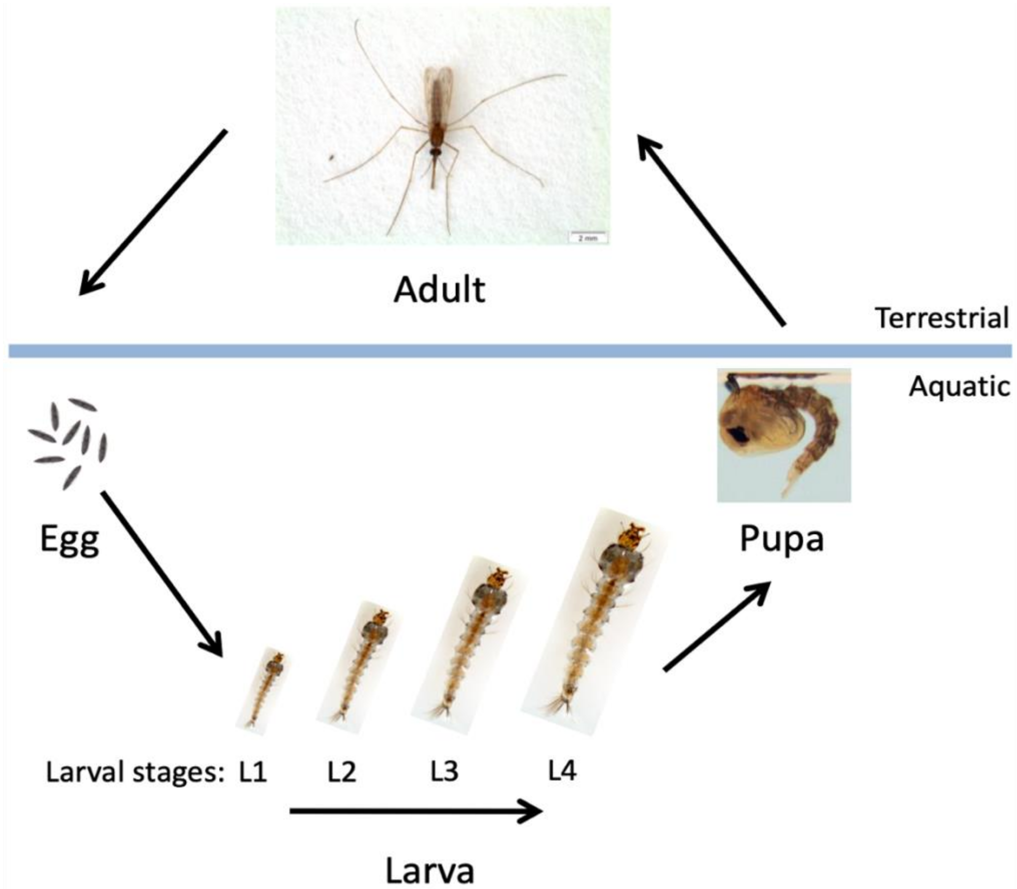


Figure 1.2. The life cycle of *Anopheles* mosquitoes.

The mosquito life cycle consists of three aquatic stages (egg, larva, and pupa) and one terrestrial stage (adult). The larval stage of mosquitoes also has four developmental states referred to as instars (L1 to L4). The schematic was adapted from [33].

[34]. *Anopheles* eggs are typically laid in stagnant fresh waters and hatch into larvae within 2 to 3, and up to 14 days [35]. Then the larvae develop into pupae in a span of 1 to 2 weeks, which rupture and release adult mosquitoes within 48 hours. Immediately after emerging, adult mosquitoes seek a sugar source and mating partners.

More than 40 *Anopheles* species, out of more than 400 identified, are described as dominant vector species (DVS) and can efficiently transmit malaria parasites to cause significant disease in humans [2, 36]. These DVS were determined as major vectors based on their vectoral capacity (ability to transmit disease), which is a combination of their population size, host feeding preferences, adult life span and capability of supporting parasite development [37]. The geographic distribution of these vectors is highly variable between malaria endemic regions across the globe (Figure 1.3). In Africa, a few DVS (primarily species of the *Anopheles gambiae* complex and *Anopheles funestus*) are distributed over most of the continent [38]. In contrast, diversity of DVS is higher in the Americas, with *An. albimanus*, *An. darlingi*, and *An. quadrimaculatus s.l.* as the main species. Asia has the highest diversity of DVS, with nearly three times and twice the number of DVS in Africa and the Americas, respectively [38].

Distinct behavioral characteristics amongst these vectors such as preferences of host [39, 40], biting time [41, 42], and biting setting (i.e., indoors vs outdoors) [41, 43] have been documented. Understanding the distribution of these vectors at the global and local scale and consideration of their behavioral differences with regards to malaria transmission is vital for developing targeted vector control strategies [38]. For example, investments in malaria control approaches that target vectors indoors (i.e., insecticides used within homes) might prove futile against vectors that mostly bite outdoors [38]. Behavioral differences

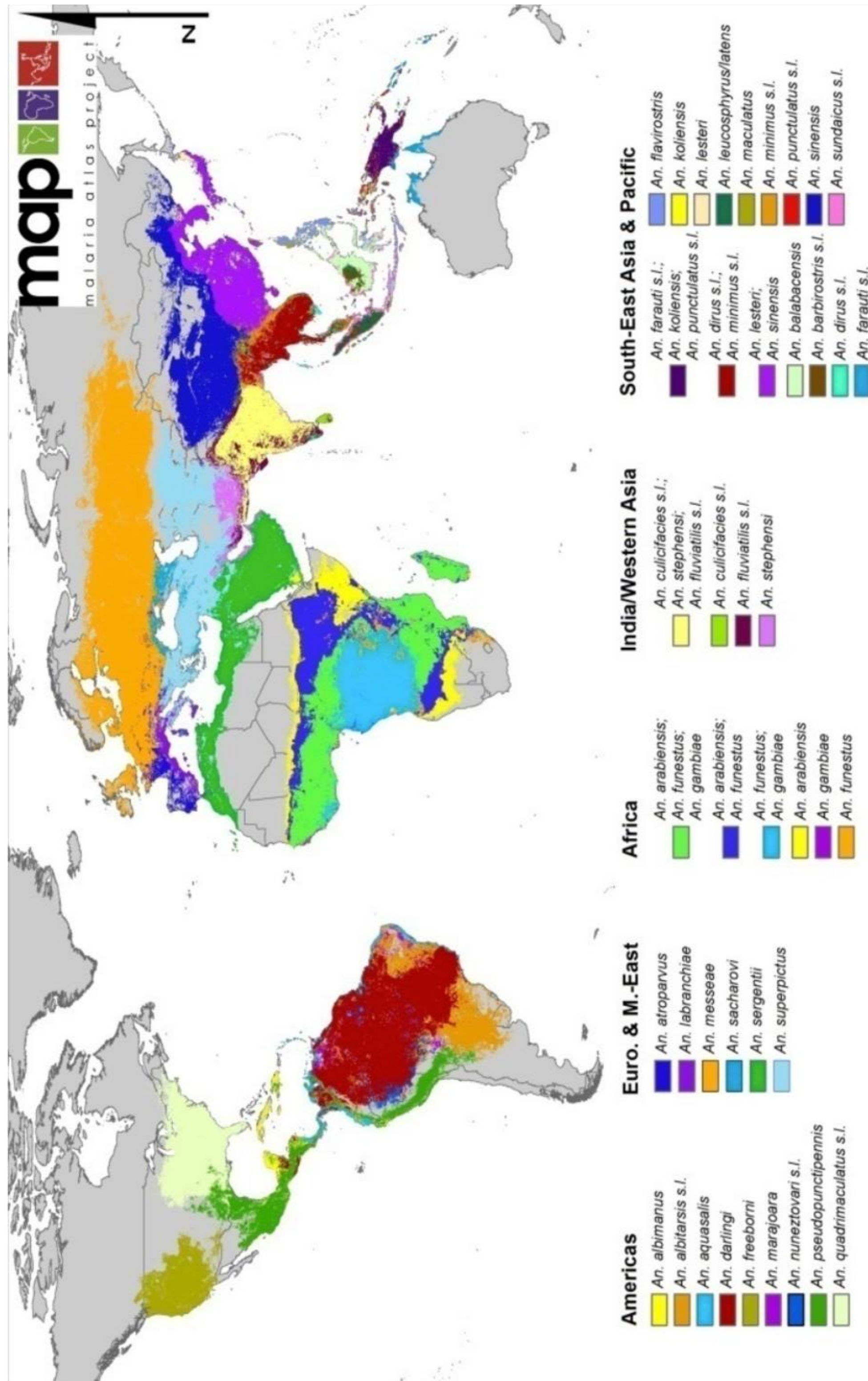


Figure 1.3. The distribution of *Anopheles* DVS across the globe.
Map was adapted from Sinka et al [38].

have been documented even within a species complex. The *An. gambiae* complex, once described as a single species, currently includes eight species that exhibit diverse ecological distribution and behavior characteristics that impact their role in malaria transmission [44].

2. Challenges of malaria elimination

2.1 Successes in disease burden reduction

In the last few decades, the mortality and morbidity associated with malaria have been significantly reduced, thanks to the global commitment to malaria elimination and eradication efforts [7, 45]. WHO reported that malaria cases decreased by 37% (42% in Africa) and deaths fell by 60% globally (66% in Africa), between 2000 to 2015 [46]. In the same time period, deaths among children have dropped by 65% [46]. This progress is accompanied by malaria elimination in many countries. In 2015, malaria incidence in 57 countries was reduced by at least 70% when compared to 2000, with 16 countries reporting zero native cases [46].

These advancements in disease burden reduction are due to improved distribution of diagnostic tests and antimalarial therapeutics, as well as the implementation of entomological control strategies [9, 45, 47]. According to the WHO's estimation, approximately 663 million malaria cases have been prevented since 2001, directly because of the dramatic increase in use of artemisinin-based combination therapies (ACTs), long-lasting insecticide-treated bed nets (ITNs), and indoor residual spraying [46].

2.2 Antimalarials

2.2.1 Antimalarial therapeutics

Antimalarial drugs have been developed to target malaria life cycle stages in the mammalian host, primarily against intraerythrocytic asexual parasites but also, to a lesser extent, hepatic schizonts, hypnozoites, and gametocytes [48]. Discovered in the 1800s, the first compound used to treat malaria was quinine, with its schizonticidal activity [49]. After the use of quinine and its derivatives to kill malaria parasites in the early twentieth century, an effective substitute, chloroquine, was discovered in 1934 [50, 51]. In the 1950s, the high efficacy of chloroquine and the rampant rate of malaria infection in developing nations led to the substantial use of the drug [51]. The success of the drug produced optimism for a WHO malaria eradication campaign, a program which was later discontinued in 1969 after malaria elimination was determined unattainable [51]. Eventually, the success of chloroquine was marred with a decrease in its efficacy due to the rise of resistance [50].

The emergence and spread of malaria parasite resistance to antimalarial drugs has had disastrous consequences, resulting in dramatic increases in malaria illnesses and deaths [52, 53], and causing a considerable financial strain on endemic regions by necessitating drug replacement programs [54]. Spontaneous genetic alterations in malaria parasites, either as a single mutation but more often a set of mutations, that interfere with an antimalarial drug's mode of action, can lead to the occurrence of resistance [55]. After the occurrence of a mutation conferring resistance, drug pressure is thought to be one of the main factors responsible for the spread of resistance, giving a survival advantage to resistant mutant parasites over non-mutants [56]. Antimalarial drugs have been shown to select for resistant parasites as a result of extensive use of a monotherapy (i.e., a single drug use in mass drug administration campaigns [56]) and misuse of therapeutic drugs [57].

The first reports of antimalarial drug resistance came from Colombia and Thailand in the late 1950s, with chloroquine-resistant *P. falciparum* parasites [51, 58]. *P. falciparum* resistance to chloroquine spread throughout South America and Southeast Asia over the next two decades and arrived in Africa in the late 1970s [51]. In contrast, the first report of chloroquine resistance in *P. vivax* parasites only occurred in Papua New Guinea in 1989, despite similar statistics of malaria cases and chloroquine exposure to *P. falciparum* [51]. A comparably inexpensive and effective substitute for chloroquine was needed to sustain the disease burden. The lack of a suitable substitute led to the resurgence of malaria morbidity and mortality across the globe, but particularly in Africa [52, 59].

As the efficacy of chloroquine decreased over the years, the search for a replacement drug ensued in a largely global effort [48, 50]. This effort has since produced a number of antimalarial drugs including: piperaquine, mefloquine, lumefantrine, pyrimethamine, sulfadoxine, primaquine, and artemisinin [31, 48]. The Chinese government is credited with developing artemisinin, a drug with a different mode of action to chloroquine [50]. Although it was used to treat malaria in China expansively starting in the 1970s, the expensive new drug was not immediately used in the West due to delays in regulatory testing, and in developing nations because of limitations in finances [50]. However, it became clear that artemisinin, and its derivatives, were the new mainstay for treating malaria due to their efficacy against drug (e.g., chloroquine) resistant parasites and low toxicity [60]. In an effort to reduce the potential rise of resistance seen with its predecessors, the strategy of using artemisinin in combination with other synthetic malaria drugs (e.g., lumefantrine and naphthoquine), known as ACT, was initiated [60]. In addition,

this combinational drug therapy provided enhanced malaria treatment effectiveness due to the synergetic effect of the partner drug with artemisinin [50, 60].

In 2006, as the transition from other therapeutics to ACTs was taking place worldwide, WHO declared for ACTs to be used as a first-line treatment of malaria [60, 61]. ACTs are currently considered the most effective therapeutics against malaria, with the WHO crediting the therapy with 21% (139 million cases) of all cases averted due to malaria interventions in sub-Saharan Africa, since 2001 [46].

Much like how chloroquine-resistance of malaria parasites rendered chloroquine much less effective against malaria, there was a fear the same fate would befall its successors. This was true for sulphadoxine-pyrimethamine (SP), which was introduced in the 1960s in several nations, but was quickly removed as a first-line antimalarial due to rapid decline in efficacy [62]. On the other hand, artemisinin and its derivates initially appeared to have avoided a similar rapid decline in efficacy against malaria. The delay of potential resistance to artemisinin can be attributed to its short half-life and its action on more than a single target (i.e., protein or cellular function) [61], as well as the fact that it is primarily used with partner drugs with a different mode of action (i.e., ACTs) [55].

Nevertheless, the rise of resistance to artemisinin was signaled by infrequent accounts of clinical failures of ACTs to clear *P. falciparum* malaria [63-65]. In 2008, the first report of resistance to artemisinin was documented in Cambodia [66]. Almost ten years later, a report identified more than 30 independent cases of resistance to dihydroartemisinin–piperaquine, an ACT used as a first-line antimalarial to treat *P. falciparum* malaria in Southeast Asia [66]. The trends observed with previous antimalarial drugs indicate that wide-spread resistance to artemisinin-based therapies could be imminent. However, these

trends also highlight the importance of a multifaceted approach to combat malaria that includes a reduction of drug pressure by, for example, implementing complementary approaches that interfere with parasite transmission (i.e., vector control).

2.3 Vector control

2.3.1 Vector control strategies

Mosquito-borne diseases are responsible for mortality and morbidity globally [67]. Vector control, a method used to reduce or prevent vector-human contact and consequently hinder transmission of vector-borne pathogens, has been historically critical in controlling mosquito-borne diseases [68]. The implementation of vector control tools, more than therapeutics, is credited for shrinking the global map of several mosquito-borne diseases [68]. Since 1955, 22 countries were certified by WHO to have eliminated malaria by means of the Global Malaria Eradication Programme (GMEP), which heavily depended on vector control measures [69, 70].

To date, a variety of vector control tools, targeting the adult and immature stages of the mosquito, and using chemicals or non-chemicals have been developed [68]. In the first quarter of the 20th century, mosquito control was mainly focused on reducing populations of immature stages of the vector, through the use of larvicidal tools and source reduction (i.e., habitat management) [71]. In the following quarters of the 20th century, in addition to the continuation of previous strategies, other approaches targeting larval and adult stages were added to the arsenal [71]. In the 1940s, DDT (dichloro-diphenyl-trichloroethane) was discovered as one of the first synthetic compounds, with larvicidal and adulticidal activity [71, 72]. Launched in 1955, the success of the GMEP, heavily relied on DDT, coupled with indoor residual spraying, for mosquito control [69, 70]. However, the fear of unintended

effects of persistent DDT use, including potential toxicity to humans and accumulation in the environment, led to the prohibition of this insecticide [71, 73].

After the ban of DDT in the 1970s, vector control measures were still dependent on insecticides, but with a shift to synthetic compounds (i.e., pyrethroids, organophosphate, and carbamates) with much less adverse effects towards humans [72]. In the 1980s, pyrethroids, synthetic compounds with potent insecticidal activity, were starting to become the primary class of insecticides utilized in vector control measures to control malaria [74]. Enabled by their low toxicity to humans, pyrethroids were incorporated in ITNs, which limit mosquito-human contact during nocturnal hours [70, 74]. In sub-Saharan African countries, the percentage of households with ITNs remarkably increased from 2% in 2000 to 58% in 2015 [46, 74], with the WHO crediting the vector control intervention for preventing 69% (457 million) of all cases in 2001 [46]. Pyrethroids remain the mainstay in current vector control approaches, as the only insecticide class recommended for use with bed nets [75].

2.3.2 Anopheles resistance to insecticides

The WHO defines resistance in vectors as the ability of the insect to survive the toxic effects of an insecticide [76]. Target-site resistance (alterations to insecticide binding site) and metabolic resistance (i.e., reduction of insecticides by metabolic enzymes) are well described mechanisms responsible for the majority of malaria vector insecticide resistance identified [77]. Resistance involving other physiological (i.e., reduced insecticide penetration through the exoskeleton) and behavioral (i.e., avoidance of lethal dose) changes are much less understood [77, 78].

Anopheles species resistance to insecticides, much like the acquisition of malaria parasite resistance to therapeutics, followed intensive use of a mostly singular approach in efforts to control malaria [79, 80]. However, the added use of related insecticides in agriculture has been critical in the selection of resistant mosquito vectors [77]. More than 50 *Anopheles* species have exhibited resistance to the main classes of insecticides (i.e., DDT and pyrethroids). *Anopheles* resistance to DDT was first reported in *An. sacharovi* in Greece, in 1953 [77]. Over the next 30 years, DDT resistance was detected across several malaria endemic regions (i.e., Africa in 1967, Central America in 1958, and Southeast Asia 1980s) [77]. Mechanisms of both metabolic (e.g., elevated levels of Cytochrome P_{450} enzymes [81]), target-site (e.g., mutations to voltage-gated sodium channels [82]), and behavioral (e.g., escape of *An. quadrimaculatus* females after contact with residual DDT [77]) DDT resistance have been described.

Resistance to pyrethroids, which was detected worldwide after intensive use in the 1990s [77], include similar, and at times identical, mechanisms (referred to as cross-resistance) to those of DDT [83]. DDT and pyrethroid cross-resistance has been shown, for example, with knockdown resistance conferred by a single amino acid mutations in insecticide target sites. While the high frequency of kdr mutations in *Anopheles* mosquitoes may not be enough to lead to failure of vector control, it is thought to have developed from aggressive past use of DDT [83]. Although their impact is much less understood, behavioral resistance to pyrethroids have been reported with changes in preferences of biting setting (indoor to outdoor) [84] and biting time (night to morning) in relation to increased use of pyrethroid-treated bed nets.

It is clear that the cycle of developing a new insecticide, followed by intense application until resistance arises, rapidly reduces available/effective insecticide-based vector controls [77]. A handful of resistance management programs that include strategies based on using insecticides rotationally, spatially separated, or simultaneously have been designed but with little success [77]. Recently, the WHO proposed a strategy that promotes the use of insecticides in combination with new/alternative (non-insecticidal) vector control approaches, named Integrated Vector Management [85].

3. New approaches for malaria control

As resistance is weakening the last lines of defense against malaria (i.e., Artemisinin-dependent therapeutics and pyrethroid-dependent insecticides), there is increasing interest in developing highly efficacious malaria vaccines and novel transmission blocking strategies [86, 87]. Transmission blocking strategies have an advantage over current vector control methods in that they allow for mosquito survival, hence, preventing the rise of resistance due to selective pressure [87]. Currently, the main strategies for transmission blocking under research focus include – gametocytocidal drugs, transmission blocking vaccines (TBV), and refractory mosquitoes [87].

3.1 Transmission blocking therapeutic and immunogenic methods

3.1.1 Transmission blocking drugs

Transmission blocking could be achieved by eliminating or inhibiting the sexual stages of *Plasmodium*. One approach is to terminate the transmission cycle in malaria infected individuals by reducing gametocyte density sufficiently to prevent infection of mosquitoes [87]. To this end, the gametocytocidal activity of currently available antimalarial

therapeutics is being explored [88]. The use of an ACT, with primaquine as a gametocytocidal agent, is credited with elimination of malaria in Cambodian villages [89]. While the relative impact of gametocyte clearance by primaquine and asexual parasite killing by ACT (eliminating potential gametocytes) is not clear [90], in 2010, the WHO recommended this drug combination for field use, acknowledging the importance of transmission reduction [87]. One limitation of current gametocytocidal drugs, as observed with primaquine, is the short half-life of the drug and the fact that targeting only late-stage gametocytes could allow immature gametocytes to survive and infect mosquitoes [91]. Therefore, gametocyte clearance rate and timing should strongly be considered if efficacious novel gametocytocidal drugs are to be developed [87].

In contrast to clearing sexual stages of *Plasmodium* in humans, a recent study theorized that antimalarials could be used to directly inhibit the parasite within the vector [92]. Exploiting similar uptake of insecticides by mosquitoes (e.g., through legs of mosquitoes in ITNs), the investigators found that exposure of *An. gambiae* mosquitoes to a cytochrome B inhibitor (atovaquone) before *P. falciparum* infection, arrested parasites development in the mosquito midgut, with no oocysts detected in treated individuals [92]. Moreover, the exposure time to atovaquone was similar to the time native mosquitoes spend on bed nets leading to the suggestion that such compounds could work synergistically with current insecticide-based approaches (e.g., ITNs and indoor residual spraying) [92]. While this approach does not eliminate the potential emergence of parasite drug resistance, malaria parasites transmission by insecticide-resistant mosquitoes would be blocked [93].

3.1.2 Immunological methods

Targeting the sexual and mosquito stages of *Plasmodium* with transmission blocking vaccines (TBV) has also gained considerable interest. In theory, immunization with TBV could prevent malaria infected individuals from infecting mosquitoes [94]. To date, surface proteins important for fertilization and other parasite processes like Pfs48/45, Pfs47, and Pfs230 (expressed on gametocytes and gametes), and Pfs25 (expressed on zygotes and ookinetes) have been identified as primary antigen candidates, with a few even in preclinical and phase I clinical trials [86, 94, 95]. Other candidates, including surface antigens on parasite stages that develop in the mosquito midgut and mosquito proteins necessary for parasite maturation in those same stages are in early developmental stages [86].

Epitopes from proteins expressed in later stages in the mosquito (ookinete and oocyst) might be even more appealing targets to TBV, as parasite numbers are low and more parasite maturation processes involving host components increase potential antigen candidates [87]. However, this rationale needs a much better understanding of the implications of reducing parasite quantity at different stages on parasite transmission and identification of more essential stage-specific proteins [87, 94].

While TBVs show much promise, there is increasing consensus that achieving and maintaining malaria elimination might require vaccines targeting multiple epitopes and stages of the parasite [96]. Pre-erythrocytic stages of *Plasmodium* present particularly appealing targets as they potentially could block parasite progression into human erythrocytes and prevent the clinical manifestation of malaria [96, 97]. Seminal studies have demonstrated attenuated whole sporozoites provide protection against malaria in rodents [98] as well as mammals [99, 100], with 90% of human volunteers immunized by

mosquito bites injecting irradiated *P. falciparum* sporozoites protected from disease [100]. As a vaccination method however, this whole organism approach presents the challenges of harvesting irradiated sporozoites at a large scale, devising an appropriate vaccine delivery technique (only multiple intravenous inoculations elicited potent immune response [101]), and testing vaccine efficacy in malaria-experienced individuals [102] need to be fully addressed [103].

At the very least, the discoveries from studying whole sporozoite vaccines have spurred the development of the first pre-erythrocytic stage vaccine targeting sporozoite proteins [97]. RTS,S, which consists of a circumsporozoite region, is the sole commercially available antimalarial vaccine and conferred up to 50% protection in vaccinated individuals over 5 months old [103]. However, protection was 20% lower in younger infants and appears to disappear around three years post vaccination [103]. In addition, the mechanisms involved with the immunogenicity of RTS,S and other pre-erythrocytic subunit-based vaccine candidates across individuals of all ages and in malaria endemic regions is not fully clear [97, 103, 104]. Even when considering the variety of hurdles present in developing efficacious malaria vaccines, their value as a long-term solution for malaria control is not lost.

3.2 Transmission blocking refractory mosquitoes

3.2.1 Transgene-dependent refractory mosquitoes

Mosquitoes exhibit natural defense mechanisms against *Plasmodium* infections, with some individuals even resistant to infection [87, 105, 106]. To exploit these phenotypes for potential malaria control approaches, in the last two decades, researchers have intensified efforts to identify genes and molecular mechanisms that i) are critical for *Plasmodium*

colonization of mosquitoes (in hopes of perturbing their function) or ii) confer resistance to infection [107, 108]. The goal is to develop or emulate *Plasmodium*-resistant phenotypes in laboratory vectors and replace susceptible mosquitoes in the field with mosquitoes refractory to *Plasmodium* infection [87].

Although the application of engineered refractory mosquitoes for malaria control is still in the early stages, a number of methods are currently under study and development [87, 107]. Population replacement with genetically modified refractory mosquitoes is one of the most promising strategies. With the advent of genetic manipulation tools for mosquitoes (i.e., germline transformation [109, 110]), the expression of proteins and peptides (transgenes/effector molecules) that hinder parasite development within the vector became possible. These tools were used to generate one of the first artificially engineered refractory mosquitoes, *An. stephensi* mosquitoes that inhibit *P. berghei* development by expressing antiparasitic transgenes in midgut epithelium [111]. After the complete sequencing of the *An. gambiae* genome and improved characterization of the mechanisms involved during the mosquito immune response mounted against *Plasmodium* infection, researchers have generated multiple refractory transgenic mosquitoes that leverage the innate immune system [112, 113]. The discovery of a variety of effector mechanisms with the potential of blocking parasite transmission and their production in the laboratory has been remarkable [114].

Despite these rapid advancements, genetically modified mosquitoes have yet to be utilized in large scale malaria control programs. This is can be attributed to a number of challenges that remain unresolved – determination of the optimal system for introducing genetically modified mosquitoes (i.e., gene drive, strategy for reduction of susceptible natural

population) and their efficacy and safety in the field, and societal and governmental acceptance of transgenic mosquito introduction to nature [114]. Regardless, enhanced understanding of the biology of *Plasmodium* parasites in the vector, will assist development of mosquito transgenics for malaria transmission-blocking strategies. In addition to parasite refractory mosquitoes, transgenic-based mosquito population reduction is in the discussion of genetic modification dependent malaria control approaches. One approach entails the mass release of male insects carrying dominant lethal transgenes that only produce male offspring [115]. One of the concerns of this strategy is that it will have limited success for malaria control because it the lethal trait is not projected to travel across large malaria endemic regions [115].

3.2.2 Microbiota-dependent approaches

3.2.2.1 Impact of microbiota on vector and Plasmodium infection

The presence of commensal microbes in the digestive tracts of larval and adult stage mosquitoes has been documented since early in the 20th century [116]. In the last few decades however, the studies of the mosquito microbial community and its influences on vector biology have broadened [116]. With regards to *Anopheles* mosquitoes, metagenomic and other conventional (i.e., culturing) approaches have described the composition and diversity of a variety of anophelines [117-119]. The mosquito microbiota evidently appears to be primarily acquired from the environment [120], but can also be transmitted horizontally (among individuals) or vertically (from parent to offspring) [121, 122]. These studies also suggest that not a particular microbe but the microbiota in its entirety is important for mosquito physiology [121].

The importance of the microbiota for development of the aquatic and terrestrial life stages of mosquitoes has been described. For example, larvae devoid of gut microbiota perish in early stages of development but are rescued with the introduction of *Escherichia coli* (and other bacterial species) [123]. In addition, the presence of antibiotics delayed larval development of *An. stephensi* mosquitoes, and this phenotype was reverted with the inoculation of antibiotic-resistant bacteria [124]. Although the processes involved in the larval microbiota dependence are not entirely clear, some studies suggest the role of gut symbionts is nutritional [121, 125]. In contrast, other findings contend that the microbiota's role is not solely as a nutrient source, reporting death of axenic larvae when fed dead bacteria along with a standard diet [123] and promotion of larval growth by bacteria-dependent gut oxygen level reduction [126].

Similarly, the microbiota appears to have importance in metabolic processes of adult mosquitoes. One study showed that *An. stephensi* fed radiolabeled bacteria exhibit radioactivity throughout their body, which is also observed in developing *P. berghei* oocysts and sporozoites, suggesting bacteria may participate in nutrition acquisition of both the vector and parasite [117]. The microbiota can also impact adult mosquitoes with adverse effects on fitness characteristics such as lifespan, fecundity, and mating behavior. The inoculation of sugar or blood meals with specific microorganisms can reduce the life span of colony *Anopheles* mosquitoes [127, 128]. *Anopheles* mosquitoes have been shown to detect bacteria in mammalian skin and water, and respond with modulation of their blood feeding [129] and oviposition behavior [130]. Recently, a study demonstrated that alteration of the mosquito microbiota due to genetic manipulation of *An. stephensi* mosquitoes impacts their mating behavior [131]. While understanding the exact

mechanisms for these observations will require more investigation, their impact on *Plasmodium* parasite transmission probability and thus potential for entomological control is understood.

The microbial community of mosquitoes is found in the mosquito midgut, which also accommodates the first stages of *Plasmodium* development in the vector. Interestingly, these stages of *Plasmodium* present a bottleneck for the parasite, with parasite numbers estimated to dwindle from thousands of gametocytes ingested into the midgut, to around a few hundred ookinetes and a handful of oocysts [132, 133]. Several studies have examined the potential role and contribution of the microbiota in this reduction of parasite density in the mosquito. In multiple different species of both *Anopheles* and *Plasmodium*, the microbiota has a deleterious effect on parasitic infection [134-137].

In our current understanding, the antiparasitic effects of the microbiota observed to interfere with *Plasmodium* infection of the mosquito midgut are mediated through mosquito immune response stimulation and synthesis of antiparasitic metabolites [138]. The immune response dependent mechanisms include: (i) the blood meal induced proliferation of bacteria in the midgut that stimulates an immune response through the immune-deficiency (Imd) pathway [139]; and (ii) immune priming dependent on the microbiota [140]. The microbiota-dependent direct antiparasitic mechanisms so far described involve the production of antimicrobial agents by specific bacterial strains [127, 134, 141].

3.2.2.2 Microbiota for vector control

The potential of the mosquito microbiota for controlling mosquito-borne diseases is illustrated by the impact the microbiota can have on the vector and the pathogens they

transmit. Out of the several parameters needed for malarial disease progression, the mosquito microbiota can potentially influence multiple vector competence components such as mosquito life span, blood feeding behavior, and capability of supporting parasite development [121, 142]. Furthermore, commensal bacteria dramatically proliferate following a blood meal by the mosquito, consequently amplifying potential microbial antiparasitic effects that inhibit *Plasmodium* parasites ingested into the midgut, which is valuable in the consideration of the mosquito microbiota for vector control [143].

In theory, vector competence can be reduced by microorganisms that naturally interfere with *Plasmodium* development in the *Anopheles* mosquitoes or the mosquitoes themselves [121]. Excellent candidates have emerged, such an *Enterobacter* strain (EspZ) that renders mosquitoes *Plasmodium*-resistant in nature [134]. *Wolbachia* bacteria have shown refractoriness to a wide range of human pathogens in the vector, including *Plasmodium* parasites [121, 144, 145]. Furthermore, *Wolbachia* shows potential for use in mosquito population control, as a process termed cytoplasmic incompatibility that benefits the propagation of the bacteria, allows for viable progeny only if both parents or just if the female are *Wolbachia*-infected [143, 145]. For malaria control, the idea would be to release *Wolbachia*-infected male mosquitoes at massive numbers, such that they mate with *Wolbachia*-uninfected females and fail to produce progeny. However, until recently, evidence of natural and laboratory *Wolbachia* infections of *Anopheles* mosquitoes was lacking, suggesting a possible incompatibly between the vectors and bacteria [145-147]. More ecological studies characterizing the interactions of *Anopheles* mosquitoes with *Wolbachia* are needed to supplement the use of the endosymbiont to the arsenal of malaria vector control [148].

Alternatively, malaria vector control could be achieved through artificially induced antiparasitic effects of the mosquito microbiota. In an approach termed paratransgenesis, genetically modified symbiont bacteria can produce effector molecules that render the vector resistant to pathogenic infection [143]. In the last decade, research focus on paratransgenesis application for malaria control has produced exciting results and potential candidates [149]. In 2012, *Pantoea agglomerans* was engineered to expresses effector molecules that inhibit *P. berghei* development in *An. gambiae* mosquitoes [150]. More recently, a strain of *Serratia* (AS1) and *Asaia* bacteria have been demonstrated to be amenable to genetic engineering induced expression of effectors that interfere with *Plasmodium* development in *Anopheles* vectors [151, 152]. Moreover, in addition to colonizing the midgut of mosquitoes, both bacteria can colonize the reproductive organs and transmit vertically and horizontally, which is advantageous for their propagation in mosquito populations [121, 143, 151, 152]. Although the approach shows promise, paratransgenesis field application for mosquito-borne disease control has yet to be performed at a large scale [143].

Entomopathogenic fungi also present an alternative option for vector control. For example, strains of *Metarhizium anisopliae* have displayed the ability to kill colony and wild mosquitoes and raise minimal safety concerns as they are already in use in agricultural settings [153]. The virulence of naturally occurring fungi strains, via the production of spores, can at times be suboptimal (i.e., insect killing achieved with high load and slowly) [153]. Addressing this shortfall, a number of groups have genetically engineered fungal isolates to induce increased lethality on insects [154-156]. The potential of fungal pathogens as a robust malaria vector control approach is exciting but will require improved

understanding of fungal pathogenesis in insects and creative approaches that implement that knowledge [154].

3.2.2.3 Influences on Anopheles microbiota

The microbial composition of mosquitoes has been characterized by classical cultivation methods and, more recently, by 16S rRNA sequencing approaches [157]. Studies using these approaches and characterizing laboratory-reared as well as field-caught mosquitoes, have described Gram-negative bacteria to dominate the microbiota in *Anopheles* species [121, 143]. Although there is much debate if anophelines have a core microbial community, members of the genera *Pseudomonas*, *Enterobacter*, and *Asaia*, among a few others, are frequently detected [121, 138, 157]. However, to date, no obligate symbiont of the *Anopheles* genus has been described [121].

Despite the account of bacterial species commonly present in *Anopheles*, the microbial community is highly variable. Differences in microbial composition have been described between different *Anopheles* species [158], among individuals in the same species [159], between different laboratories rearing the same species [138, 160], and in the overall diversity between colony and wild-caught mosquitoes of the same species [121, 138]. The mosquito factors thought responsible for the microbial variation have been recognized in numerous studies and include: larval habitat, blood-feeding behavior, seasonality, diet, host genetics [118, 119, 138, 159, 161]. Mosquito microbial community is mainly acquired in the aquatic life stages, and the microbial diversity is lower in adult mosquitoes and can change remarkedly to a degree that does not show correlation with larval origins [121, 162]. The driving force of the microbial composition of adult mosquitoes is still under some debate, with attention focused on mosquito intrinsic and extrinsic factors.

4. Outstanding questions and concluding remarks

In the last decades, development of antimalarial therapeutics and entomological controls have markedly reduced the malaria mortality rate and brought malaria eradication within sight [7, 8]. However, the emergence and spread of insecticide resistance, as well as the potential of malaria resurgence from areas where it had been eliminated, threaten global malaria elimination efforts [45, 47]. These challenges highlight the need for innovative entomological control approaches as well as a better understanding of malaria transmission dynamics, in order to successfully eradicate the disease.

While meeting the goal of malaria eradication comes with many hurdles and required multi-pronged approaches, addressing the following key gaps in our current understanding of *Plasmodium* biology in the mosquito and factors influencing its transmission to the mammalian host could prove vital: 1) *Plasmodium* numbers can be at their lowest at the oocyst stage, however, the relative influence of the mosquito microbiota and other mosquito factors (e.g. immune response) on the ookinete-oocyst bottleneck is unclear; 2) only a subset of sporozoites are injected into the mammalian host, and the maturational and regulatory mechanisms that accompany *Plasmodium* parasites as they their egress from ruptured oocysts to the salivary glands, which could explain this phenomenon, have not been comprehensively investigated; 3) while more light is being shed on the mosquito-microbiota interactions, little remains known about the factors shaping the composition of bacterial communities of mosquitoes. Here, I address elements of these outstanding questions in researched aimed at:

1) Determining the relative contribution of mosquito genetic and environmental factors on the mosquito microbiota

2) Characterizing the transcriptional variations and heterogeneity among *Plasmodium* sporozoites

Chapter 2. Relative Contributions of Various Endogenous and Exogenous Factors to the Mosquito Microbiota[1]

Introduction

Aedes, *Culex*, and *Anopheles* mosquitoes can transmit eukaryotic parasites and viruses to humans and are responsible for devastating diseases - such as malaria, lymphatic filariasis, dengue, West Nile, and Zika that affect hundreds of millions of people worldwide and cause hundreds of thousands of deaths annually [67]. Successful disease elimination campaigns have focused on interrupting transmission of these diseases by targeting their mosquito vectors. Thus, in the early twentieth century, vector control approaches primarily relied on the use of chemicals and larviciding tools (i.e., petroleum oils and larvivorous fish) to eliminate larval and adult stages of the mosquito [71, 73]. Environmental and human health concerns brought by the persistent use of chemicals like Dichlorodiphenyltrichloroethane (DDT) over the years have partly led to the increased use

[1] Bogale, H.N., Cannon, M.V., Keita, K. et al. Relative contributions of various endogenous and exogenous factors to the mosquito microbiota. Parasites Vectors 13, 619 (2020).

of pyrethroids, which are safer alternatives, in vector control measures [73]. In the last three decades, entomological control strategies based on pyrethroid-treated bed nets and indoor residual spraying have been extensively used in the fight against vector-borne diseases with considerable success in reducing disease burden [9, 45, 163]. However, wide-spread use of these approaches, combined with exposure to agricultural pest control chemicals, have led to the emergence and rapid spread of insecticide-resistance alleles in many areas [164, 165]. In addition, several populations of mosquitoes have modified their behaviors (e.g., their host biting time [166, 167] and location [84, 168] or their host species preference [169, 170]) upon exposure to insecticides. This acquisition of chemical and behavioral resistance to insecticides threatens the advances made in control of mosquito-borne diseases and highlight the need for alternative measures.

One alternative to chemicals is to leverage biological agents to control mosquito populations (sometimes referred to as biological controls). For example, *Bacillus thuringiensis*, a spore-forming bacterium with larvicidal characteristics, is used extensively against disease-transmitting insects as well as agricultural pests [171]. In recent years, the use of *Wolbachia* as a biological control agent has also gained momentum, with studies revealing its ability to promote pathogen interference and to reduce the life span of mosquitoes [172-174]. Furthermore, recent findings have highlighted how modifications of the bacterial communities present in the midgut of mosquitoes could decrease or inhibit transmission of pathogens. For example, studies have demonstrated the importance of gut microbiota in mosquito larval development and shown that bacteria are required for *Aedes* mosquitoes to survive to the adult stages [123, 175]. Similarly, elimination of native microbiota resulted in delayed growth in *Anopheles* larvae [124]. Functional studies on

adult stage mosquitoes showed that the gut microbiota can increase the resistance of mosquitoes to human pathogens by modulating the mosquito innate immune response [176, 177] or directly through production of anti-pathogen molecules from specific microbial species [127, 134]. Overall, these studies demonstrate the potential of microbiota manipulations for inhibiting pathogen transmission and/or reducing vector competence.

However, while these laboratory and field studies highlight the role of the mosquito midgut microbiota in regulating the development and transmission of human pathogens, very little is known about the factors that shape the diversity of the bacterial composition in wild mosquitoes. Some studies have suggested that mosquito collection location is associated with the composition of the microbiota [178, 179] while others showed that the microbial composition differed between mosquito species, even when they are closely related and collected at the same location [180], or reared under the same conditions [123]. In addition, it is possible that genetic resistance to insecticides may also influence the microbial composition of field-collected adult mosquitoes (e.g., resistance could result in differential exposure of the gut microbiota to insecticides, which can alter the composition of the microbiota, see e.g., [181]). Lastly, the source of the blood-meal has also been shown to impact gut microbiota composition, with mammalian blood-meal source altering the gut bacterial composition of adult mosquitoes [182]. However, since these studies typically addressed only one of those factors at a time (without correcting for confounding effects), the relative contribution of each of these parameters to shaping the midgut microbiota composition of wild-caught mosquitoes remain unclear.

Here, we analyzed 665 individual *Anopheles* mosquitoes collected in Guinea and Mali. We characterized the microbial composition of these mosquitoes and screened them for a large

variety of eukaryotic parasites and viruses. We also characterized the species of all mosquitoes, genotyped them at a major associated insecticide resistance locus and examined the source of their last blood meal. We then tested how these endogenous and exogenous factors influenced the bacterial diversity, and simultaneously estimated the relative contribution of these factors to the mosquito microbiota.

Materials and Methods

Sample collections

Mosquitoes were collected from six sites in Guinea by Human Landing Catches (HLC) and Pyrethrum Spray Catches (PSC) in August-November of 2016 and August of 2017 (Table 2.1 and Figure 2.1). The captured mosquitoes were placed in Eppendorf tubes containing ethanol and shipped to the University of Maryland School of Medicine for analysis. Mosquitoes were collected in Mali from homes in Bandiagara using PSC in July of 2011. The captured mosquitoes were placed in Carnoy's solution (1 volume of acetic acid for 3 volumes of ethanol) and shipped to the molecular biology laboratory of the Malaria Research and Training Center (MRTC) in Bamako, for DNA extraction. See Table 2.1 for details on the collection sites and the collection method used.

Guinea mosquito DNA extraction

DNA was extracted from each mosquito using a modified version of the Qiagen 96-well extraction protocol. Briefly, whole individual mosquitoes were randomly placed in each well of a 96-well plate with five 1mm RNASE free oxide beads for homogenization. 11-14 extraction controls were also included on each plate. Each mosquito was homogenized using a TissueLyser for 6 minutes at 20 m/s in the lysis buffers provided by the Qiagen

DNeasy 96 Blood & Tissue Kit (Qiagen N.V., Hilden, Germany). The Plates were then centrifuged at 1,500 x *g* for 3 minutes. The homogenates were then incubated for 1 hour at 55°C, centrifuged again, and incubated overnight at 55°C. After a final centrifugation step, the supernatant was transferred to a 96-Well DNeasy plate to bind the DNA. The columns

Country	Site	Ecoregion	Collection methods used	# of mosquitoes	# of species	Collection dates
Guinea	Kissidougou	Guinean forest savanna mosaic	HLC	118	2	August 2017
	Kankan	West Sudanian Savanna	HLC/PSC	178	3	August 2017
	Faranah	Guinean forest savanna mosaic	HLC	79	3	August/September 2016
	Dabola	Guinean montane forest	HLC	79	4	August 2017
	Boffa	Guinean forest savanna mosaic	HLC	89	4	July 2017
	Mamou	Guinean forest savanna mosaic	HLC	41	1	October/November 2016
Mali	Bandiagara	West Sudanian Savanna	PSC	81	2	July 2011

Table 2.1. Mosquito collection locations, ecoregions, methods, dates, and numbers. Abbreviations: HLC, human landing catch; PSC, pyrethrum spray catch.

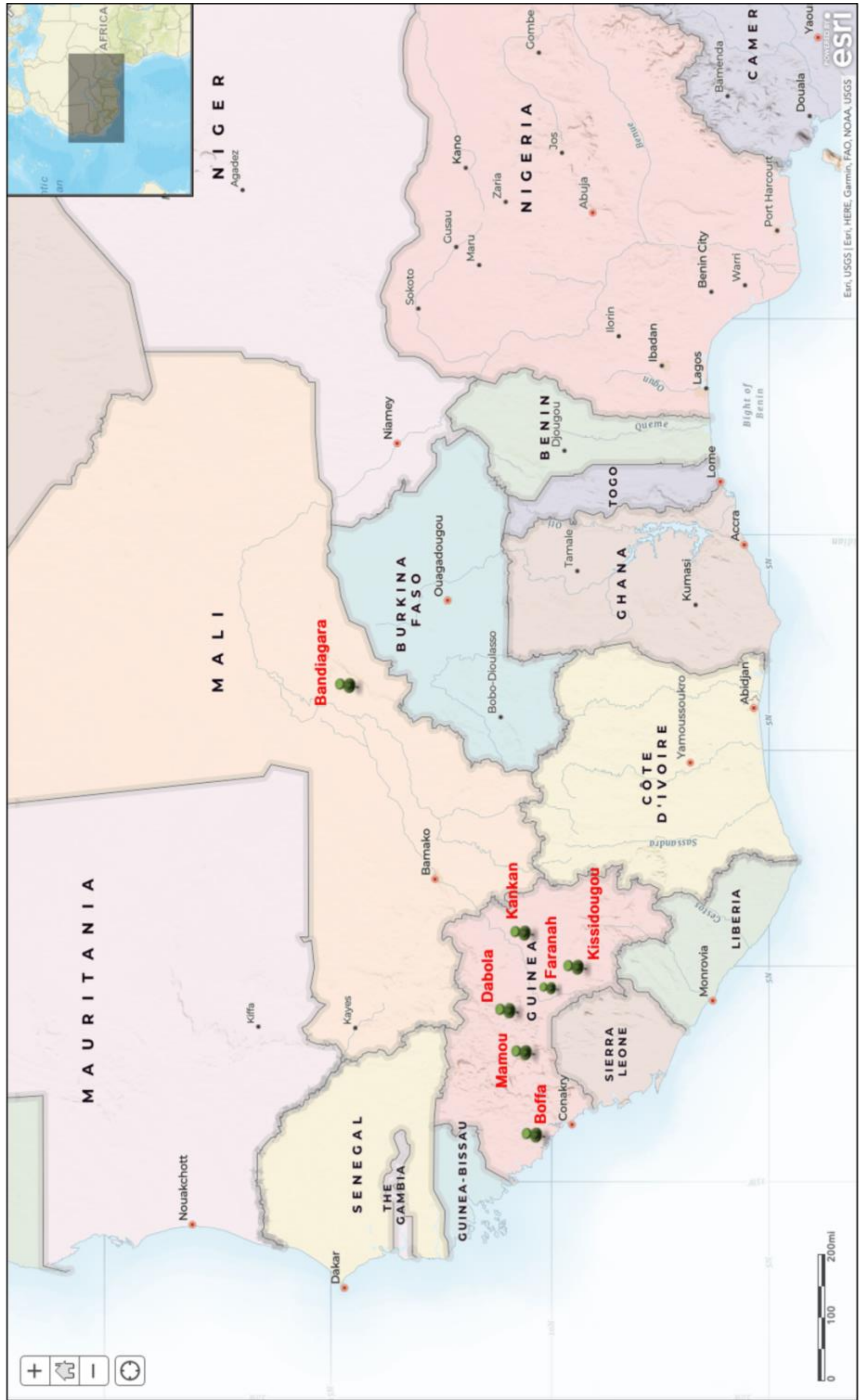


Figure 2.1. Geographical locations (green pins) of mosquito collection sites in Guinea and Mali.

Map image was prepared using the online ArcGIS® software by Esri.

were washed twice before elution of the DNA with 100 μL of Qiagen buffer AE (Qiagen N.V., Hilden, Germany). Nanodrop was used to determine DNA concentration.

Mali mosquito DNA extraction

DNA was extracted from the body section of each mosquito using Chelex-100 (Bio-Rad Laboratories, Hercules, California) protocol. Briefly, a dissecting needle was used to separate out the thorax and abdomen sections of each individual mosquito. Both sections of each mosquito were then placed in 1.5 mL tubes containing deionized water. Pipette tips were used to grind each sample in the tube. Each sample was further homogenized in PBS (1X)/1% saponin solution, shaking gently for 20 minutes. The homogenates were then incubated at room temperature (25°C) overnight. The tubes were then centrifuged at 20,000 x *g* for 2 minutes and supernatants were discarded. After washing with PBS (1X), each pellet was resuspended in 75 μL of deionized water and 25 μL of 20% Chelex-100 resin solution. This mixture was placed on a heating block for at least 10 minutes and stirred every 5 minutes. DNA was transferred into a new tube after a final centrifugation step for 1 minute.

PCR primers

DNA extracted from each mosquito was amplified using primers targeting bacterial 16S rRNA primers for microbiota analysis [183], mosquito *kdr*-west (L1014F) for insecticide resistance genotyping [184], cox1 and S200X6.1 [185] loci for mosquito species determination, mammalian mitochondrial 16S rRNA sequences for blood meal analysis, as well as eukaryotic parasite and virus primers (targeting 18S rRNA and NS5 loci respectively) [186] for identification of parasite and virus species (Table 2.2). The rationale for targeting these specific loci and the primer design are described in detail in the

respective references. Briefly, all these primers were designed or selected to simultaneously fulfil the following conditions: i) allow amplification of all sequences of the taxon targeted, ii) avoid off-target amplification, iii) provide sufficient sequence information for resolving the genotype or taxonomy and iv) be sufficiently short to be sequenced using Illumina technology and with paired end overlaps to allow for sequencing error-correction.

DNA amplification

DNA extracted from 665 individual mosquitoes and from the 95 extraction control samples were amplified separately with each primer pair using the Promega GoTaq DNA Polymerase with the following conditions: initial denaturing step at 95°C for 2 minutes followed by 40 cycles of 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 30 seconds, and a final extension of 5 minutes. Only 35 cycles were used to amplify bacterial 16S rRNA.

RNA virus detection

Viral sequences were analyzed by first synthesizing cDNA from carry-over RNA present in DNeasy extracted samples using M-MLV Reverse Transcriptase (Promega, Madison, Wisconsin). Briefly, 2 µl of mosquito DNA extract from each sample was incubated with 1 µl of random hexamers (0.5 ug) and 12 µl of RNA-free water at 70°C for 5 minutes. After this denaturation step, 1.25mM of each dNTP, 25 units of recombinant RNasin® Ribonuclease Inhibitor, and 200 units of M-MLV RT are added and the cDNA synthesis is carried out at 37°C for 60 minutes. The cDNA products were amplified using the same conditions as described above.

Barcoding and sequencing

We then pooled together the PCR products generated from one DNA sample (i.e., individual mosquito): the products from all parasite and viral amplifications were pooled at equal molar concentration (pool1) and the products of the remaining amplifications were pooled together (pool2) in various proportions reflecting the sequence diversity expected at each locus (75% Bacteria_16S rRNA, 5% S200X6, 5% CulicCox1, 10% KDR, 5% mammalian_16S rRNA). 384 pools (i.e., products from a single mosquito or water control) were then randomly assigned to a well of a 384-well plate and reamplified in a second PCR to add a unique barcode and the Illumina sequencing adaptors [186]. Finally, the barcoded product pools from all mosquitoes were combined (pool1:pool2 1:3 ratio) and sequenced simultaneously on Illumina HiSeq 2500 using a protocol allowing the generation of 300 bp paired-end reads[187].

Bioinformatic analyses

First, we used the sequences of the barcodes incorporated in the second PCR to assign each read to an individual sample. Next, we used the first 18-27 nucleotides of each read to identify the sequence of the PCR primers used to amplify a given sequence and separated the reads by loci. Further analyses were performed for each locus separately as indicated below.

Microbiota assessment

All reads carrying bacterial 16S rRNA primers (see above) were analyzed in DADA2 [188] (v1.6.0) by first trimming low-quality bases at the end of each read pair (>250 bp for forward, >210 for reverse reads) using the following parameters: *maxN = 0*, *maxEE = 2*, *truncQ = 2*. Dereplication was done by combining identical reads and assigning the number

of reads belonging to each unique read (*derepFastq*). Next, the dereplicated data was analyzed with the *dada* core sample inference algorithm followed by the merging of read pairs that overlap by at least 12 bases (*mergePairs*). An amplicon sequence variant table (ASV) table was constructed with *makeSequenceTable* for all samples and chimeras were removed using *removeBimeraDenovo*. For taxonomic assignment, Silva (v128) [189] was used as a training set (using *assignTaxonomy* and *addSpecies*) to create taxonomy data. Finally, the R (v3.4.0) package phyloseq (v1.25.2) [190] was used to combine the ASV table, taxonomy data, and sample metadata for downstream microbiome data analyses. Samples with less than 5,000 reads were discarded from further analysis, as they likely represent low-level cross contamination. PCoA with Bray-Curtis and weighted UniFrac distance matrices were calculated in phyloseq. Finally, Adonis analyses were performed to simultaneously evaluate the contribution of each factor to the bacterial composition: this multivariate analysis provided a statistical assessment of the association of each factor (i.e., collection site, mosquito species, *kdr*-w genotype, blood-meal status, and infection status, see below for details) with bacterial diversity, as well as an estimate of the proportion of the variance explained.

Eukaryotic parasites, viruses, and blood meal composition assessment

First, reads that did not contain the exact barcode and primer sequences were discarded. The rest of the reads were assigned to a given sample according to their unique barcode sequence. In order to remove low-quality bases and sequences that were likely primer dimers, each sequence was searched for the forward and reverse primers and trimmed after the reverse primer (if both primers are found). Sequences where the forward primer was found but the reverse primer was missing were left untrimmed. Untrimmed sequences that

belong to a primer with an expected amplicon length of <300 bp were trimmed from 50 bp from the 5' end for further quality filtering. Afterwards, filtered paired-end sequences from each read pair were merged using FLASH [191] to generate a consensus sequence of the overlapping region. Each correctly merged sequence was trimmed of both amplification primers (forward and reverse) and kept only if it was longer than 90 bp. Then all concatenated sequences amplified with the same primer pair (from all samples) were compared to each other and a single copy of each unique sequence was kept (while the number of times it is observed in each sample was recorded). Unique sequences that were observed less than 10 times across all samples were removed as they likely represent instances of sequences carrying errors [186, 192]. The remaining unique sequences were compared against all DNA sequences annotated on the NCBI nr database using BLAST [186]. We then retrieved the taxonomic information of the most similar sequence(s) if it had at least 70% identity over the entire sequence length. Finally, we summarized the parasite and virus species identified, the percent identity (i.e., similarity to the most similar sequence(s) on NCBI), and the number of reads observed in each sample.

When evaluating the blood meal composition of each mosquito, the same procedure was applied but only samples with at least 1,000 reads were considered (to avoid including possible cross-contamination or sequences mis-assigned to one sample due sequencing errors in the barcode) [193]. The distribution of reads generated from the mammalian_16S rRNA primers for all mosquito and negative control samples is presented in Figure 2.2.

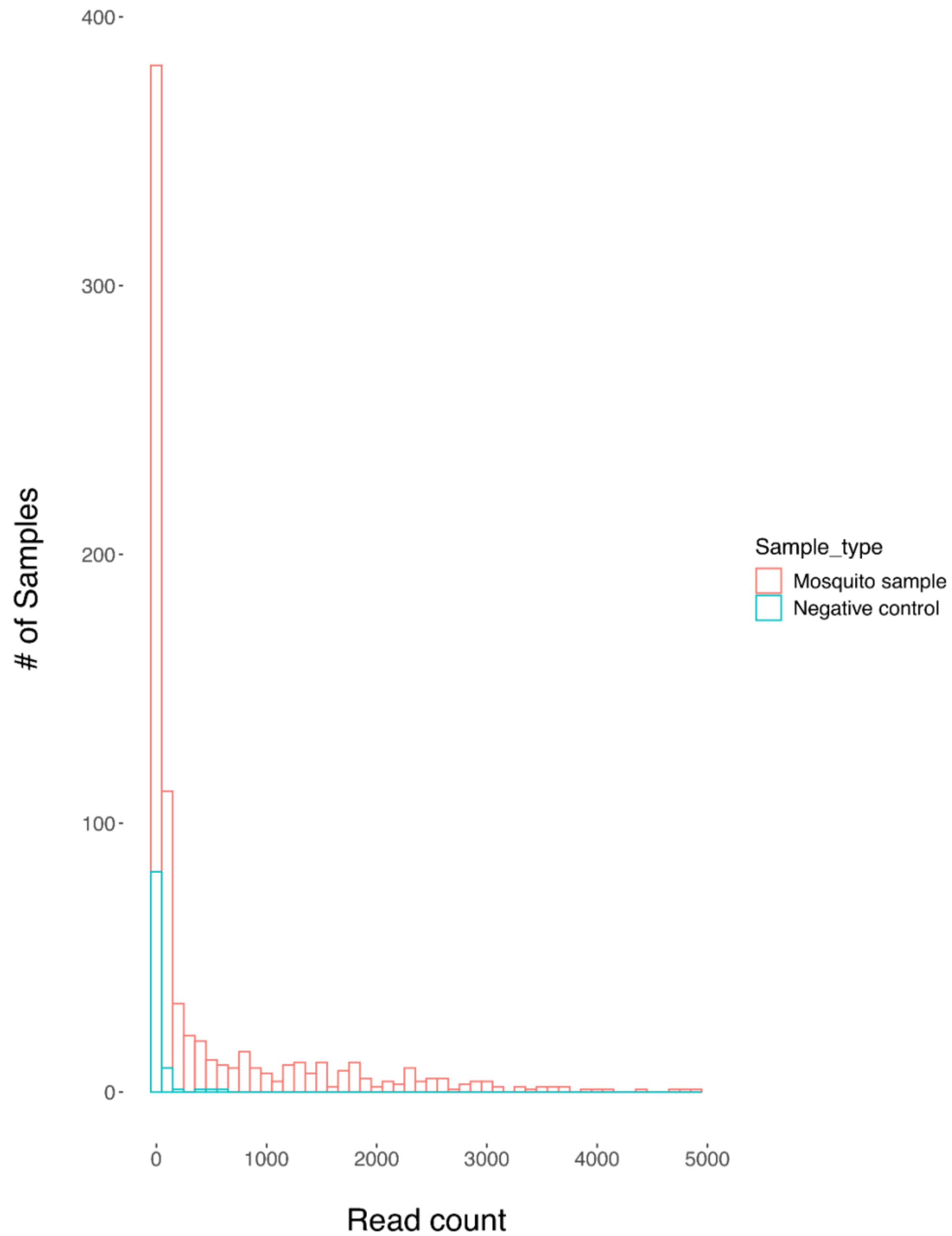


Figure 2.2. Distribution of reads counts for the mammalia_16S primer across mosquito samples and negative control samples.

***kdr* genotype (L1014F) and species determination (S200X6.1 and COX1)**

Reads amplified from the *kdr* and S200X6.1 primer pairs were processed as described above, with sequences assigned to their specific sample, filtered for quality, and merged with FLASH.

For the L1014F locus, the top two most abundant unique sequences in each sample (Seq1 and Seq2) were considered for further analysis. If one sample is homozygous, the second most abundant sequence will be a read carrying sequencing error(s) and should account for a small fraction of the Seq1 reads (i.e., Seq2/(Seq1+Seq2) ~ 0). Alternatively, if one sample is heterozygous, we would expect the number of Seq2 reads to be very close to Seq1 and Seq2/(Seq1+Seq2) ~ 0.5. We calculated the ratio Seq2/(Seq1+Seq2) for all samples and, based on the distribution (Figure 2.3) determined cutoffs for homozygous and heterozygous genotypes (taking into account small deviation from expectation due to sequencing errors). Only samples with at least 1,000 reads were considered for further analysis.

For the S200X6.1 locus [185], the most abundant sequences for each sample were compared against DNA sequences on NCBI as described above. For samples with greater than or equal to 1,000 reads, the species level taxonomy was retrieved and the *Anopheles* species as well as the total read count were summarized. Sequences generated from the primers targeting the COX1 gene (CulicCox1) were used to identify *An. nili* species in samples as this species is not successfully amplified with the S200X6.1 primers [185, 194].

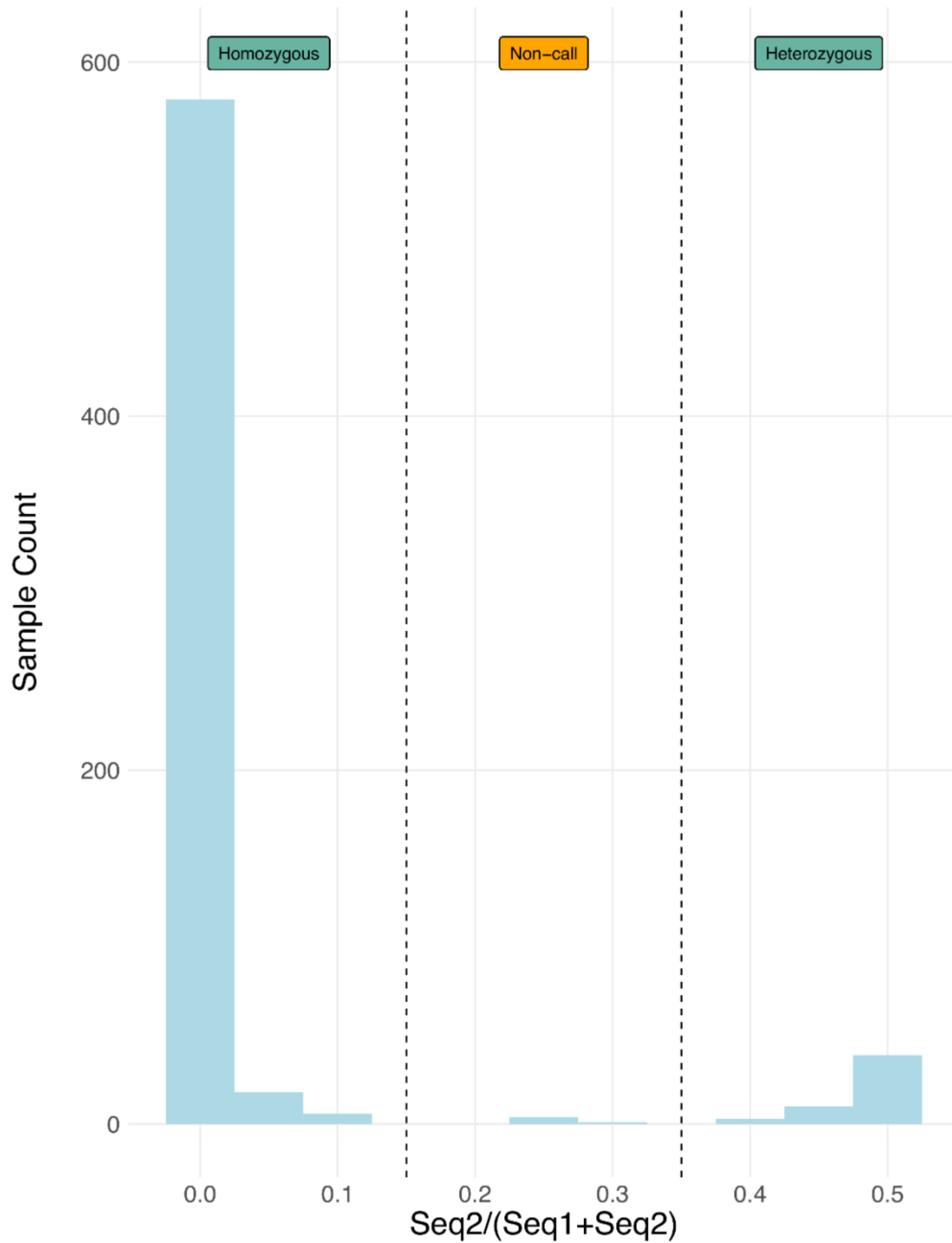


Figure 2.3. Distribution of the Seq2/(Seq1+Seq2) ratio across samples with one or more reads for the KDR primer used to determine genotype for the kdr locus.

Samples with a ratio < 0.15 (left dashed line), between 0.15 and 0.35 (between dashed lines), and > 0.35 (right dashed line) were deemed homozygous, non-called, and heterozygous, respectively.

Results

Bacterial composition of *Anopheles* mosquitoes from West Africa

We extracted DNA from 665 individual *Anopheles* mosquitoes collected in Guinea (N=584) and Mali (N=81). To characterize the microbiota of each mosquito, we amplified and sequenced the V2 variable region of the bacterial 16S ribosomal RNA genes (see Materials and Methods for details). We obtained a total of 8,467,703 sequences derived from 760 samples (665 mosquitoes and 95 extraction controls). On average, each mosquito sample yielded 11,730 sequences (minimum = 259, maximum = 29,908) compared to 5,984 sequences on average per extraction control (minimum = 31, maximum = 15,946). We assigned these sequences to 21,527 amplicon sequence variants (ASVs, analogue of operational taxonomic units [188]), representing 37 phyla including *Proteobacteria* (6,692 ASVs accounting for 64% of all reads), *Firmicutes* (26%), *Actinobacteria* (6%) and *Bacteroidetes* (2%) (Figure 2.4 and Figure 2.5).

To investigate differences in bacterial composition among mosquitoes, we calculated β-diversity estimates using weighted UniFrac and Bray-Curtis dissimilarity matrices. Principal coordinate analyses (PCoA) conducted using Bray-Curtis dissimilarity or weighted UniFrac distances showed that the microbial composition separates mosquitoes into distinct clusters (Figure 2.6a and Figure 2.7a, respectively). These clusters appeared to group mosquitoes collected in the same sites (Figure 2.6), and this observation held true when we restricted our analyses to mosquitoes only collected from sites in Guinea (Figure 2.6b and Figure 2.7b). The details of all the ASVs identified and their taxonomy are provided in Table 2.3.

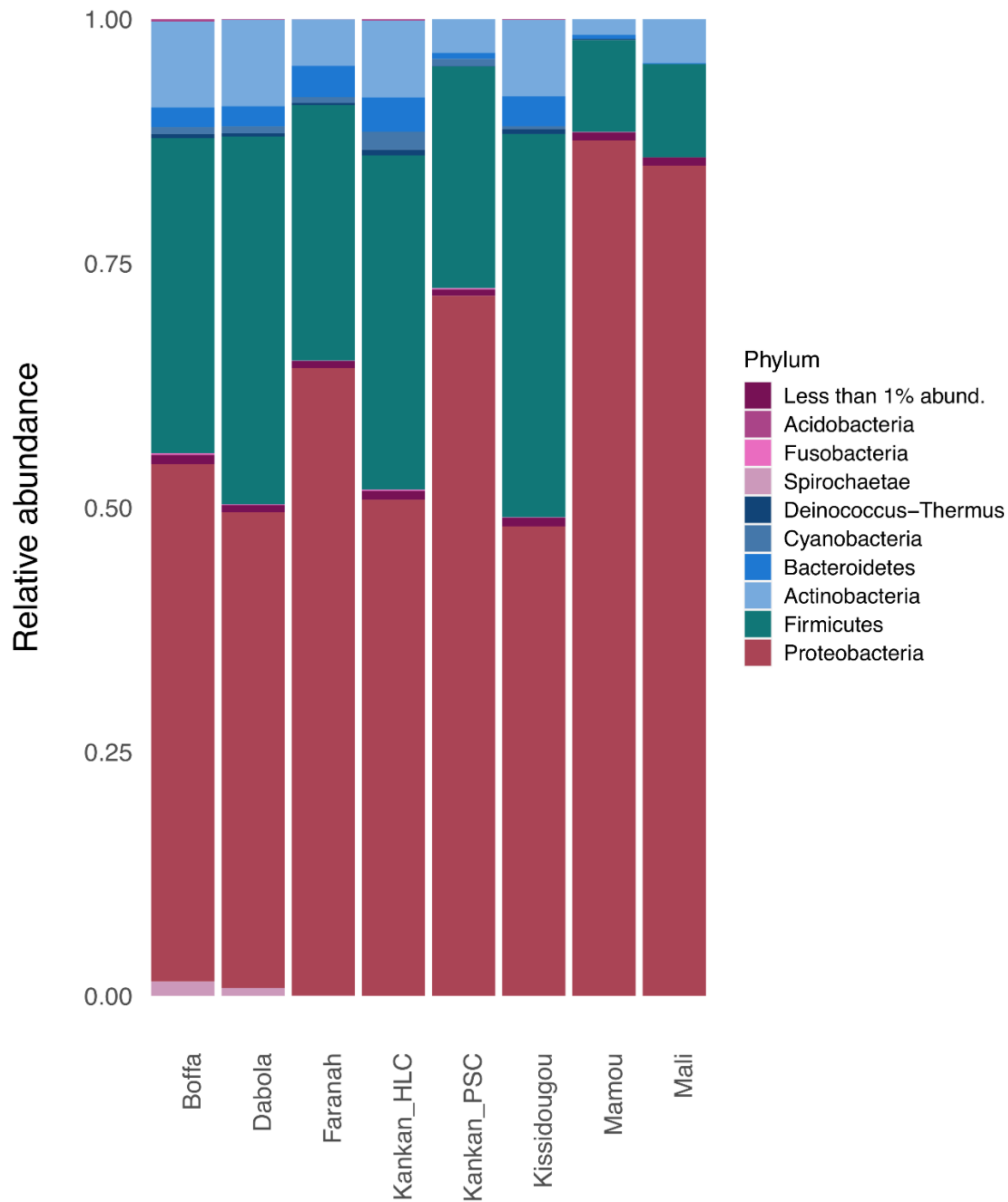


Figure 2.4. Average relative abundance of bacterial phyla from each mosquito collection site in Guinea and Mali.

Bacterial species from the Proteobacteria phylum are the most abundant, followed by Firmicutes and Actinobacteria. Less than 1% abund. represents the aggregate of all phyla that make up < 1% of all bacteria.

Figure 2.5. Average relative abundance of bacteria at the family (a) and genus (b) level in terms of taxonomic classifications from each mosquito collection site in Guinea and Mali.

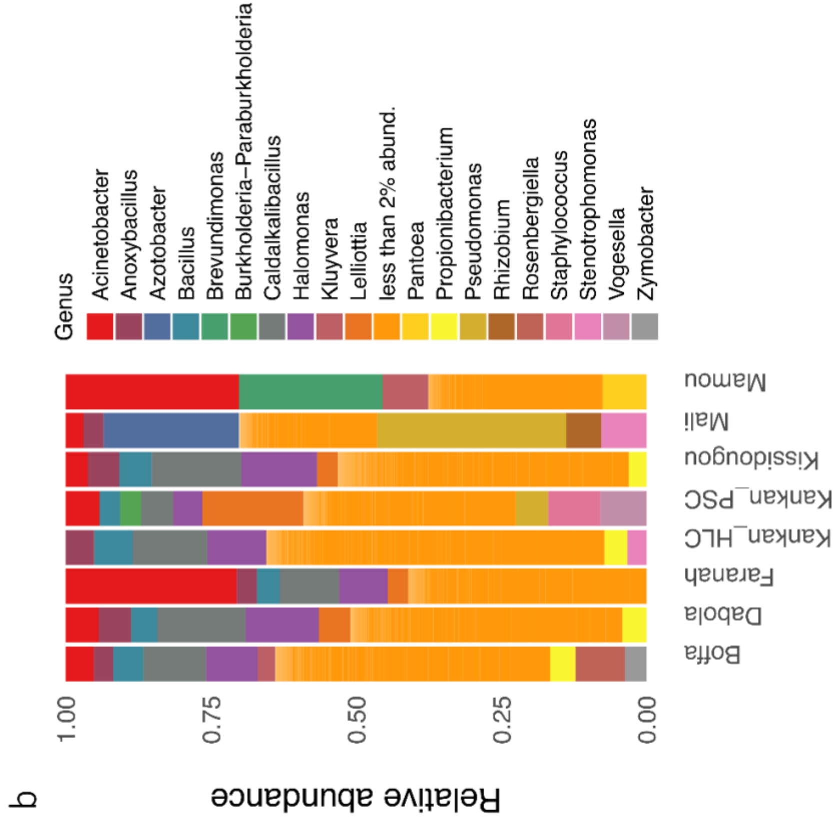
Less than 2% abund. Phyla that make up < 2% of all bacteria.

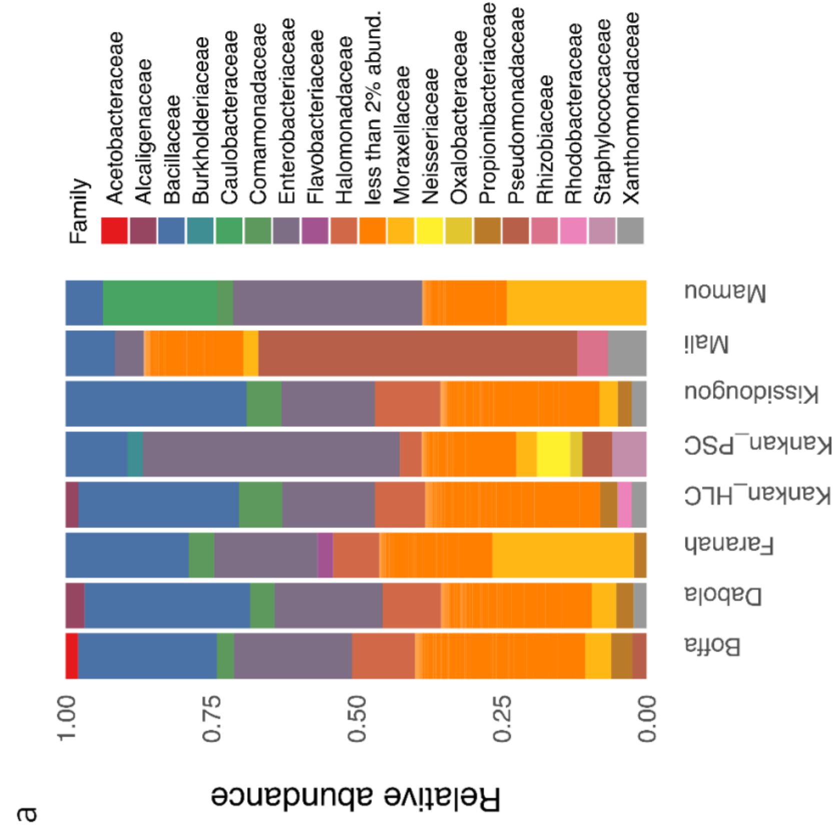
Figure 2.6. PCoA plot showing the dissimilarity between the microbial composition of individual mosquitoes based on the Bray-Curtis dissimilarity metric for sites in Guinea and Mali (a) and Guinea only (b).

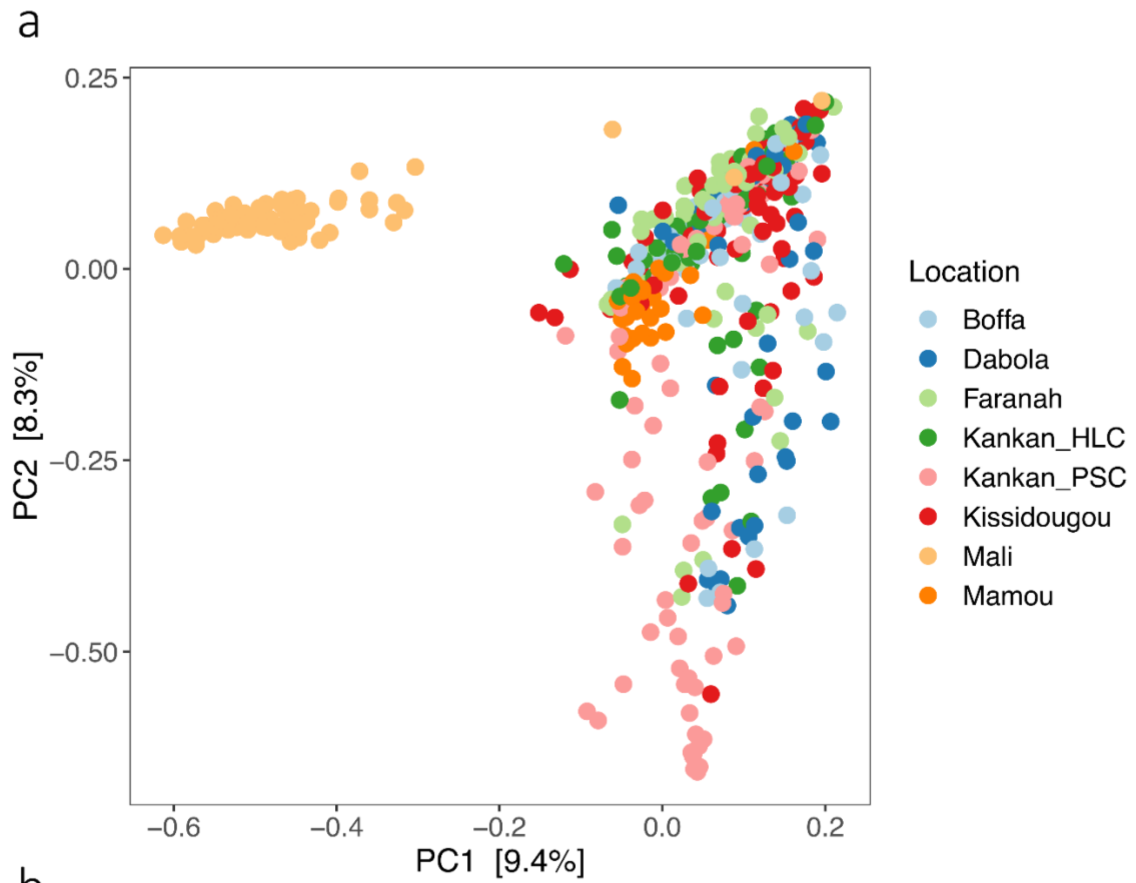
Each dot represents the bacterial composition of a single mosquito. The numbers in brackets near the axes indicate the proportion of the variance explained by the principal components 1 and 2 (PC1, PC2, respectively).

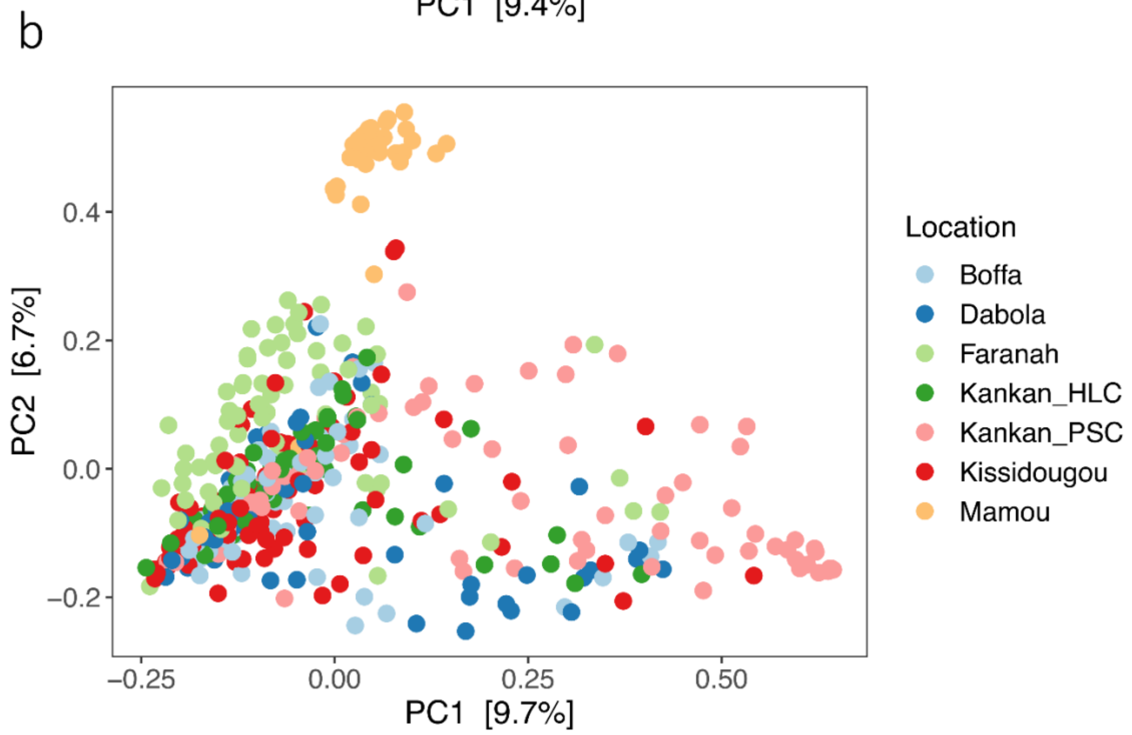
Figure 2.7. PCoA plot showing the dissimilarity between the microbial composition of individual mosquitoes based on weighted UniFrac metric for sites in Guinea and Mali (a) and Guinea only (b).

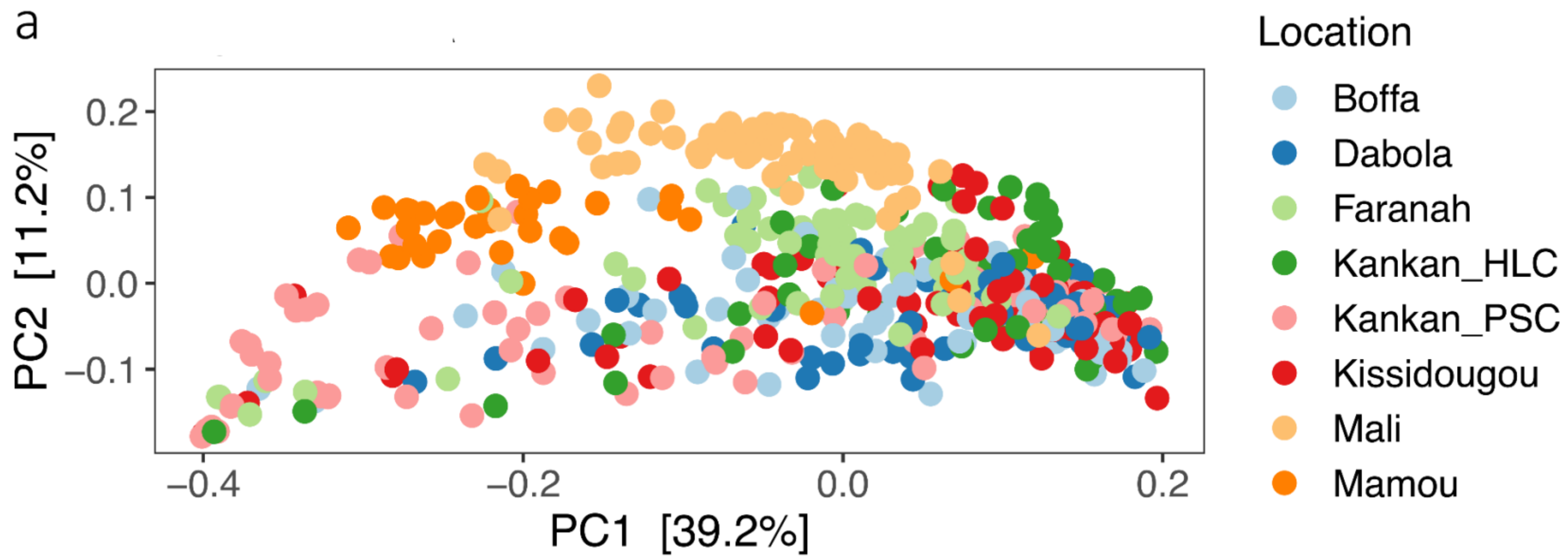
Each dot represents the bacterial composition of a single mosquito. The numbers in brackets near the axes indicate the proportion of the variance explained by the components 1 and 2.

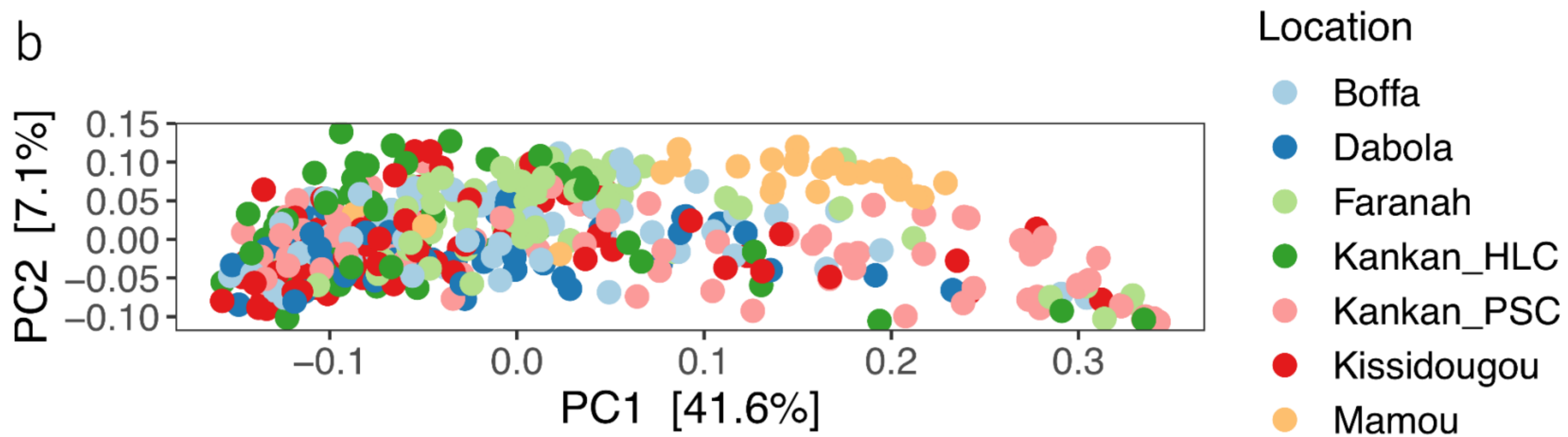

b
Relative abundance
1.00
0.75
0.50
0.25
0.00
Boffa
Dabola
Faranah
Kankan_HLC
Kankan_PSC
Kissidougou
Mali
Mamou
Genus
Acinetobacter
Anoxybacillus
Azotobacter
Bacillus
Brevundimonas
Burkholderia–Paraburkholderia
Caldalkalibacillus
Halomonas
Kluyvera
Lelliottia
less than 2% abund.
Pantoea
Propionibacterium
Pseudomonas
Rhizobium
Rosenbergiella
Staphylococcus
Stenotrophomonas
Vogesella
Zymobacter


a
Relative abundance
1.00
0.75
0.50
0.25
0.00
Boffa
Dabola
Faranah
Kankan_HLC
Kankan_PSC
Kissidougou
Mali
Mamou
Family
Acetobacteraceae
Alcaligenaceae
Bacillaceae
Burkholderiaceae
Caulobacteraceae
Comamonadaceae
Enterobacteriaceae
Flavobacteriaceae
Halomonadaceae
less than 2% abund.
Moraxellaceae
Neisseriaceae
Oxalobacteraceae
Propionibacteriaceae
Pseudomonadaceae
Rhizobiaceae
Rhodobacteraceae
Staphylococcaceae
Xanthomonadaceae


a
0.25
0.00
−0.25
−0.50
PC2 [8.3%]
−0.6
−0.4
−0.2
0.0
0.2
PC1 [9.4%]
Location
Boffa
Dabola
Faranah
Kankan_HLC
Kankan_PSC
Kissidougou
Mali
Mamou


b
0.4
0.2
0.0
−0.2
PC2 [6.7%]
−0.25
0.00
0.25
0.50
PC1 [9.7%]
Location
Boffa
Dabola
Faranah
Kankan_HLC
Kankan_PSC
Kissidougou
Mamou


a
PC2 [11.2%]
0.2
0.1
0.0
−0.1
−0.4
−0.2
0.0
0.2
PC1 [39.2%]
Location
Boffa
Dabola
Faranah
Kankan_HLC
Kankan_PSC
Kissidougou
Mali
Mamou


b
PC2 [7.1%]
0.15
0.10
0.05
0.00
−0.05
−0.10
−0.1
0.0
0.1
0.2
0.3
PC1 [41.6%]
Location
Boffa
Dabola
Faranah
Kankan_HLC
Kankan_PSC
Kissidougou
Mamou

Assessment of mosquito species and *kdr* mutation

We simultaneously genotyped the same mosquitoes at loci informative of their species and insecticide-resistance status by high-throughput sequencing (see Materials and Methods).

Out of 665 mosquitoes, 551 (82.9%) were successfully genotyped for the S200X6.1 [185] and *cox1*[194] loci. We primarily used the S200X6.1 locus to identify the species of each mosquito as i) this locus was more robustly amplified and sequenced than the *cox1* locus (with an average read count of 2,917 and 1,181 per mosquito, respectively) and ii) provided clearer taxonomic resolution (with, for example, 233-234 (mean of 233.67) nucleotides differentiating the sequences from *Anopheles gambiae s.s.* from those of *Anopheles coluzzii*, compared to 0-4 (mean of 1.71) nucleotide differences using the cox1 locus) (Table 2.4). However, the S200X6.1. locus systematically failed to yield sequences for some mosquitoes that were identified at *Anopheles nili* using the cox1 sequences. Overall, we identified that the mosquitoes belonged to five *Anopheles* species. 404 mosquitoes (74.5%) were identified as *Anopheles gambiae s.s.,* while the remaining mosquitoes consisted of *Anopheles coluzzii* (61 mosquitoes or 11.3%), *Anopheles melas* (with 57 mosquitoes or 10.5%), *Anopheles arabiensis* (8 mosquitoes or 1.5%), and *Anopheles nili* (7 mosquitoes or 1.3%) (Figure 2.8). We also identified 5 mosquitoes that were heterozygous for the S200X6.1 locus and likely represented F1 hybrids of *An. gambiae s.s.* and *An. coluzzii* species. The species distribution varied extensively between locations, with *An. gambiae s.s.* accounting for more than 90.00% of all mosquitoes collected in five out of six locations in Guinea, while *An. melas* was the most abundant species (79.2%, 57/72) in Boffa, a coastal region in western Guinea, and *An. coluzzii* predominated in Bandiagara, Mali (86.3%, 44/51) (Figure 2.8).

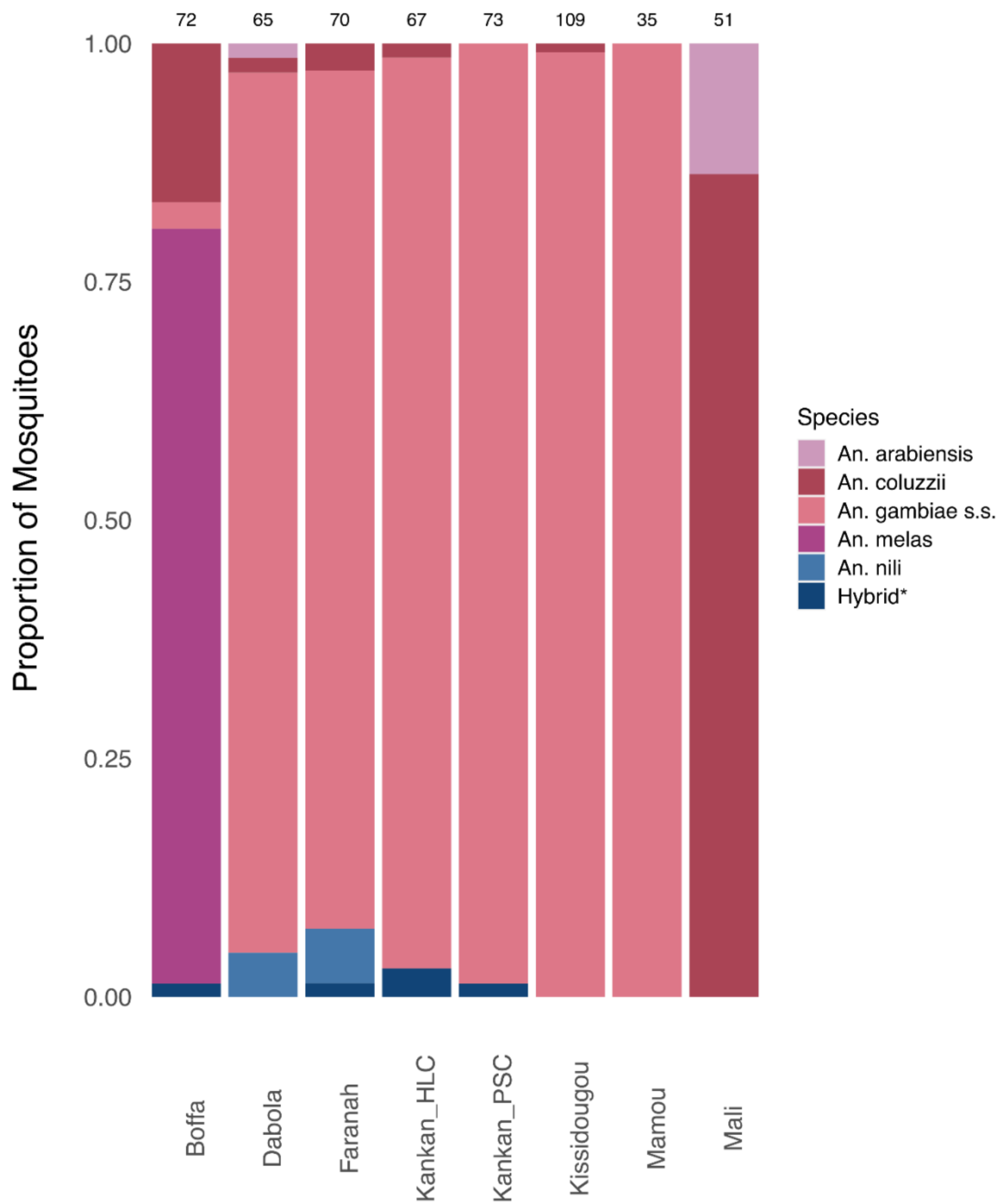


Figure 2.8. Mosquito species diversity across collection sites in Guinea and Mali.

Hybrid* represents samples identified as heterozygous for Anopheles gambiae and An. coluzzii at the S200X6.1 locus. Numbers above each bar represent the total number of mosquitoes with successfully characterized species from each site.

Pyrethroid resistance is often due to a point mutation in the voltage gated sodium channel gene, described as knockdown resistance (*kdr*) [184]. 550 (82.7%) of the mosquitoes were successfully genotyped at this locus (with an average coverage of 2,436 reads per mosquito). In Guinea, with the exception of mosquitoes collected in Boffa, most mosquitoes (>92.6%) were homozygous for the *kdr*-w (L1014F) alleles that is associated with resistance to pyrethroids [184] (Figure 2.9). In Boffa, where *An*. *melas* is the predominant species, most mosquitoes were homozygous for the wild-type allele (L1014L). In Mali, the distribution was more heterogeneous, with roughly equal proportions of mosquitoes homozygous for the wild-type, resistant allele or heterozygous. Across mosquitoes, the genotype at the *kdr*-w locus correlated almost perfectly with the mosquito species, with *An. gambiae* carrying primarily L1014F alleles while *An. arabiensis* and *An. melas* were essentially wild-type. Only *An. coluzzii* showed high proportion of both alleles (Figure 2.10). The details of all the genotypes and sequences amplified from each mosquito are provided in Table 2.5.

Determination of the blood meal composition

To characterize the composition of the last blood meal of each these mosquitoes, we used the same DNA extract to amplify and sequence a short fragment of the mammalian mitochondrial 16S rRNA gene. 133 mosquitoes yielded >1,000 reads and were considered blood fed in later analyses. 126 mosquitoes carried human DNA, 14 mosquitoes cow DNA, and 2 sheep DNA (Figure 2.11 and Table 2.6). Nine mosquitoes fed on more than one mammalian host species (Figure 2.11). The blood meal composition differed between sites with, for example, 12 mosquitoes (20.1%) from Kankan that fed, at least

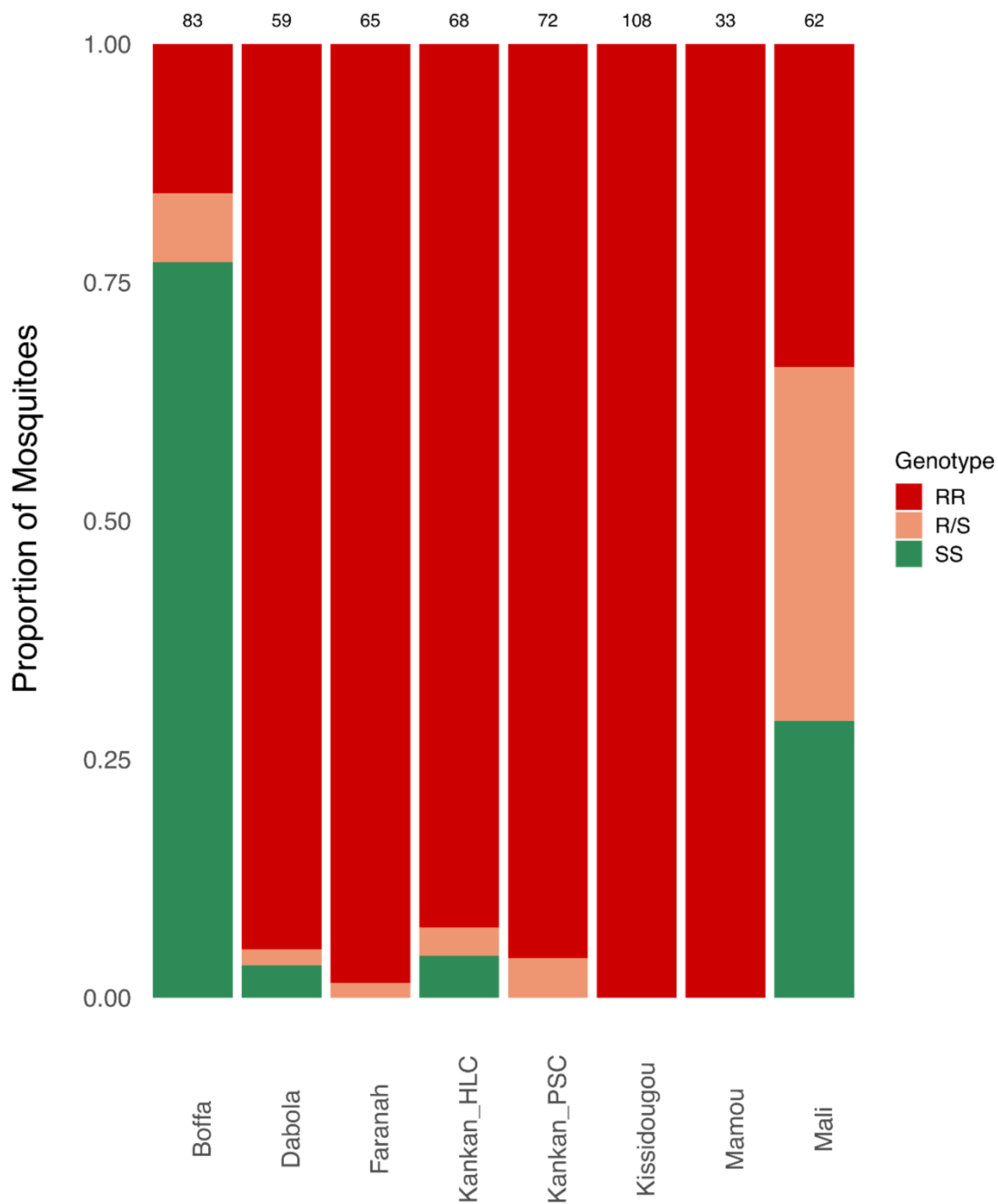


Figure 2.9. Distribution of mosquito knockdown resistance west (kdr-w [L1014F mutation variant]) in mosquitoes collected across Guinea and Mali.

Numbers above each bar represent the total number of mosquitoes successfully genotyped at the kdr-w genotype, per site. RR Homozygous resistant, SS homozygous sensitive, R/S heterozygous.

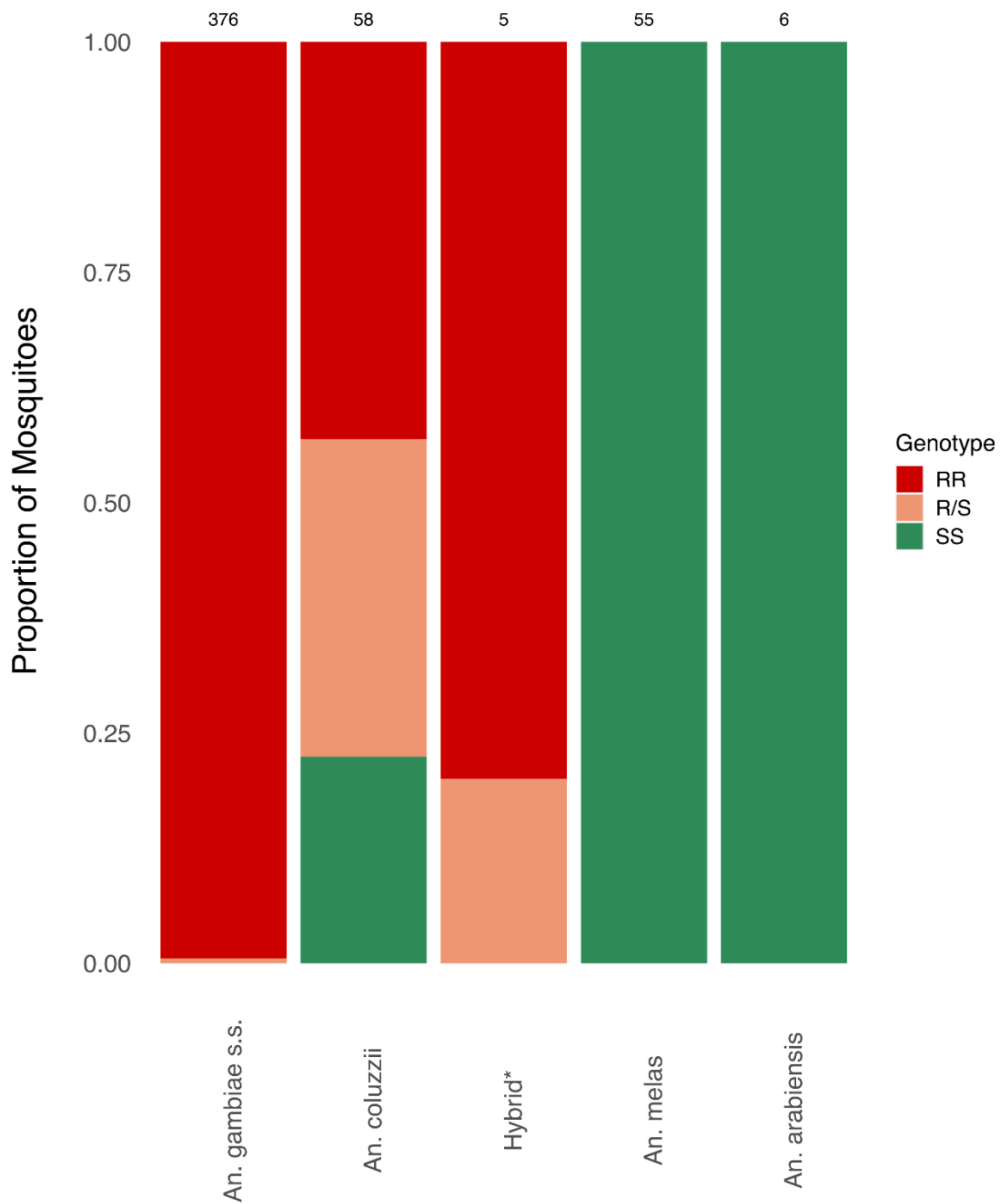


Figure 2.10. Distribution of L1014F mutation (kdr-w) in mosquitoes grouped by Anopheles species.

Numbers above each bar represent the total number of mosquitoes successfully genotyped at the kdr-w genotype, per site. RR Homozygous resistant, SS homozygous sensitive, R/S heterozygous.

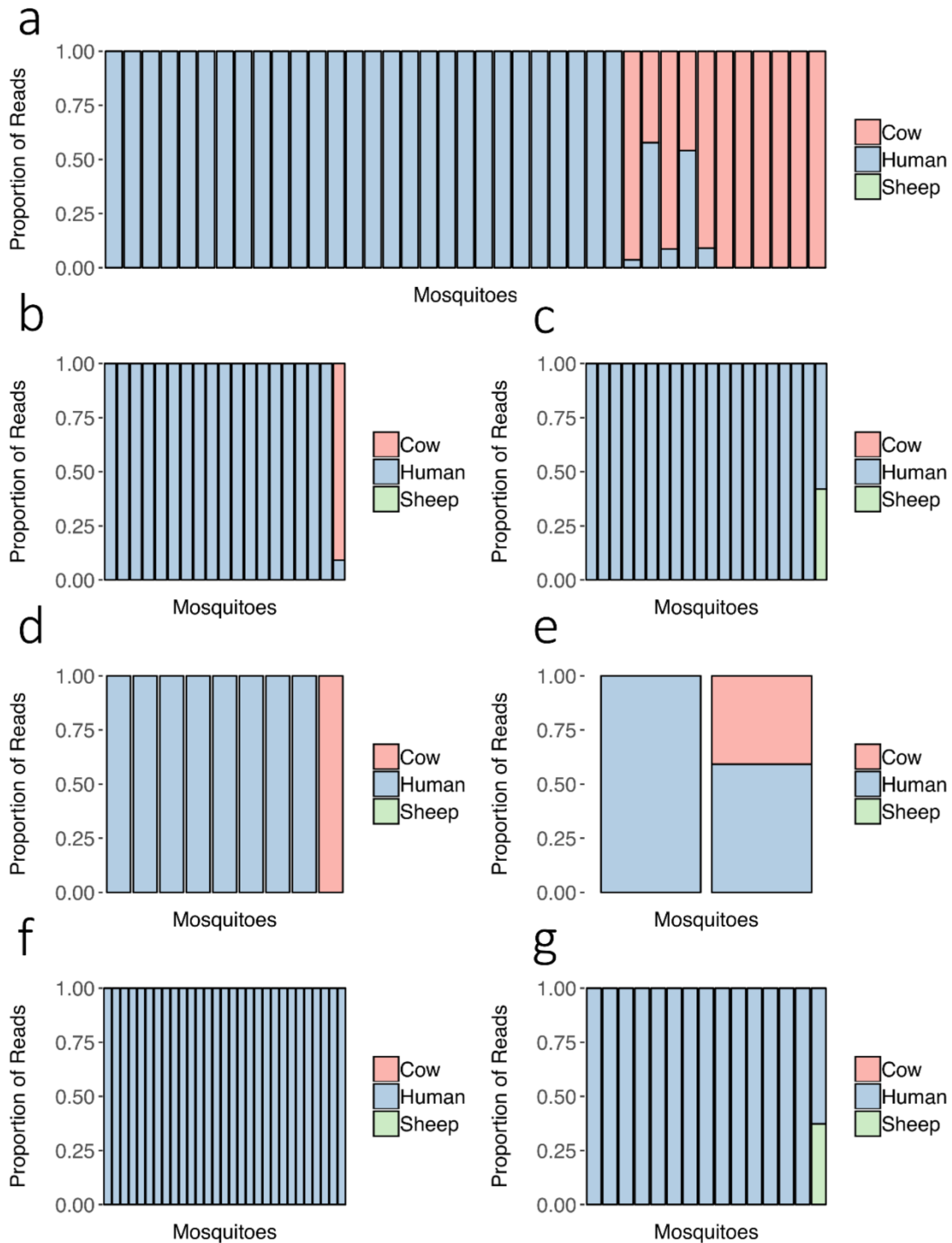


Figure 2.11. Host blood-meal composition of individual mosquitoes collected from Kankan using pyrethrum spray catches (a), Kankan using human landing catches (b), Kissidougou (c), Dabola (d), Faranah (e), Boffa (f), Mali (g).

Each bar represents an individual mosquito.

Mammal	# of samples detected	% Identity	Average # of reads
Human	126	100	2,038
Cow	14	100	1,409
Sheep	2	100	1,065

Table 2.6. Summary of host blood-meal composition.

Table shows, the number of mosquitoes carrying mammalian DNA, the percent match of sequence to the NCBI database, and average count of reads per mammal.

partially, on cow while mosquitoes from all other sites, in Mali and Guinea, fed almost exclusively on human.

Identification of eukaryotic parasites and viruses from individual mosquitoes

Finally, we determined whether each mosquito carried a eukaryotic parasite and/or arbovirus using a sequencing-based assay recently developed in our laboratory [186]. After PCR amplification, and sequencing of DNA extracted from individual mosquitoes, we identified DNA sequences from eukaryotes and viruses from 127 mosquitoes, with on average, 1,876 reads supporting each identification in each mosquito. Nine out of 95 extraction controls (water samples that have been processed simultaneously) were also positive for one or more parasites, but with an average of 54 reads per parasite (Table 2.7). The low read counts in those water controls could be explained by low-level cross-contamination during extraction, or sequence mis-assignment due to sequencing errors in the Illumina index sequences [186].

Eight mosquitoes (1.2%), from 3 sites (6 of them identified as *An. gambiae*), yielded DNA sequences identical to *Plasmodium falciparum,* the primary cause of human malaria in Africa. We detect DNA belonging to *Theileria* species in a relatively high number of mosquitoes (27/665 or 4.1%). *Theileria* species are protozoan parasites that can be infective to domestic (i.e. cattle) and wild (buffalo) animals and transmitted by ticks [195]. Interestingly, from the fourteen samples for which *Bos indicus* (cow) was identified with greater than 500 reads, we detect *Theileria* species in twelve (86%), suggesting the tick-transmitted parasite may have been ingested by these mosquitoes during their last blood-meal. Seven mosquitoes yielded a DNA sequence identical to *Loa loa* and to several other filarial worms (and were further characterized as deriving from *Mansonella perstans* after sequencing of longer amplification products, M. Cannon. personal communication). Several DNA sequences were closely related to known parasites of mosquitoes themselves, belonging to microsporidia [196] (e.g., *Parathelohania sp.*), mosquito-transmitted nematodes (e.g., *Setaria sp.* [197, 198]), as well as two recently discovered *Anopheles* flaviviruses (e.g., *Anopheles flavivirus variant 1* and *variant 2*) [199] (Table 2.8). The details of all parasite sequences amplified and their taxonomic information for each mosquito are provided in Table 2.7.

Evaluation of the factors influencing microbial composition of wild mosquitoes

The characterization of the mosquito species, insecticide-resistance associated genotype, blood meal status and infection from the same mosquitoes that have been examined for their microbial diversity enables a rigorous assessment of the relative contribution of these factors to the microbial composition. Note that to avoid possible biases introduced by sample storage or DNA extraction, we restricted this analysis to mosquitoes collected in

Taxon targeted	Species identified (# positive)	% Positive	% Identity
Apicomplexa A	*Theileria sp*. (24)	3.60	100
Apicomplexa B	*Plasmodium falciparum* (8)	1.20	100
Apicomplexa C	*Theileria sp*. (3)	0.45	100
Microsporidia	*Parathelohania anopheles* (38)	5.71	92.47
	Hazardia milleri (1)	0.15	97.38
	Culicospora magna (6)	0.90	99.7
	Microsporidium sp. 3 NR-2013 (34)	5.11	97.01
Nematoda A	*Acanthocheilonema viteae* (12)	1.80	100
	Loa loa/Dipetolenma sp. (7)	1.05	99.64
	Setaria labiatopapillosa (11)	1.65	100
	Auanema rhodensis (1)	0.15	98.21
Nematoda B	*Setaria yehi/Setaria digitate* (4)	0.60	99.72
	Acanthocheilonema viteae (1)	0.15	99.72
	Dipetolenma sp./Filariodea sp. (3)	0.45	98.94
Flavivirus	*Anopheles flavivirus* variant 2 (2)	0.30	99.06
	Anopheles flavivirus variant 2/ variant 1 (1)	0.15	88.26
	Culex flavivirus (1)	0.15	99.06

Table 2.8 Eukaryotic parasites and viruses identified from screening mosquito samples.

Table shows the parasite and viral taxon targeted by each primer, the species identified and the number of mosquitoes positive for that species, the percent of total mosquitoes positive, and the percent match of the sequences amplified to that of the NCBI database.

Guinea that were all processed identically and simultaneously. We implemented an analysis of variance [200] to simultaneously test the contribution of each factor, while accounting for the others (multivariate analysis). The geographical location of the samples explained most of the variation in microbial composition ($R^2 = 0.200$, $\rho = 0.001$), whereas the mosquito species ($R^2 = 0.004$, $\rho = 0.208$), *kdr-w* genotype ($R^2 = 0.006$ $\rho = 0.438$), and

feeding status ($R^2 = 0.003$, $\rho = 0.173$) were not statistically associated with the bacterial composition. The mosquito infection status was significantly associated with the microbial composition but had a very marginal effect ($R^2 = 0.015$, $\rho = 0.001$) (Table 2.9). To further investigate the relative roles of geography and species that are partially confounded in this dataset, we examined PCoAs of the bacteria composition, restricting the analysis to i) all *An. gambiae* collected in seven sites (Figure 2.12a) and, separately, ii) mosquitoes from all *Anopheles* species collected in Boffa (Figure 2.12b). Together, these analyses validated the results of the statistical testing and confirmed that geographical location of the mosquitoes had a much greater influence on the bacterial composition than the species of the mosquitoes.

Factor	**Df**	**R^2**	**P-Value**
Location	7	0.2	0.001
Mosquito species	4	0.004	0.208
kdr-w genotype	2	0.006	0.438
Blood-meal	1	0.003	0.173
Infection status	1	0.015	0.001
Residuals	440	0.772	

Table 2.9 Relative contribution of mosquito factors on microbial variation.

Table shows, for each factor, the Df, R2 (percent of variation explained), and P-value (significance value) calculated by Adonis. Abbreviations: Df, Degrees of freedom.

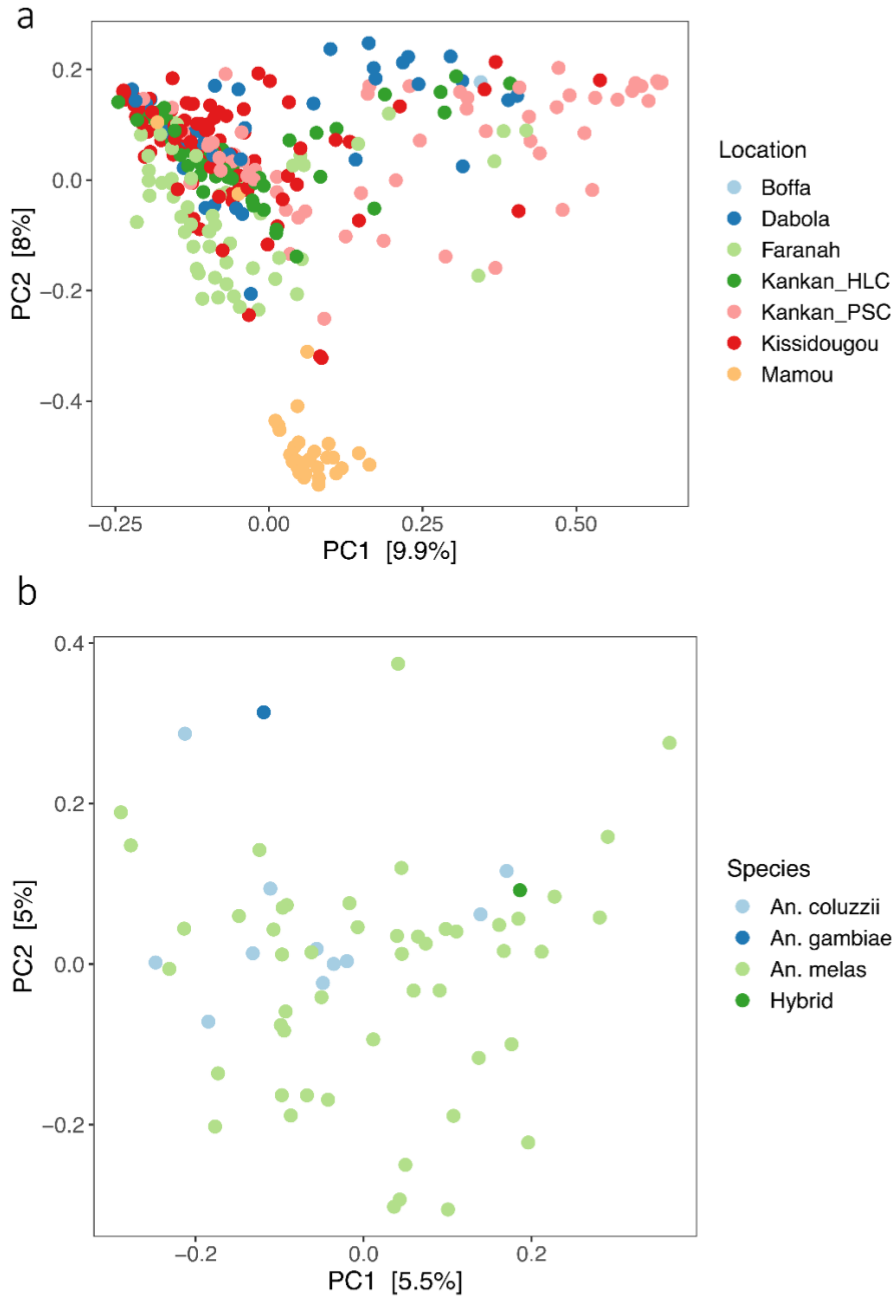


Figure 2.12. PCoA plot showing the dissimilarity between the microbial composition of individual mosquitoes based on Bray-Curtis dissimilarity metric for An. gambiae mosquitoes only, from sites in Guinea (A) and all Anopheles mosquitoes identified in Boffa, Guinea (B).

Each dot represents the bacterial composition of a single mosquito. The numbers in brackets near the axes indicate the proportion of the variance explained by the PC 1 and 2.

Discussion

The importance of the mosquito microbiota on vector biology and pathogen transmission has been well recognized, with several studies demonstrating the role of endogenous bacteria on the vector's development [123, 175], immunity [176, 177], and competency [201]. However, few studies have examined the factors that shape the bacterial composition of mosquitoes and most of those used laboratory-reared mosquitoes to assess influences on bacterial communities [123, 182, 202]. This latter limitation could be especially problematic since bacterial diversity of wild-caught *Anopheles* mosquitoes has been shown to be greater than that of mosquitoes reared in the laboratory [203]. In addition, these studies typically focus on testing the influence of a single factor on the bacterial composition without accounting for confounding factors. In this study, we examined the microbial diversity of 665 individual wild-caught *Anopheles* mosquitoes collected in six sites in Guinea and one site in Mali. Consistent with previous studies, the bacterial composition of mosquitoes was dominated by bacteria from the *Proteobacteria*, *Firmicutes*, and *Actinobacteria* phyla [178-180]. *Pseudomonas*, bacterial genus commonly found in mosquito larvae and larval habitats [179] was one of the most abundant genera detected across all samples (Figure 2.5 and Table 2.3). Despite these overall similarities, we observed significant differences in bacterial composition among mosquitoes and examined the contribution of various factors to this diversity. For each mosquito, we characterized their species, *kdr-w* genotype, blood-meal status, and infection with various eukaryotic parasites and viruses. We then simultaneously estimated the relative contribution of each of those endogenous and exogenous factors on the microbial composition of the mosquitoes. In this analysis, the mosquito collection site accounted for

~20% of the variation in bacterial composition, whereas the other factors showed marginal or non-significant contribution (Table 2.9).

Our findings are consistent with previous studies that showed that collection site was a major contributor to the microbial diversity of field-caught *Anopheles* mosquitoes [159, 178, 204, 205]. For example, Muturi *et al.* found that sampling site has a strong effect on microbial composition and diversity, even when examining nine different mosquito species [179]. Note however that "collection site" in our study summarizes many parameters. In particular, the mosquitoes were collected from very different ecoregions (grasslands and canopy forests, mountainous forests or savanna, Table 2.1) and the influence of the "collection site" on the microbial composition could reflect the effect of differences in larval habitats, flora the mosquitoes rely on for nectar feeding, and/or local population differences. In this regard, it is worth noting that the adult mosquito bacterial composition has been shown to vary depending on the larval breeding sites and the bacterial composition of these aquatic habitats [118]. In addition, sugar source appears to have a pronounced influence on the vectoral capacity of *An. sergentii* mosquitoes [206] and impacts the microbial composition of laboratory-reared adult mosquitoes [207]. Future studies using a denser, more local sampling of wild-caught mosquitoes, will be required to better understand the individual contribution of these local parameters.

On the other hand, our analyses provided new insights on the role of other factors on the microbial composition of mosquitoes. We did not observe any significant contribution of feeding status on microbial variation of the wild mosquitoes. This finding contrasts with a previous study that described that the bacterial diversity of *Aedes aegypti* mosquitoes fed on human, chicken, or rabbit blood was significantly lower than that of newly emerged

unfed mosquitoes [182]. This discrepancy could reflect differences between mosquito species/genera, or more likely, differences between wild-caught mosquitoes (that might have had prior blood meals) and laboratory-reared mosquitoes with less variable microbial composition. Similarly, our study did not reveal any significant contribution of genetic factors (i.e. mosquito species, *kdr-w* genotype) on mosquito microbial variation. These observations contrast with a previous study that described distinct bacterial compositions in two species of *Culex* mosquitoes collected from the same site and with identical larval aquatic environment [180], possibly due to differences among *Culex* species in their larval feeding habits [208]. The L1014F *kdr* allele frequency was reported high or near fixation in Kankan and Kissidougou sites of Guinea and low in Boffa in a previous study [209], consistent with our findings. In theory, insecticide-resistant mosquitoes could display a different microbial composition since this resistance may allow them to survive insecticide exposure that could impact the bacterial populations. Here, we did not see evidence of L1014F resistant allele influence on adult mosquito microbial composition, although the lack of information on whether these mosquitoes might have been exposed to insecticides limits the conclusions that can be drawn from this observation. Note however that resistance allele genotypes and mosquito species only represent a small fraction of the genetic factors that could impact the mosquito microbiota and that, given our observation that the collection site is strongly associated with the bacterial composition, it would be interesting to further investigate whether genetic diversity is associated with the microbiota of mosquitoes [157].

Interestingly, we observed a marginal but statistically significant association between infection status (infected n=127 vs. non-infected n=513) and the mosquito microbial

composition. Modification of insect gut microbiota by parasitic [210] or viral [211] infections has been demonstrated in few studies. Pathogenic or non-pathogenic (e.g., insect-specific viruses) species could be involved in crosstalk with insect metabolism pathways or immune system to influence the microbiota [212]. It is worth noting that, due to the low infection rate with parasitic and viral species we found in the mosquitoes (<5.0%), we assessed the influence infection has on the microbiota using an aggregate of all the parasite and viruses we detected (as opposed to individual parasite and virus species) and it is possible that the effect of one organism on the microbiota might be diluted down and undetected once analyzed together with other parasites and viruses that have no influence on the bacterial communities. For example, *Theileria sp.* are transmitted by ticks and unlikely to be viable in mosquitoes and therefore, have probably little to no contribution on the influence of infection on the mosquito microbiota. Future studies assessing the direct influence of some of the parasites found in abundance in this study (e.g., *Parathelohania sp.*, *Microsporidium sp.*,) and the recently discovered virus (*Anopheles flavivirus*) could further elucidate the tripartite relationship between the mosquito, microbiota, and mosquito infecting agents. One important caveat of our study is that we screened for RNA virus sequences from DNA extracts and, while this approach successfully detected multiple Flaviviruses, the extraction was not optimized for RNA molecules and many sequences might have been lost, leading to an underestimation of the number of viruses.

Finally, it is noteworthy that the approach described in this study is easily adaptable to other disease vectors (e.g., ticks and sandflies) or insects important in agriculture (such as

bees) and easily customizable to examine specific factors of interest by simply adding or replacing PCR primers.

Conclusions

In summary, this study provides a comprehensive assessment of the microbial composition and diversity of 665 wild mosquitoes and a simultaneous examination of the relative contribution of five different mosquito factors on microbial variation. This approach enables rigorous estimation of the importance of these factors to shaping the bacterial composition, while correcting for their often confounding effect. Our results highlight the prominent role of the mosquito collection site and, to a lesser extent, the parasitic and viral infection, on shaping the bacterial composition of wild-caught mosquitoes. These findings provide a solid foundation to implement further investigations and examine the specific components of the environment (e.g., bacterial communities of the larval habitats, source of nectar, genetic diversity) shaping the microbial composition of wild mosquitoes and the mechanisms mediating these effects.

Table 2.2. Summary of all primers used in the study.

Table shows, for each primer pair, the loci targeted, the base pair length of amplicon, and the forward and reverse primer sequences. The file is found at https://static-content.springer.com/esm/art%3A10.1186%2Fs13071-020-04491-7/MediaObjects/13071_2020_4491_MOESM3_ESM.xls.

Table 2.3. ASV taxonomy.

Table shows, for each ASV, the sequence identified, the taxonomic information (Kingdom to Species), and abundance values. NA represents when an ASV is unknown at that taxonomic rank. The file is found at https://static-

content.springer.com/esm/art%3A10.1186%2Fs13071-020-04491-7/MediaObjects/13071_2020_4491_MOESM8_ESM.xls.

Table 2.4. Pairwise comparison of cox1 and S200X.6 primers for resolution of mosquito species.

Table shows, for each pairwise-comparison between two Anopheles species, the mean nucleotide difference and range for cox1 and S200X.6 loci. The file is found at https://static-content.springer.com/esm/art%3A10.1186%2Fs13071-020-04491-7/MediaObjects/13071_2020_4491_MOESM9_ESM.xls

Table 2.5. Summary of species and kdr-w determination.

Table shows, for each sample, the mosquito collection site, the number of reads belonging to kdr alleles and Anopheles species and their sequences. kdr_w Knockdown resistance west (mutant), WT wildtype, H2O water controls. Seq sequence. The file is found at https://static-content.springer.com/esm/art%3A10.1186%2Fs13071-020-04491-7/MediaObjects/13071_2020_4491_MOESM11_ESM.xls

Table 2.7. Eukaryotic parasite and virus identification.

Table shows the primer name, sample name, collection site, mosquito species, and taxonomic information per sequence identified. Table also gives, for each sequence, the frequency per sample (count), percentage match to NCBI database (% Identity), length of sequence (in bp), and length of the match (in bp) to NCBI sequence. The file is found at https://static-content.springer.com/esm/art%3A10.1186%2Fs13071-020-04491-7/MediaObjects/13071_2020_4491_MOESM13_ESM.xls.

Chapter 3. Single Cell Analysis Reveals Transcriptional Heterogeneity and Patterns of Regulated Gene Expression in *Plasmodium* Sporozoite Development[2]

Introduction

Malaria is a disease caused by unicellular parasites of the *Plasmodium* genus that are transmitted to humans by the bites of infected female *Anopheles* mosquitoes. Socioeconomic developments [7, 213-215], combined with the use of extensive entomological controls [216, 217] and improved antimalarial drug development and distribution [218, 219], have significantly decreased the mortality associated with malaria over the last 50 years. However, the disease remains a heavy burden on many human populations, affecting more than 200 million people and responsible for half a million deaths per year [1]. An efficient malaria vaccine remains elusive, but encouraging progress has been achieved in recent years with the development and testing of vaccines using recombinant proteins [220, 221] or attenuated parasites [102, 222, 223] from the infectious stage, the sporozoites. However, despite renewed interest on *Plasmodium* mosquito stages for vaccine development and their critical importance for reducing malaria transmission, much of the molecular processes regulating sporozoite development remains unclear.

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[2] Bogale, H.N., Pascini, T.V., Kanatani, S. et al. Single Cell Analysis Reveals Transcriptional Heterogeneity and Patterns of Regulated Gene Expression in Plasmodium Sporozoite Development. PNAS 10, 118 (2021).

Following the ingestion of male and female *Plasmodium* gametocytes during a blood feeding, fertilization occurs in the mosquito midgut, producing zygotes that undergo meiosis and develop into motile ookinetes. The ookinete traverses the midgut epithelium and matures into an oocyst. On the basal surface of the midgut epithelium, the sessile oocyst undergoes meiosis and multiple cycles of DNA replication and cell divisions, which leads to the production of thousands of sporozoites that are released into the mosquito's hemolymph. The crescent-shaped sporozoites are then transported by the hemolymph and approximately 20% of them successfully enter the salivary glands, where they wait to be inoculated into a mammalian host, remaining viable for days to weeks [224]. Transmission to the mammalian host occurs when a small number of sporozoites, typically less than 100, are ejected with the mosquito saliva into the dermis during the probing phase of a bite [225]. Once in the skin, sporozoites move rapidly to locate blood vessels and enter the blood circulation which carries them to the liver, where they enter hepatocytes and develop into the next developmental stage. During their migration, from the mosquito midgut to the salivary glands, into the skin and to the liver, sporozoites do not show major morphological changes but appear to go through important developmental changes. For example, sporozoites collected from oocysts or from the hemolymph can cause successful mammalian infections but these sporozoites are, overall, much less infectious than salivary gland sporozoites [226-228]. Conversely, sporozoites collected from a salivary gland and injected into the hemolymph show reduced infectivity for the mosquito salivary glands [229]. These changes in infectivity are mirrored by changes in mRNA expression and protein abundance that have been characterized for rodent parasites and, to a lesser extent, for *P. falciparum* [230, 231]. Combined with elegant reverse genetic experiments, these

analyses have highlighted some of the molecular processes underlying sporozoite maturation, and revealed key *Plasmodium* genes involved in sporozoite egress from the oocyst (e.g., SERA5 [232], SIAP-1 [233] or PCRMPs [234]), and in the recognition and invasion of the mosquito salivary glands (e.g., TREP [235] or MAEBL [236]). However, despite these important studies, several outstanding questions remain regarding the regulation and development of *Plasmodium* sporozoites in mosquitoes. For example, it is not clear whether the maturation of sporozoites is primarily driven by extrinsic (e.g., the location of the sporozoites in the mosquito) or intrinsic factors (e.g., the age of the sporozoites) as these parameters are confounded in most studies. Similarly, we do not know whether all sporozoites at the same anatomical location are identically regulated or whether they mature asynchronously. These questions have been difficult to rigorously address due to technical limitations of available methodologies. In order to have sufficient amount of material for analysis, genomic and proteomic approaches typically rely on molecules extracted from many parasites, only providing "averages" on these populations and masking potential heterogeneity among individual parasites. The advent of single cell technologies allows us to examine the regulation of individual cells and has already provided exciting new insights on the biology of *Plasmodium* parasites [237-240]. Here we describe the analysis of the transcriptomes of 36,958 individual sporozoites representing three Plasmodium species, and collected throughout their development, from oocysts to salivary glands, and from forced salivation experiments. We examine the similarities and specificities of sporozoite gene expression of different *Plasmodium* species and describe the molecular changes, and their variations, occurring during the development

and maturation of the sporozoites as they migrate from midgut oocysts to the salivary glands.

Materials and Methods

Parasite and mosquito strains

Infections of *An. stephensi* mosquitoes (Nijmegen strain [241]) were performed with P. falciparum (NF54), P. cynomolgi (M strain), and *P. berghei* ANKA parasites expressing the green fluorescent protein (GFP)($PbGFP_{CON}$) [242] at the insectary of the Laboratory for Malaria and Vector Research of the NIAID/NIH. Infections of *An. stephensi* mosquitoes (Liston strain) were performed with *P. berghei* ANKA line 159cl1 that constitutively expresses mCherry [243] at the Johns Hopkins Bloomberg School of Public Health.

An. stephensi mosquitoes were reared under standard insectary conditions at 80% humidity, and 12h/12h light/dark cycle and maintained with cotton pads soaked either in 10% sucrose solution or 10% corn syrup solution (Karo®, ACH Food Companies).

Animal handling and ethics protocol

This study was performed following the recommendations from the Guide for Care and Use of Laboratory Animals of the National Institutes of Health (NIH). The animal use was approved by the Johns Hopkins Institutional Biosafety Committee (IBC) and Johns Hopkins University Institutional Animal Care & Use Committee (MO17H325), and by the National Institute of Health Animal Ethics Proposal and registered in the Standard

Operating Procedures of Laboratory of Malaria and Vector Research (NIAID/NIH-SOP LMVR 22).

Infection conditions

Three- to four-week-old Swiss-Webster mice were infected with *P. berghei* from frozen stocks, and the mouse parasitemia was monitored daily by light microscopy analysis of methanol-fixed blood smear stained with 10% Giemsa. Four- to five-day-old *An. stephensi* females were starved for 12 hours before being allowed to feed for 30 minutes on an anesthetized mouse infected with *P. berghei* with an ~1% parasitemia and an exflagellation rate of 1:10 fields. Only fully engorged mosquitoes were kept and maintained at 18°C (JHSPH) or 19°C (NIAID) with 10% sugar solution *ad libitum* until sporozoite collection.

Isolation of *P. falciparum*, *P. cynomolgi*, and *P. berghei*-GFP sporozoites

To isolate sporozoites from oocysts, 50 female mosquitoes were dissected 14 days after the infected blood meal. Mosquitoes were anesthetized on ice and the midguts were dissected under a stereomicroscope in phosphate buffered saline (PBS). The midguts were placed in an excavated petri dish with PBS under a fluorescent microscope, opened, and the oocysts were disrupted with a needle to release the sporozoites. The released sporozoites were collected with a Sigmacote®-coated pipette tip (Sigma) and transferred to a low-retention tube (Protein LoBind®, Eppendorf).

Hemolymph sporozoites were collected by perfusion of 50 ice-cold anesthetized females at day 16 post-infection. Mosquitoes were gradually injected with 10 µL of PBS into the thorax, and the sporozoites were collected from the flow-through by an incision made in

the abdominal wall using forceps. The samples were collected with a Sigmacote®-coated tip and transferred to a low-retention tube (Protein LoBind® Tube, Eppendorf) on ice.

Sporozoites from salivary glands were collected at 21 days post-infection. For *P. berghei* ($PbGFP_{CON}$), one day before sporozoite collection, salivary gland infection was confirmed by the accumulation of parasite-expressed GFP on the mosquito thoracic cavity under a fluorescent microscope (MZ10 F, Leica). Fifty female mosquitoes were anesthetized on ice and their salivary glands dissected in PBS under a stereomicroscope. The salivary glands were transferred to a low-retention tube (Protein LoBind® Tube, Eppendorf) containing PBS, homogenized with a disposable pestle, and kept on ice.

Salivated sporozoites were collected from 60 infected female mosquitoes at day 21 post-infection by forced probing in a Sigmacote®-coated tip filled with 10 µL of PBS. Briefly, mosquitoes were anesthetized on ice for 5 minutes, the wings were removed, and the mosquito proboscis was gently inserted into the tip. The mosquitoes were left to salivate for 30 minutes, and the salivated sporozoites were pooled in a low-retention tube (Protein LoBind® Tube, Eppendorf). After collection, all the samples were homogenized, passed through a 20 µm Pluriselect® filter (Cell Strainer) to remove cellular debris, and counted using a hemocytometer (C-Chip™, Chemglass Life Sciences). The excess volume was removed by centrifugation at 15,000 g for 10 minutes, and the sporozoite final concentration adjusted to 500 to 2,000 sporozoites/µl in PBS.

Isolation of *P. berghei*-mCherry sporozoites from mosquito saliva and salivary glands

One day prior to sporozoite collection, mosquitoes were fluorescently sorted and mosquitoes with fluorescence in the salivary gland area were kept for sporozoite harvest. Salivary gland- and salivated sporozoites were collected from mosquitoes 21- or 22 days

post infection. Briefly, mosquitoes were anesthetized on ice for 5 minutes and immobilized on a glass plate by placing their wings on double-sided tape. The mosquito proboscis was gently inserted into a low-adhesion P10 pipet tip containing 2 µl of immersion oil (ZEISS, Immersol 518F) or 4 µl of PBS. Salivation was induced by applying 1% pilocarpine (Sigma, P6503) / 0.2% Tween 20 (Sigma, P1379) in PBS to the mosquito abdomen. Following this, mosquitoes were allowed to salivate for 30 minutes. Salivated sporozoites were collected from 60-78 mosquitoes by pooling pipet tips containing saliva sporozoites in a low-retention 0.6 ml tube (Thermo Fisher, 3446). For sporozoites salivated into oil collection, pooled oil was mixed with 20 µl of PBS and spun at 1,000 x g for 5 minutes to separate saliva from oil.

Library preparation and sequencing

The number of sporozoites isolated from each sample was crudely determined using a hemocytometer and light microscopy. An estimated 1,000-5,000 sporozoites per sample were loaded on a 10X Genomics Chromium controller and 12 individually barcoded 3'-end scRNA-seq libraries were prepared according to the manufacturer's protocol. Each library was then sequenced one an Illumina high-output sequencer to generate a total of 2,296,539,124 75-bp paired-end reads (Table 3.1).

Single-cell transcriptome analysis

A custom analysis pipeline, similar to the Cell Ranger single-cell software [244], was developed to process all scRNA-seq reads [239]. Briefly, we identified all reads containing the 10X Genomics barcodes, trimmed of sequences downstream of 3' polyadenylation tails, and only kept for further analyses reads longer than 40 bp. We mapped all reads to the *P. berghei* ANKA genome [245] using HISAT2 (version 2.0.4 [246]), allowing for a

maximum intron length of 5,000 bp, and to the *An. stephensi* genome (AsteS1 [247]). To identify sequences that represent PCR duplicates resulting from the library preparation, we identified reads with identical sequences for the 16-mer 10X Genomics barcode, 12-mer unique molecular identifier sequence, and mapped to the same genomic location of DNA and on the same strand, and only kept one of them. We then used the 10X Genomics barcodes to assign reads to individual parasites and tallied the number of reads mapped to each annotated *P. berghei* gene (i.e., from the annotated transcription start site to 50 bp after the 3'-end).

We then combined all scRNA-seq libraries and used the scran (v1.14.6, [248]) addPerCellQC function to identify and remove outlier cells based on library size and gene count while accounting for variations between libraries (e.g., sequencing depth). We then normalized the transcriptomes of these filtered transcriptomes and calculated size factors by deconvolution using the scran functions quickCluster and computeSumFactors. The calculated size factors were used to compute normalized counts per cell via logNormCounts function in scater (v1.14.6) [249]. Finally, we selected the most variable genes in the dataset using the scran modelGeneVar and getTopHVGS functions (var.threhsold=0) and performed principal component analysis with runPCA.

Estimation of the pseudotime and differential expression analysis

The dataset analyzed with scran/scatter - including raw counts, normalized counts, reduced dimensionality (PCA) and metadata - was then imported to slingshot (v1.4.0) [250] to calculate pseudotime for all individual parasites.

To identify genes that were differential expressed along the estimated pseudotime trajectory, we used the fitGAM function (k=7) of tradeSeq (v1.1.19, [251]). Due to the low

mRNA transcript abundance observed in salivated sporozoites, we only included in the statistical testing data from sporozoites collected from oocyst, hemolymph, and salivary gland. We tested for differentially expressed genes according to the calculated trajectory using the associationTest function and corrected p-values for multiple testing using false discovery rates [252, 253].

Analysis of ribosomal RNA genes

All scRNA-seq reads were also mapped to the four chromosomal locations of the *P. berghei* genome containing the 18S-5.8S-28S rRNA genes (on chromosomes 5, 6 ,7 and 12) using Hisat2 but without allowing for spliced alignments (--no-spliced-alignment mode) to avoid mismapping caused by the high sequence homology among loci. Very few reads mapped to the 5S rRNA gene cluster on chromosome 10 and those were not included in these analyses. We then tallied the number of reads unambiguously mapped to each chromosomal location for each individual parasite. To control for variations in the number of informative reads derived from each chromosome, we split the reference sequence of each rRNA genes into 75 bp (with a shift of 1 bp) and determined the proportion of reads, from each gene, mapped unambiguously to its original location.

Results and Discussion

Gene expression profiling of individual *Plasmodium* sporozoites

We analyzed sporozoites collected from the salivary glands of *Anopheles stephensi* mosquitoes infected with *P. falciparum* (n=1), *P. cynomolgi* (n=1) and *P. berghei* (n=9). We also analyzed *P. berghei* sporozoites collected throughout their development in An.

stephensi mosquitoes and collected from punctured late-stage oocysts (n=2), mosquito hemolymph (*P. berghei*, n=3), and forced salivation experiments (*P. berghei*, n=3). Overall, these 19 samples derive from three *Plasmodium* species, two *P. berghei* strains, and used two different *An. stephensi* colonies (Table 3.1**,** see Material and Methods for details).

From each sample, we prepared a 3'-end 10X Genomics scRNA-seq library [244] and sequenced 51-257 million paired-end reads of 75 base pairs (Table 3.1). 0.6% to 67.2% of the reads mapped to the corresponding *Plasmodium* genome (*P. falciparum* 3D7 [254], *P. cynomolgi* M strain [255] and *P. berghei* ANKA [245]) providing, on average, 20.5 million *Plasmodium* reads per sample. Most of the remaining reads mapped to the *An. stephensi* genome and represented contamination by cell-free mosquito RNA released during the sample collection and/or mRNA from individual *Anopheles* cells. After stringent quality filters (see Material and Methods), we obtained between 84 and 8,412 single *Plasmodium* cell transcriptomes from each library, for a total of 36,958 individual sporozoite transcriptomes, each characterized by an average of 1,033 reads per parasite 2,713 unique reads (235-24,507) (Table 3.1).

Single-cell salivary gland sporozoite transcriptomes differ between *Plasmodium* species

We first evaluated the gene expression profiles of 1,390 *P. falciparum*, 5,867 *P. cynomolgi*, 5,397 *P. berghei* individual sporozoites collected from the salivary glands of *Anopheles stephensi*. Overall, we detected expression of 2,775 *P. falciparum*-, 3,844 *P. cynomolgi*-, and 4,800 *P. berghei* genes, with averages of 32, 28, and 214 genes expressed per individual cell, respectively for each species. The circumsporozoite protein (CSP,

PBANKA_0403200) [256] and the cell traversal protein for ookinetes and sporozoites (CelTOS PBANKA_1432300) [257] were the most highly expressed genes in all three species (Table 3.2), while the sporozoite surface protein essential for liver stage development (SPELD, PBANKA_0910900), the gamete egress and sporozoite transversal protein (GEST, PBANKA_1312700), the thrombospondin-related anonymous protein (TRAP, PBANKA_1349800) and the sporozoite protein essential for cell traversal (SPECT1, PBANKA_1355600) were amongst the 20 most expressed genes in all species. By contrast, some of the most highly expressed genes in one species had no known orthologs in the other *Plasmodium* species (Table 3.2), potentially representing genes underlying species-specific sporozoite features, such as differences in motility [243, 258] or in the mammalian cell invasion specificity.

To further investigate differences in gene expression among species, we compared the expression profiles of these 12,654 single-cell salivary gland sporozoites using 4,202 annotated *Plasmodium* genes with one-to-one ortholog between the three species (see Material and Methods). Principal component analysis (PCA) revealed clear clustering of the sporozoites into distinct clusters according to their species, with 8-10% of the variance in overall gene expression explained by species differences (Figure 3.1). Note that despite these apparently dissimilar gene expression profiles, sporozoites from all three species clustered tightly together when analyzed jointly with blood-stage *P. berghei* parasites [240], indicating that the differences in sporozoite gene expression between species are much smaller than those between sporozoites and blood-stage parasite of the same species (Figure 3.2). Out of 587 orthologous genes that were robustly expressed in at least 20% of the parasites from one species, 157 were deemed statistically differentially expressed in

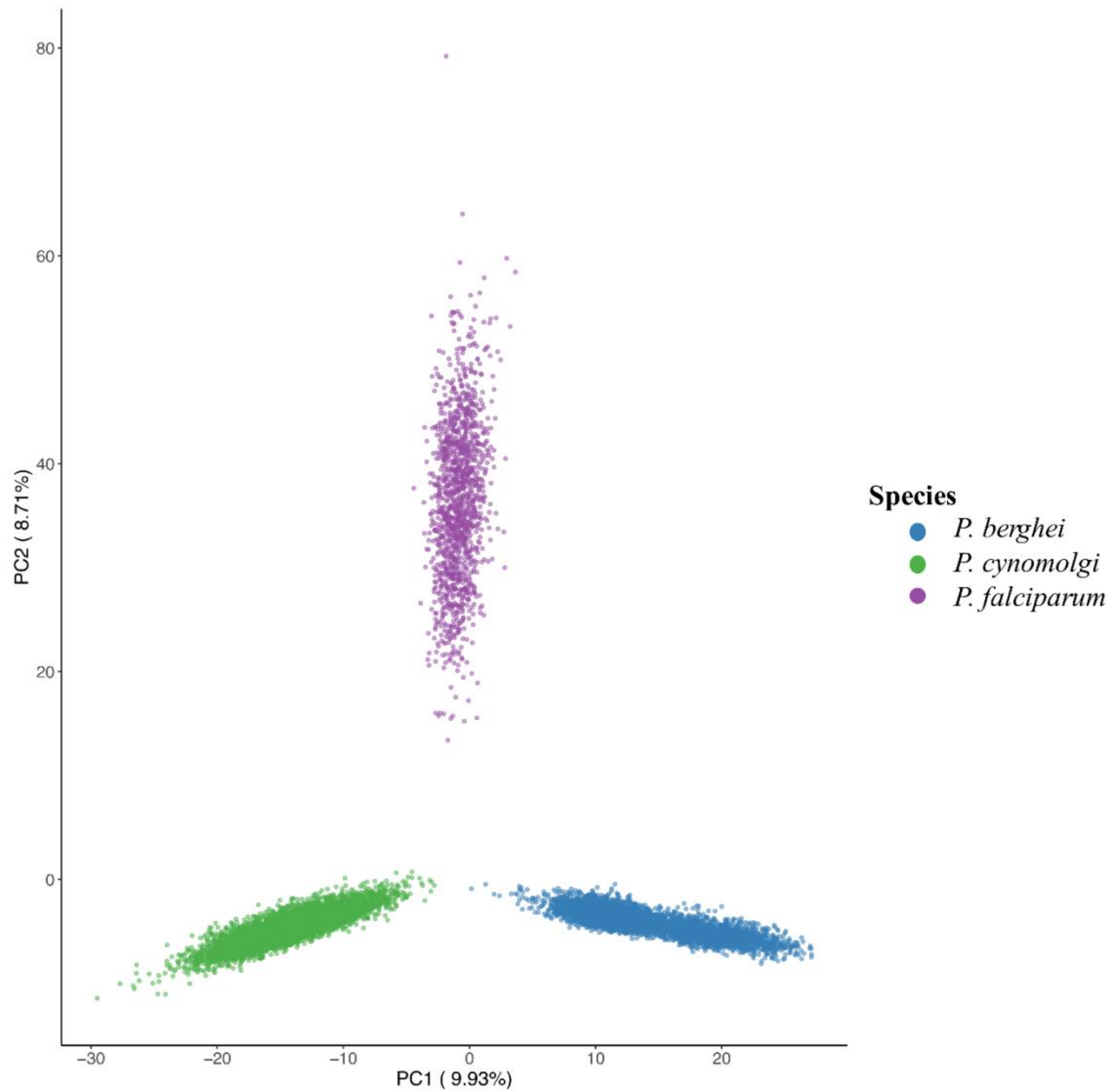


Figure 3.1. PCA showing the relationships between individual salivary gland sporozoites isolated from *P. berghei* (n=5,397, in blue), *P. falciparum* (n=1,390, in purple) and *P. cynomolgi* (n=5,867, in green) based on the expression of 4,202 genes with one-to-one orthologs in the three species.

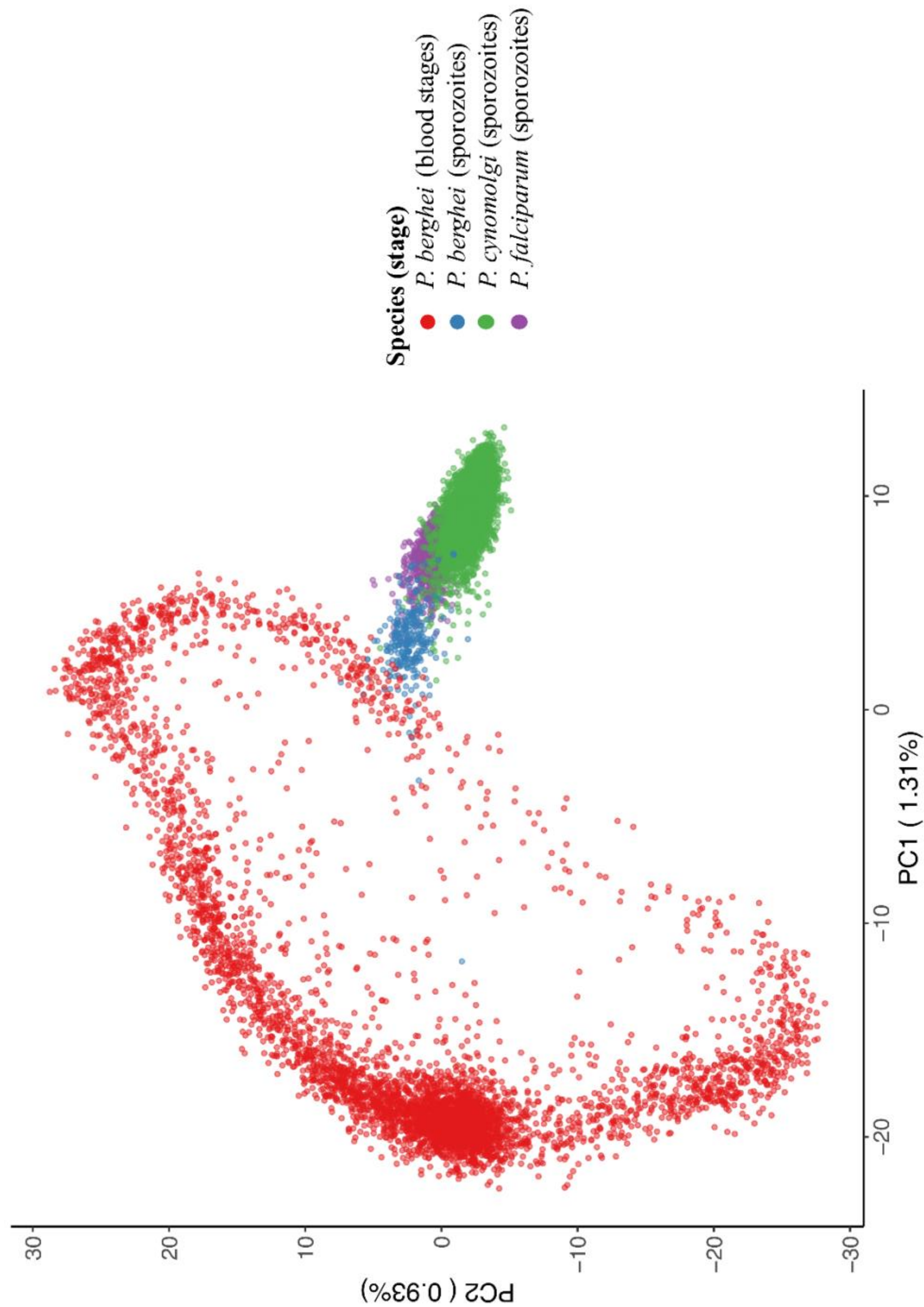


Figure 3.2. PCA showing the relationships between individual salivary gland sporozoites isolated from *P. berghei* (in blue), *P. falciparum* (in purple) and *P. cynomolgi* (in green) and blood-stage *P. berghei* parasites (in red) based on the expression of 4,202 genes with one-to-one orthologs in the three species.

Note that despite clear species differences (Fig 3.1), the sporozoites from all species clustered tightly together and distinctly separated from the asexual blood-stage parasites.

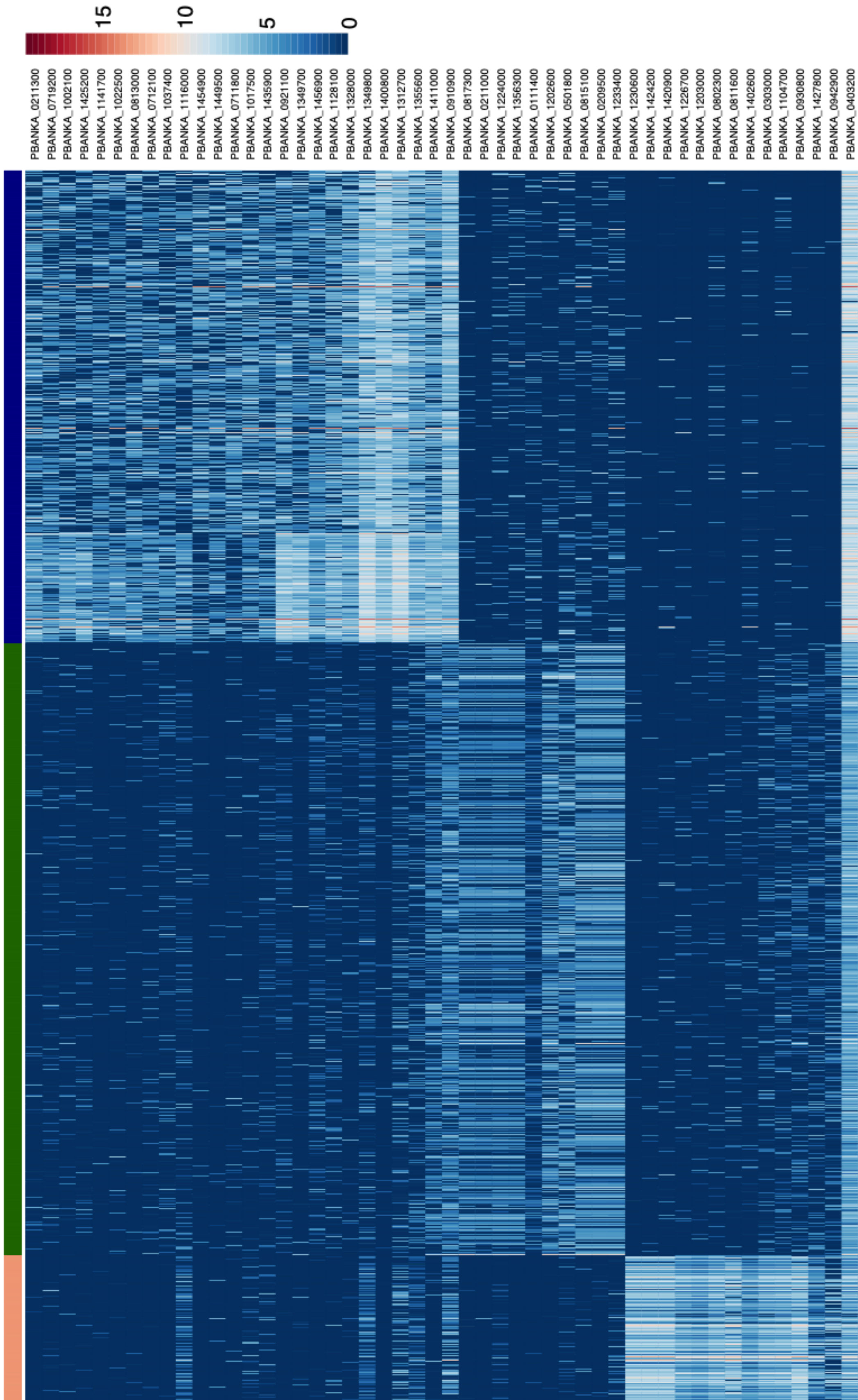
one of the three species (FDR=0.1, Table 3.3). Interestingly, these include several genes responsible for inhibition of protein degradation (PBANKA_0813000) and translation inhibition (PBANKA_0719200, see also below) that were significantly upregulated in *P. berghei* salivary gland sporozoites (Figure 3.3), possibly related to the slower development of the sporozoites of this species and/or the longer infectivity of mosquitoes infected with *P. berghei* compared to *P. falciparum*. We would however caution against overinterpreting these data since the *P. falciparum* and *P. cynomolgi* salivary gland sporozoites derive from a single sample collected from a few mosquitoes: it is therefore possible that these differences reflect stochastic variations or differences in the maturity of the sporozoites rather than genuine species differences, and more studies will be required to rigorously identify and validate species-specific regulatory differences.

scRNA-seq reveals heterogeneous and overlapping changes in gene expression during *P. berghei* sporozoite development

We characterized the transcriptomes of 16,038 sporozoites: 614 sporozoites collected from disrupted oocysts, 2,147 sporozoites isolated from the mosquito hemolymph, 5,979 sporozoites dissected from mosquitoes' salivary glands, as well as on 7,298 sporozoites obtained after forced salivation experiments (Table 3.1). After excluding the transcripts of genes detected in less than 300 cells, we obtained expression data from 1,763 genes (out of the 5,120 protein-coding genes annotated in the *P. berghei* genome) for further analysis (Materials and Methods). To examine transcriptomic changes occurring during sporozoite development, we first compared the gene expression profiles of individual sporozoites using principal component analysis (PCA). Interestingly, while *P. berghei* sporozoites primarily clustered according to the anatomical site from which they were collected, there

Figure 3.3. Heatmap showing the most 50 differentially expressed genes between the salivary gland sporozoites of the three species.

Each row shows the expression of one protein-coding gene and each column one individual sporozoite organized from left to right by species (salmon - *P. falciparum*, green - *P. cynomolgi*, blue - *P. berghei*). The heatmap color shows the number of a given transcript per cell from dark blue (not detected) to white and red (most highly expressed).

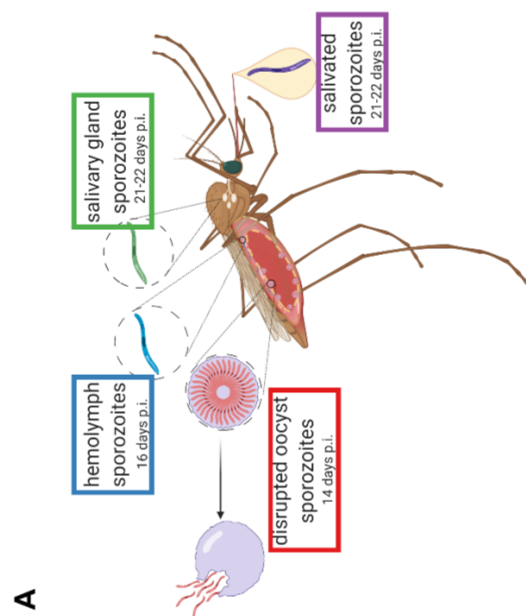

15
10
5
0
PBANKA_0211300
PBANKA_0719200
PBANKA_1002100
PBANKA_1425200
PBANKA_1141700
PBANKA_1022500
PBANKA_0813000
PBANKA_0712100
PBANKA_1037400
PBANKA_1116000
PBANKA_1454900
PBANKA_1449500
PBANKA_0711800
PBANKA_1017500
PBANKA_1435900
PBANKA_0921100
PBANKA_1349700
PBANKA_1456900
PBANKA_1128100
PBANKA_1328000
PBANKA_1349800
PBANKA_1400800
PBANKA_1312700
PBANKA_1355600
PBANKA_1411000
PBANKA_0910900
PBANKA_0817300
PBANKA_0211000
PBANKA_1224000
PBANKA_1356300
PBANKA_0111400
PBANKA_1202600
PBANKA_0501800
PBANKA_0815100
PBANKA_0209500
PBANKA_1233400
PBANKA_1230600
PBANKA_1424200
PBANKA_1420900
PBANKA_1226700
PBANKA_1203000
PBANKA_0802300
PBANKA_0811600
PBANKA_1402600
PBANKA_0303000
PBANKA_1104700
PBANKA_0930800
PBANKA_1427800
PBANKA_0942900
PBANKA_0403200

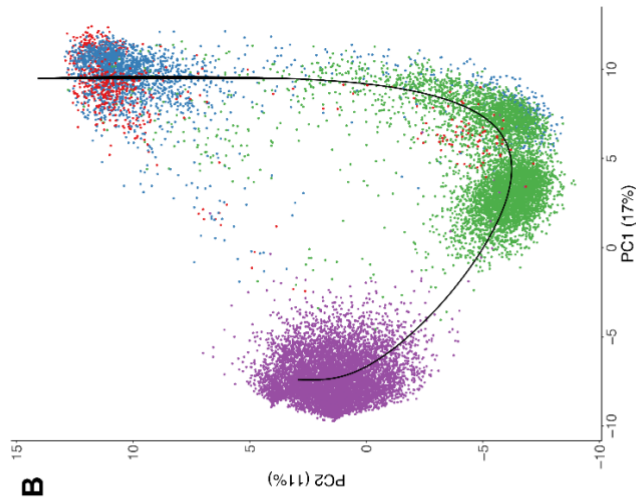
were extensive variations within, and overlap between, sporozoites isolated from different anatomical sites (Figure 3.4B). For example, the gene expression profiles of hemolymph sporozoites (in blue on Figure 3.4B) ranged from profiles indistinguishable from those of oocyst sporozoites (in red) to profiles of gene expression similar to those of salivary gland sporozoites (in green), consistent with the range of phenotypes observed in this population. Similarly, the gene expression profiles of some of the salivated sporozoites (in purple) were similar to those of salivary gland sporozoites, while others showed a very distinct profile (Figure 3.4B).

To further examine changes in gene expression during *P. berghei* sporozoite development, while accounting for the apparent heterogeneity of each sample, we estimated the position of each individual sporozoite along a putative developmental trajectory (or pseudotime) calculated solely using the gene expression data [250]. This unsupervised analysis sequentially organized sporozoites starting with those collected from disrupted oocysts and ending with salivated sporozoites, but with significant overlaps between sporozoites collected at different points of their development (with the possible exception of salivated sporozoites that displayed a more distinct gene expression profile) (Figure 3.4C). Some of this transcriptional heterogeneity could be accounted for by slight differences between biological replicates (Figure 3.5). Some of this transcriptional heterogeneity could be accounted for by slight differences between biological replicates (Figure 3.5). In particular, we observed a shift between the modes of the distributions obtained from mCherry- and GFP-labeled salivary gland sporozoites, which could be due to differences in the times of collection or in the insectary temperatures, or the effect of the fluorophore and/or its level of expression in these genetically modified parasites. However, variations between

Figure 3.4. Changes in gene expression during sporozoite development.

(A) Schematic summarizing sporozoite populations included in this study and their time of collection in days post-infection (figure created with BioRender.com). **(B)** PCA showing the relationships among individual *P. berghei* sporozoites based on their expression profiles. Each dot represents a single sporozoite and is colored according to its collection site (red – disrupted oocysts, blue – hemolymph, green – salivary glands, purple – forced salivation). The black line shows the trajectory of the developmental pseudotime inferred based on the gene expression profiles. **(C)** Distribution of the pseudotime values (x-axis) inferred for each individual sporozoite (each represented by a dot colored as in B) according to their collection site (y-axis). Note the overlap between the distributions of each group.


A
hemolymph
sporozoites
16 days p.i.
salivary gland
sporozoites
21-22 days p.i.
salivated
sporozoites
21-22 days p.i.
disrupted oocyst
sporozoites
14 days p.i.


B
PC2 (11%)
PC1 (17%)

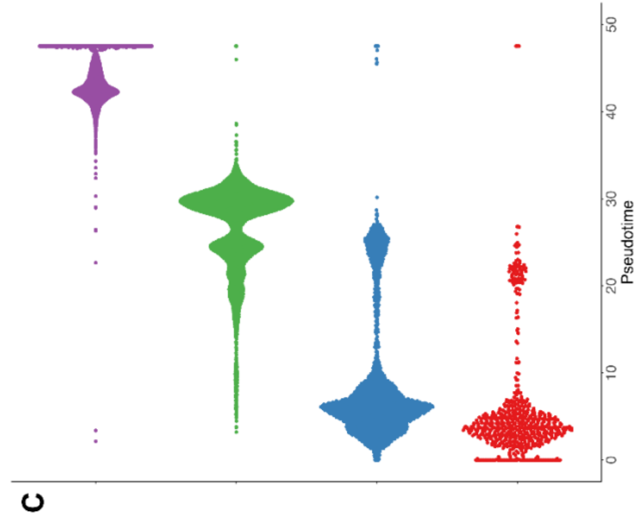

C
Pseudotime

Figure 3.5. Distribution of the pseudotime values inferred for each individual sporozoite (x-axis) according to the sample they derive from and colored by their collection site (y-axis).

Note that the overlaps between the distributions of pseudotimes are consistently observed across replicates (with some variations).

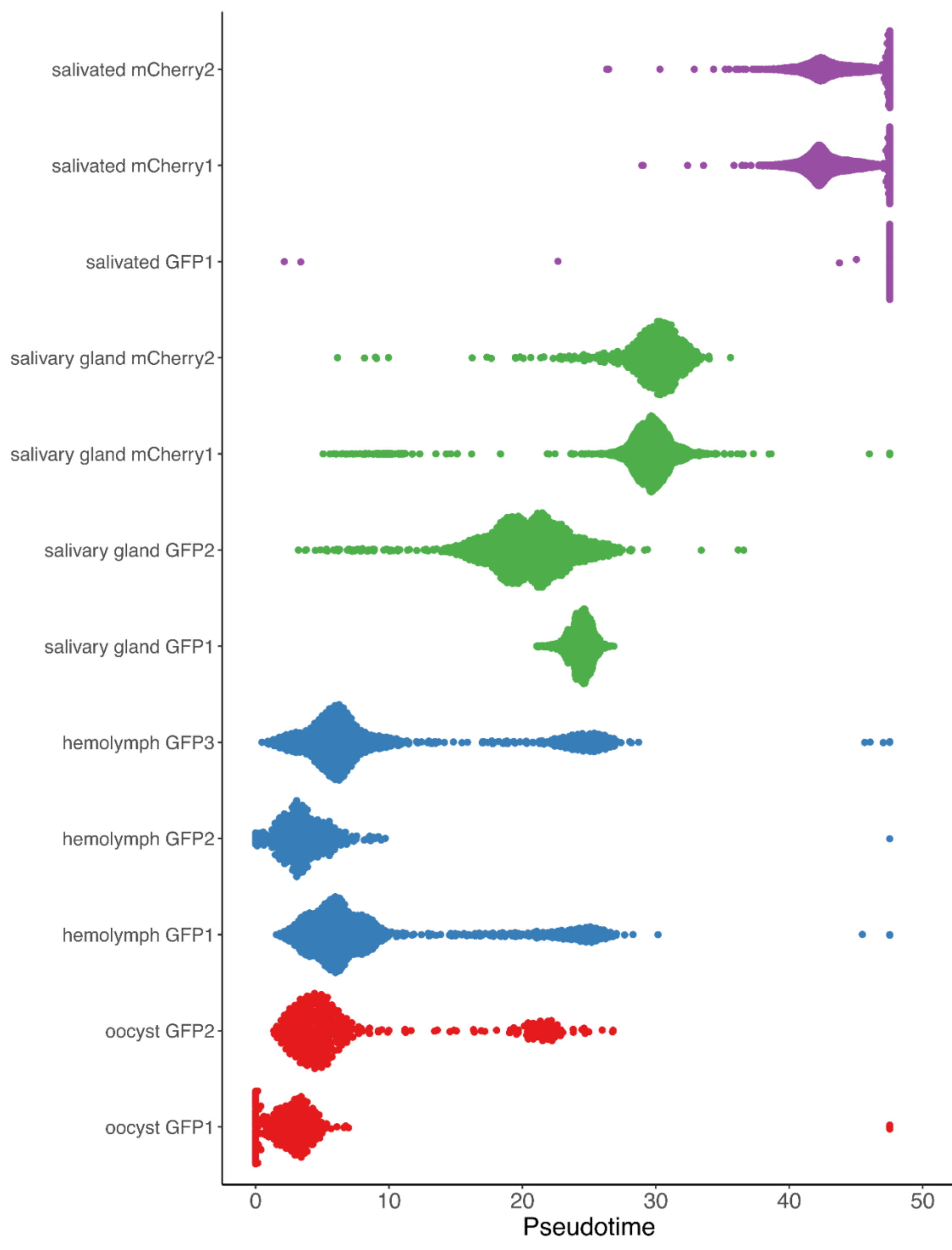
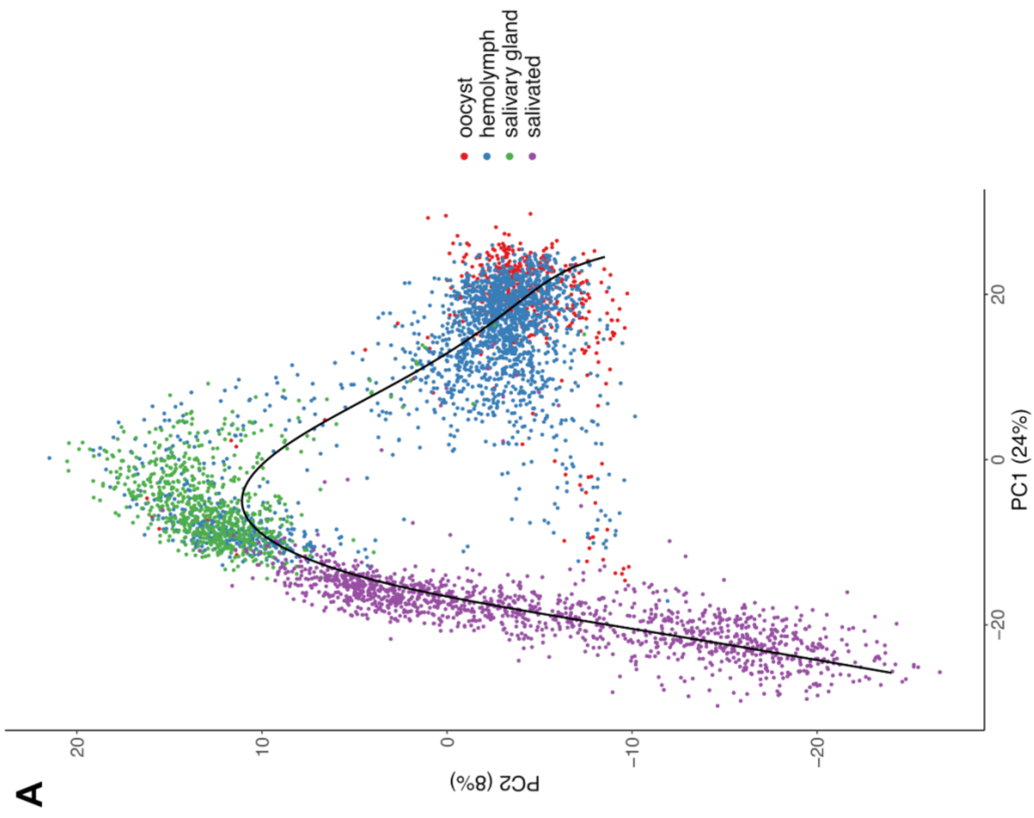
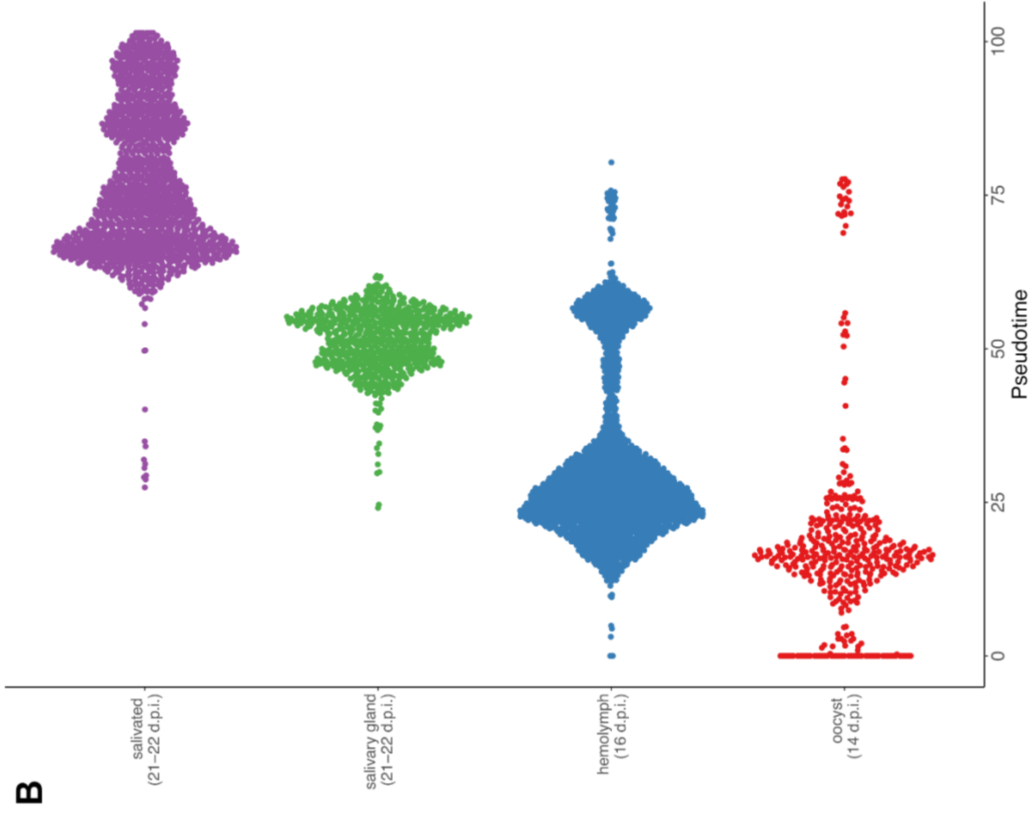

salivated mCherry2
salivated mCherry1
salivated GFP1
salivary gland mCherry2
salivary gland mCherry1
salivary gland GFP2
salivary gland GFP1
hemolymph GFP3
hemolymph GFP2
hemolymph GFP1
oocyst GFP2
oocyst GFP1
0
10
20
30
40
50
Pseudotime

Figure 3.6. Characterization of gene expression during sporozoite development by more than 1,000 unique reads.

(A) PCA showing the relationships among individual *P. berghei* sporozoites based on their expression profiles using the 4,462 cells characterized by more than 1,000 unique reads. Each dot represents a single sporozoite and is colored according to its collection site (red – disrupted oocysts, blue – hemolymph, green – salivary glands, purple – forced salivation). The black line shows the trajectory of the developmental pseudotime inferred based on the gene expression profiles. **(B)** Distribution of the pseudotime values (x-axis) inferred for each individual sporozoite (each represented by a dot colored as in **B**) according to their collection site (y-axis). Note the overlap between the distributions of each group.



A
PC2 (8%)
PC1 (24%)
20
10
0
−10
−20
oocyst
hemolymph
salivary gland
salivated



B
salivated
(21−22 d.p.i.)
salivary gland
(21−22 d.p.i.)
hemolymph
(16 d.p.i.)
oocyst
(14 d.p.i.)
0
25
50
75
100
Pseudotime

replicates did not entirely explain the wide range of transcriptional heterogeneity observed among sporozoites collected at the same anatomical site, as distinct gene expression profiles were consistently observed within each biological replicate (Figure 3.5). Furthermore, to evaluate whether the "outlier cells" might represent technical artefacts caused by low signal, we repeated the analysis considering only cells characterized by more than 1,000 unique reads. The gene expression patterns observed were qualitatively similar to those from the entire dataset and confirmed high heterogeneity among sporozoites collected at the same site as well as overlaps between those collected at different sites (Figure 3.6).

Taken together, these analyses supported previous findings from microarray and RNA-seq studies that showed that gene expression changes over the course of sporozoite development [230, 231, 259], resulting in differences between parasites collected from different anatomical locations (since those were also correlated with the time post infection, Figure 3.4). However, the analysis of individual parasites enabled identifying heterogeneity among sporozoites at the same location, which was previously masked in bulk analyses. This heterogeneity could derive from differences in the rate of oocyst maturation and the time since sporozoite egress: *P. berghei* oocysts, within the same mosquito, develop asynchronously and can reach maturity at different times [260]. It is therefore possible that the differences in gene expression observed among sporozoites reflect variations in their time since egress. Alternatively, these findings could indicate that the rate of sporozoite development stochastically varied between individual parasites, resulting in similar gene expression profiles between, for example, fast maturing sporozoites in the hemolymph and slower maturing salivary gland parasites. Lastly, this

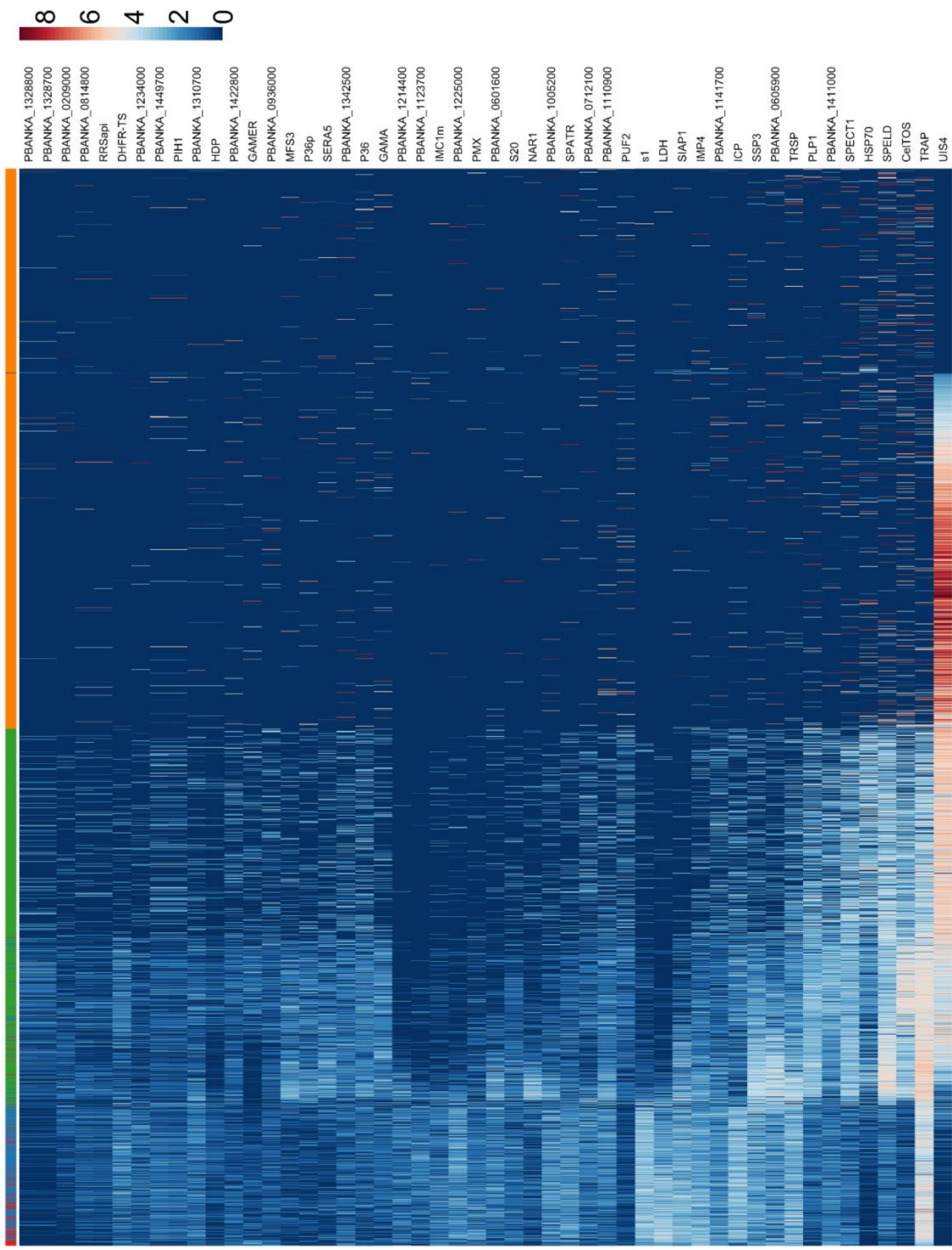
analysis relied on parasites collected from multiple mosquitoes (see Table 3.1) which could account for some of the transcriptional heterogeneity: while each experiment used mosquitoes from the same colony and of the same age, it is possible that mosquito-specific factors influenced the rate of *Plasmodium* development and that the sporozoites with unusual transcriptional profiles (either delayed or accelerated) all derive from the same mosquito. Irrespective of the underlying reason for the transcriptional heterogeneity, the overlap observed between the gene expression profiles of sporozoites collected at different sites clearly indicated that *Plasmodium* development in the mosquito is not solely regulated by signals from their tissue environment: a small fraction of the oocyst and hemolymph sporozoites looked identical, transcriptionally, to salivary gland sporozoites suggesting that the development of the parasites does not entirely depend upon their anatomical location. This observation could explain why a few sporozoites collected from oocysts or hemolymph can induce mammalian infections [226-228]: these successful infections would be caused by the minority of oocyst- or hemolymph sporozoites with advanced maturation (i.e., those with greater pseudotimes on Figure 3.4C).

Tightly regulated changes in gene expression during sporozoite development

To systematically identify genes differentially expressed during sporozoite development, we tested, for each individual *P. berghei* gene, whether the expression level was significantly associated with the developmental pseudotime, from oocyst- to salivary gland sporozoites. Since salivated sporozoites displayed low mRNA abundance compared to sporozoites collected from other anatomical sites (see below), we excluded these parasites from the differential analysis reported here (but the results using all parasites is shown in Table 3.4 and Figure 3.7). This differential gene expression analysis

Figure 3.7. Heatmap showing the expression patterns for the 50 most differentially expressed genes during sporozoite development in mosquitoes (from oocyst- to salivated sporozoites).

Each row shows the expression of one protein-coding gene. Each column shows the data for one individual sporozoite, organized from left to right according to its pseudotime and colored by its anatomical location in the track above the heatmap (red – oocyst, blue – hemolymph, green – salivary gland, orange – salivated sporozoites). The heatmap color shows the number of a given transcript per cell from dark blue (not detected) to white and red (most highly expressed).


8
6
4
2
0
PBANKA_1328800
PBANKA_1328700
PBANKA_0209000
PBANKA_0814800
RRSapi
DHFR-TS
PBANKA_1234000
PBANKA_1449700
PIH1
PBANKA_1310700
HDP
PBANKA_1422800
GAMER
PBANKA_0936000
MFS3
P36p
SERA5
PBANKA_1342500
P36
GAMA
PBANKA_1214400
PBANKA_1123700
IMC1m
PBANKA_1225000
PMX
PBANKA_0601600
S20
NAR1
PBANKA_1005200
SPATR
PBANKA_0712100
PBANKA_1110900
PUF2
s1
LDH
SIAP1
IMP4
PBANKA_1141700
ICP
SSP3
PBANKA_0605900
TRSP
PLP1
PBANKA_1411000
SPECT1
HSP70
SPELD
CelTOS
TRAP
UIS4

recapitulated much of our current knowledge of sporozoite biology. CSP [256] and TRAP [261] showed high and sustained level of expression from oocyst- to salivary gland sporozoites (consistent with previous reports, see e.g., [259]) before an abrupt decreased in expression (discussed below). Many other genes with validated roles in oocyst egress and gliding motility (SSP3 [262]), salivary gland invasion (MAEBL [236], SIAP-1 [233], TREP [235], ICP [263]) or skin passage and liver development (UIS4 [264], UIS3 [265], CelTOS [266], UIS2 [267], TRSP [268], GEST [269], PL [270]) were among the 50 most differentially expressed genes and displayed a timing of expression consistent with their known function (Table 3.5, Figure 3.8). In addition to these well-characterized genes, our analysis also highlighted the potential role of genes not previously known to be important in sporozoites: for example, Plasmepsin X (PM-X, PBANKA_1222500), that has been shown to be involved in merozoite egress [271], was highly expressed during early sporozoite development, suggesting a possible role in oocyst egress, while bergheilysin (PBANKA_1137000) was expressed later, consistent with a putative role in salivary gland invasion or skin passage (Figure 3.9A). Similarly, several genes without any functional annotation, predicted domain or reported knock-out mutants, showed high level of expression and tightly regulated timing of expression (Figure 3.9B) consistent with a role in sporozoite development, and it will be exciting to further examine these potential candidates with functional studies. Finally, only a few AP2 domain transcription factor genes were consistently detected in the scRNA-seq data, including a poorly studied gene (PBANKA_0521700) that was specifically expressed in salivary gland sporozoites (Figure 3.10). The complete list of genes is available in Table 3.4.

Figure 3.8. Heatmap showing the expression patterns for the 50 most differentially expressed genes during sporozoite development in mosquitoes (from oocyst- to mature salivary gland sporozoites).

Each row shows the expression of one protein-coding gene. Each column shows the data for one individual sporozoite, organized from left to right according to its pseudotime and colored by its anatomical location in the track above the heatmap (red – oocyst, blue – hemolymph, and green – salivary gland). The heatmap color shows the number of a given transcript per cell from dark blue (not detected) to white and red (most highly expressed). The black arrow at the bottom indicates the corresponding pseudotimes.

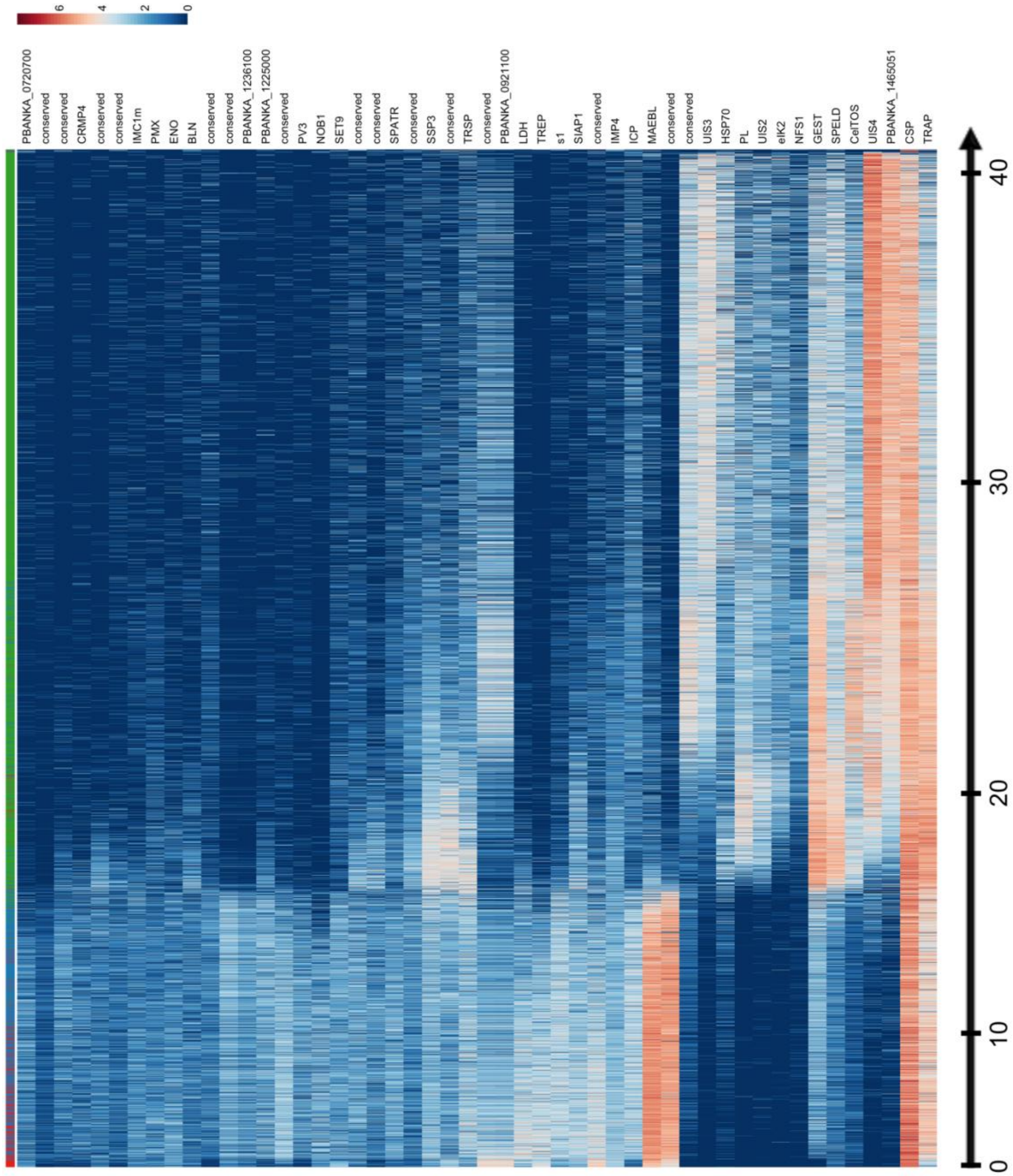
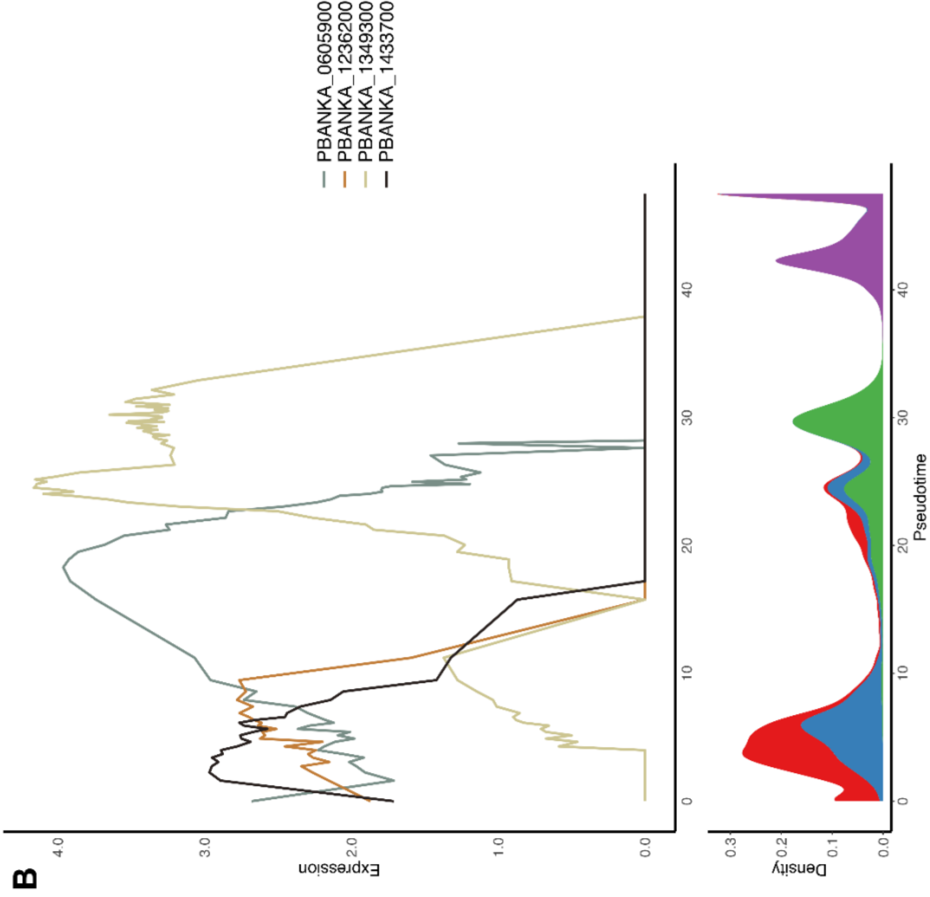

6
4
2
0
PBANKA_0720700
conserved
conserved
CRMP4
conserved
conserved
IMC1m
PMX
ENO
BLN
conserved
conserved
PBANKA_1236100
PBANKA_1225000
conserved
PV3
NOB1
SET9
conserved
conserved
SPATR
conserved
SSP3
conserved
TRSP
conserved
PBANKA_0921100
LDH
TREP
s1
SIAP1
conserved
IMP4
ICP
MAEBL
conserved
conserved
UIS3
HSP70
PL
UIS2
eIK2
NFS1
GEST
SPELD
CelTOS
UIS4
PBANKA_1465051
CSP
TRAP
0
10
20
30
40

Figure 3.9. Novel candidate genes that could play a role in sporozoite development.

(A) Changes in expression of Plasmepsin-X (PMX, red) and Bergheilysin (BLN, blue) during sporozoite development. **(B)** Changes of expression of five conserved unannotated *P. berghei* genes with distinct expression patterns throughout sporozoite development. Each plot shows the median gene expression of 100 individual parasites (y-axis) binned according to their pseudotime (x-axis). The plot under each graph summarizes the distribution of the pseudotimes obtained for oocyst- (red), hemolymph- (blue) and salivary gland (green) sporozoites.


B
Expression
Density
Pseudotime
PBANKA_0605900
PBANKA_1236200
PBANKA_1349300
PBANKA_1433700

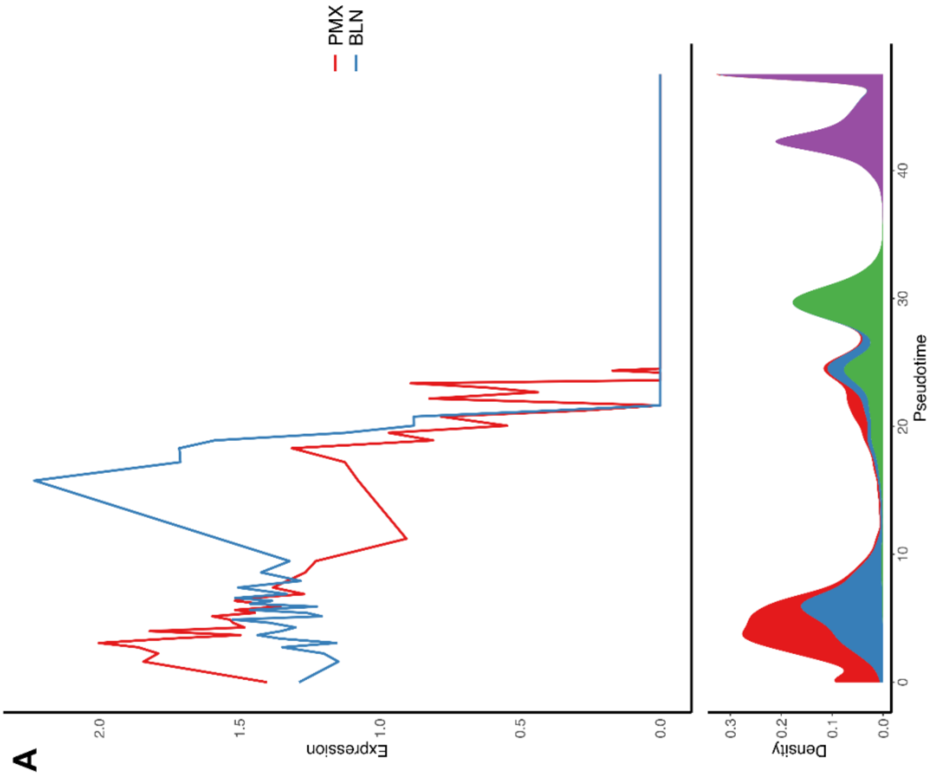
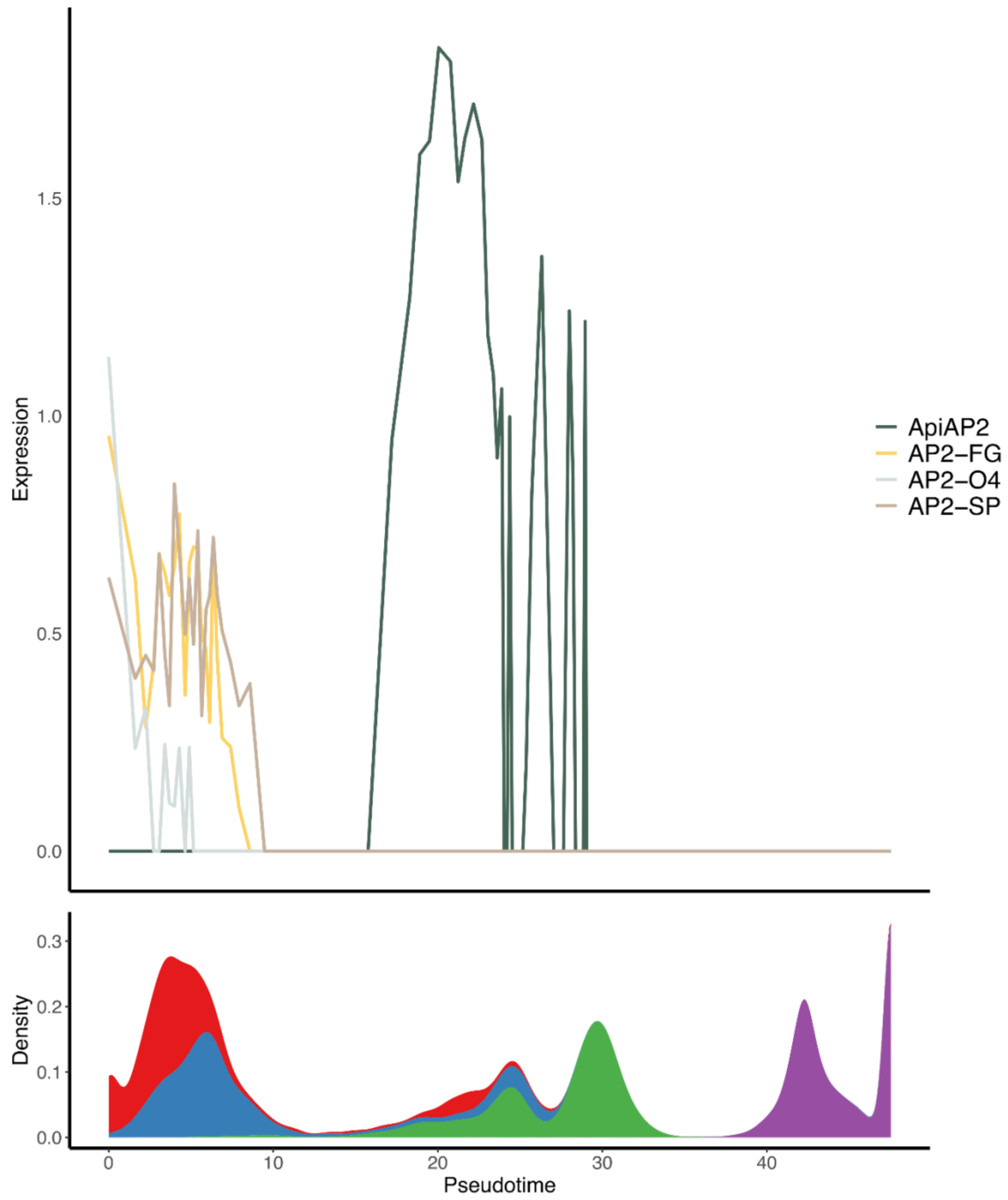

A
Expression
Density
Pseudotime
PMX
BLN

Figure 3.10. Expression level of AP2-domain transcription factor genes detected in the sporozoite scRNA-seq data.

See legend of Figure 3.8 for details.


Expression
1.5
1.0
0.5
0.0
ApiAP2
AP2−FG
AP2−O4
AP2−SP
Density
0.3
0.2
0.1
0.0
0
10
20
30
40
Pseudotime

Despite the clear separation of salivated- and salivary gland sporozoites based on their overall gene expression profiles (Figure 3.4), few protein-coding genes appeared to be consistently expressed in salivated sporozoites (Table 3.6 and discussion below). A few notable exceptions were the early transcribed membrane protein (UIS4, PBANKA_0501200), which reached even higher level of expression in salivated sporozoites than those observed in salivary gland sporozoites (Figure 3.11A), and an uncharacterized exported protein (PBANKA_1465051, Figure 3.11B), which contains a predicted circumsporozoite-related antigen domain and would be fascinating to functionally evaluate.

Transcription and translation are dynamically regulated in sporozoites

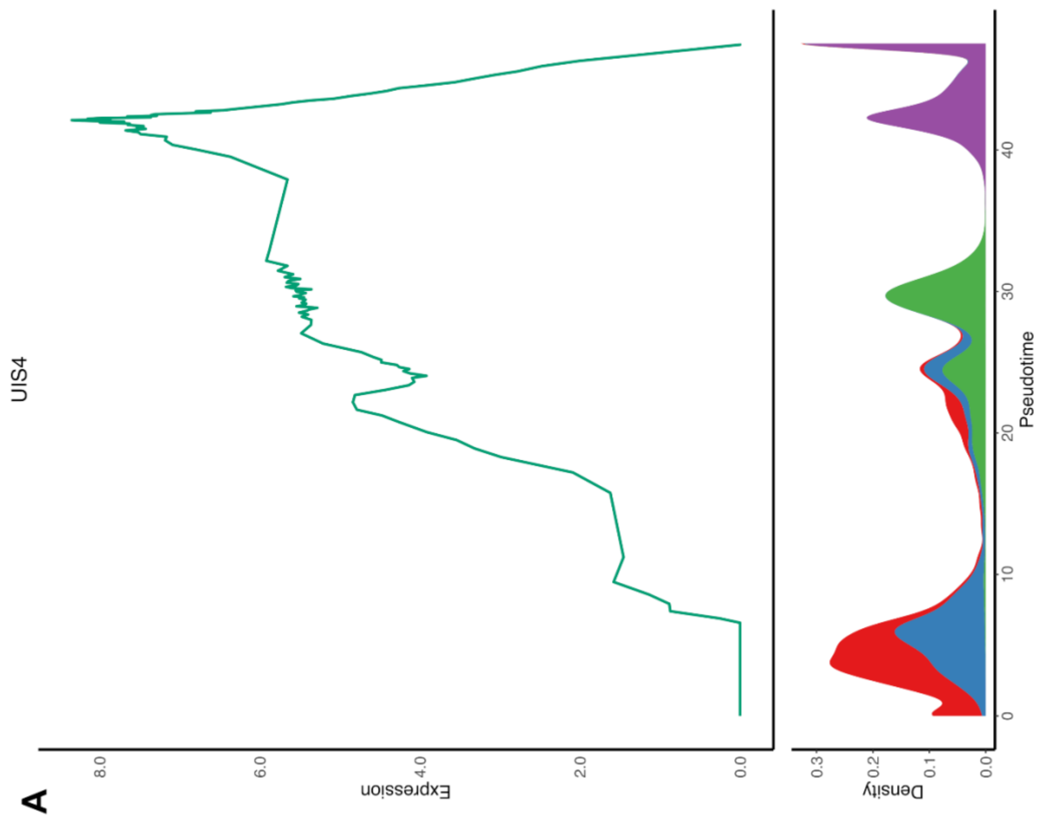
Once they reached the salivary glands, *Plasmodium* sporozoites can remain quiescent for several weeks [224] before being injected into the mammalian host and, where they quickly become motile and able to invade host cells [243]. A proposed molecular mechanism underlying this quiescent state of salivary gland sporozoites and their rapid reactivation upon salivation is translational repression of the mRNAs encoding the proteins required later in the mammalian host [231, 272-274]. This global translational repression results from the phosphorylation of the eukaryotic translation initiation factor 2-alpha by eIK2 (PBANKA_0205800) [272], while the Pumilio protein (PUF2, PBANKA_0719200) binds to the matching phosphatase-encoding transcripts (UIS2, PBANKA_1328000) and blocks their translation (reviewed in [273, 275, 276]). Consistent with this mechanism, we observed a clear peak of expression of eIK2 and PUF2 in salivary gland sporozoites (Figure 3.12A) but not in oocyst sporozoites, as has recently been described [231]. Some of the

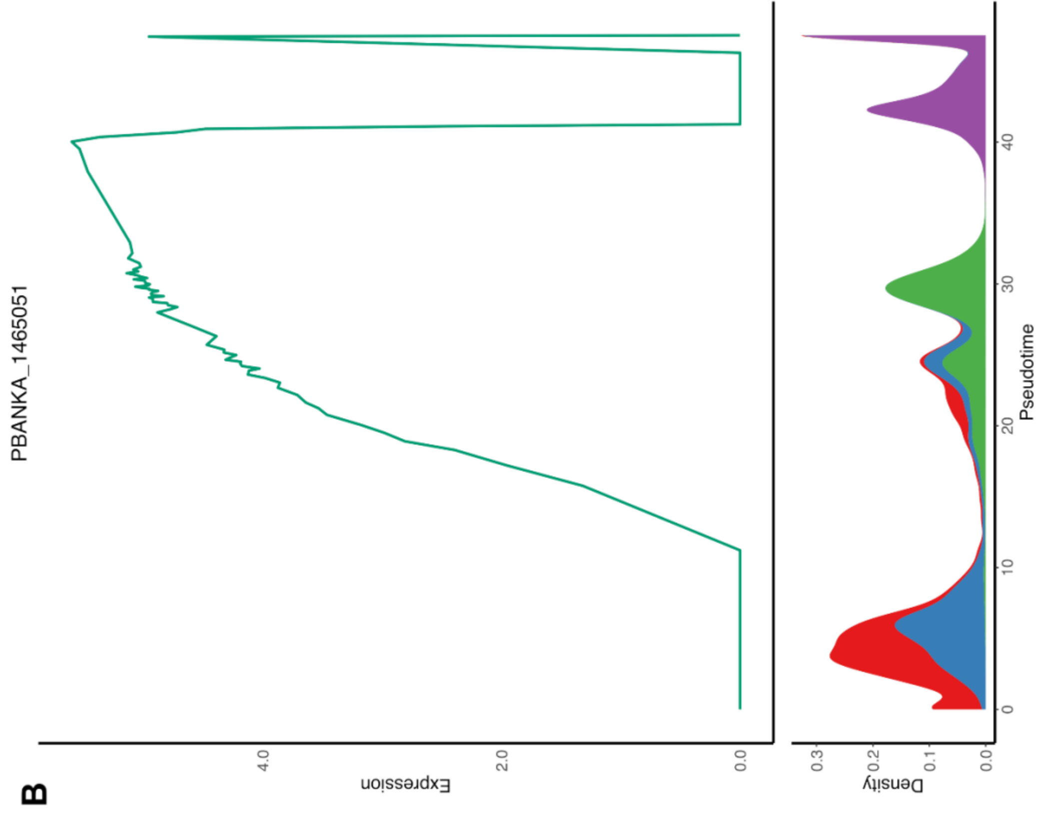
Figure 3.11. Genes highly expressed in salivated sporozoites.

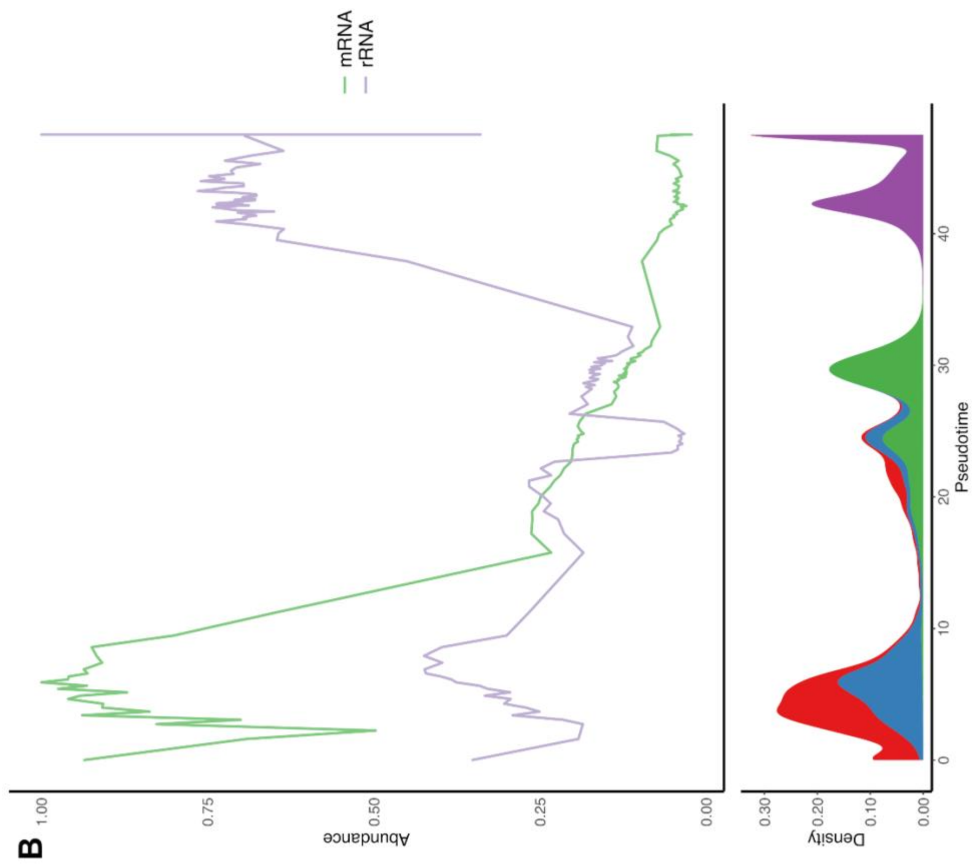
(A) early transcribed membrane protein UIS4 (PBANKA_0501200) and **(B)** an unannotated exported protein (PBANKA_1465051). The plot under each graph summarizes the distribution of the pseudotimes obtained for oocyst- (red), hemolymph- (blue) and salivary gland (green) sporozoites.

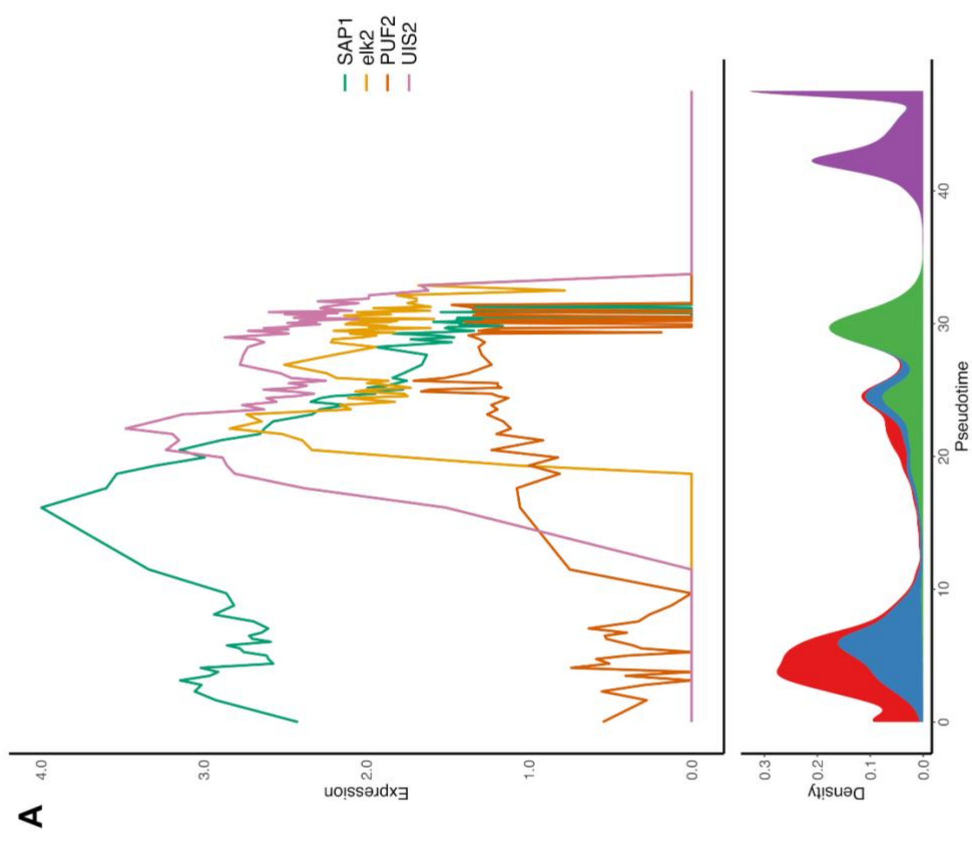
Figure 3.12. Regulation of transcription and translation in sporozoite.

(A) Changes in expression of *Plasmodium* genes involved in mRNA stability (SAP1 – green) and translation inhibition (eIK2 – orange, PUF2 – red, UIS2 – pink). **(B)** Changes in relative abundance of mRNA (green) and ribosomal RNAs (purple) during sporozoite development. Both the mRNA and rRNA curves show the proportion of RNA at a given pseudotime compared to the maximum observed (fixed arbitrarily at 1). See legend of Figure 3.9 for deltails.


A
UIS4
Expression
8.0
6.0
4.0
2.0
0.0
Density
0.3
0.2
0.1
0.0
0
10
20
30
40
Pseudotime


B
PBANKA_1465051
Expression
4.0
2.0
0.0
Density
0.3
0.2
0.1
0.0
0
10
20
30
40
Pseudotime


B
Abundance
1.00
0.75
0.50
0.25
0.00
mRNA
rRNA
Density
0.30
0.20
0.10
0.00
0
10
20
30
40
Pseudotime


A
Expression
4.0
3.0
2.0
1.0
0.0
SAP1
elk2
PUF2
UIS2
Density
0.3
0.2
0.1
0.0
0
10
20
30
40
Pseudotime

proteins regulating the long-term storage of the mRNAs in stress granules in female gametocytes have been characterized (e.g., [274, 277]), but these genes do not seem to play a similar role in sporozoites. Indeed, aside from eIK2 and PUF2, none of the proteins involved in translational repression in gametocytes (e.g., DOZI or CITH [278]), nor the proteins associated with stress granules in humans or yeasts [279], were detectable in our dataset. Only the Sporozoite Asparagine-rich Protein 1 (SAP-1) that has been shown to regulate mRNA-stability in sporozoites [280, 281]) was robustly detected and showed a pattern of expression akin to those of eIK2 and PUF2, although with an earlier peak of expression (Figure 3.12A). Interestingly, among the genes expressed at the same time as eIK2, we observed one RNA helicase (PBANKA_1103800) that was lowly but specifically expressed in salivary gland sporozoites and it will be interesting to test whether it may play a role in translational repression.

Finally, the increased expression of genes involved in translational repression appeared to be preceded by an overall and steady decrease in mRNA transcription (Figure 3.12B). Fluctuations in overall mRNA abundance have been described in blood-stage Plasmodium parasites [282, 283] and the decrease in mRNA transcription during sporozoite development is consistent with the hypothesis that mature salivary gland sporozoites are quiescent and their transcriptional activity reduced. Conversely, we observed a 2- to 3-fold increase in the number of ribosomal RNA (rRNA) molecules synthesized in sporozoites collected by forced salivation compared to the amount observed in oocyst- to salivary gland sporozoites (Figure 3.12B). While rRNA molecules are not poly-adenylated in *Plasmodium* and should theoretically not pass the polyA-selection used during the 10X scRNA-seq library preparation, they are extremely abundant in cells and are often detected

in transcriptomic experiments. Indeed, we observed very specific and consistent expression of the 5.8S, 18S and 28S rRNA genes (Figure 3.13) across the entire sporozoite development but with a clear increase upon salivation. This observation would be consistent with the abrupt reactivation of quiescent sporozoites and the restarting of the entire transcriptional/translational machinery upon salivation. In contrast to multicellular eukaryotes that typically have hundreds of copies of ribosomal RNA genes in their genome, *P. berghei* (and most *Plasmodium*) only carry four copies of the 18S-5.8S-28S ribosomal unit and the expression of these rRNA genes is developmentally regulated [284, 285]: the rRNA genes located on chromosomes 5 and 6 are primarily expressed in mosquito stages (S-forms), while the rRNA genes from chromosomes 7 and 12 are the dominant form in blood stages (A-forms). Analyses of the rRNA sequences that carried sufficient genetic information to reliably differentiate the chromosomal origin of each rRNA transcript (see Materials and Methods) indicated that they were roughly twice as many transcripts derived from chromosome 6 (S-form) than the combined number of transcripts derived from chromosome 7 and 12 (A-form) while no transcripts from chromosome 5 (also S-form) could be detected (Figure 3.14). Interestingly, these proportions remained relatively constant throughout sporozoite development and, in particular, did not change upon salivation, suggesting that the switch to the A-form rRNAs observed in blood-stage parasites occurs during the pre-erythrocytic cycle.

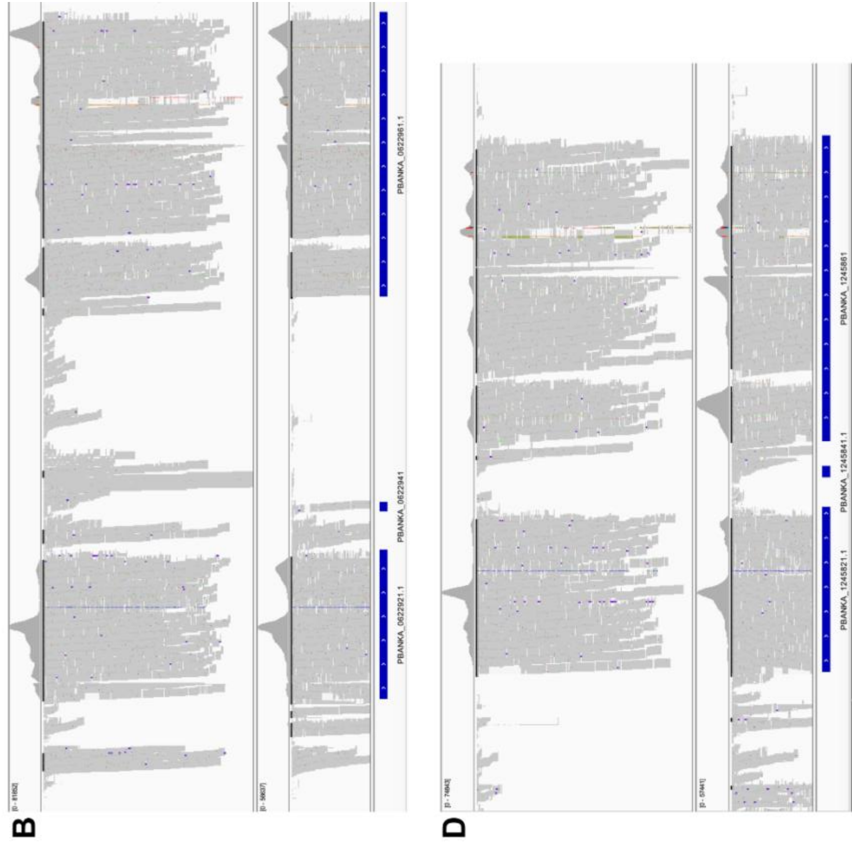
Conclusions

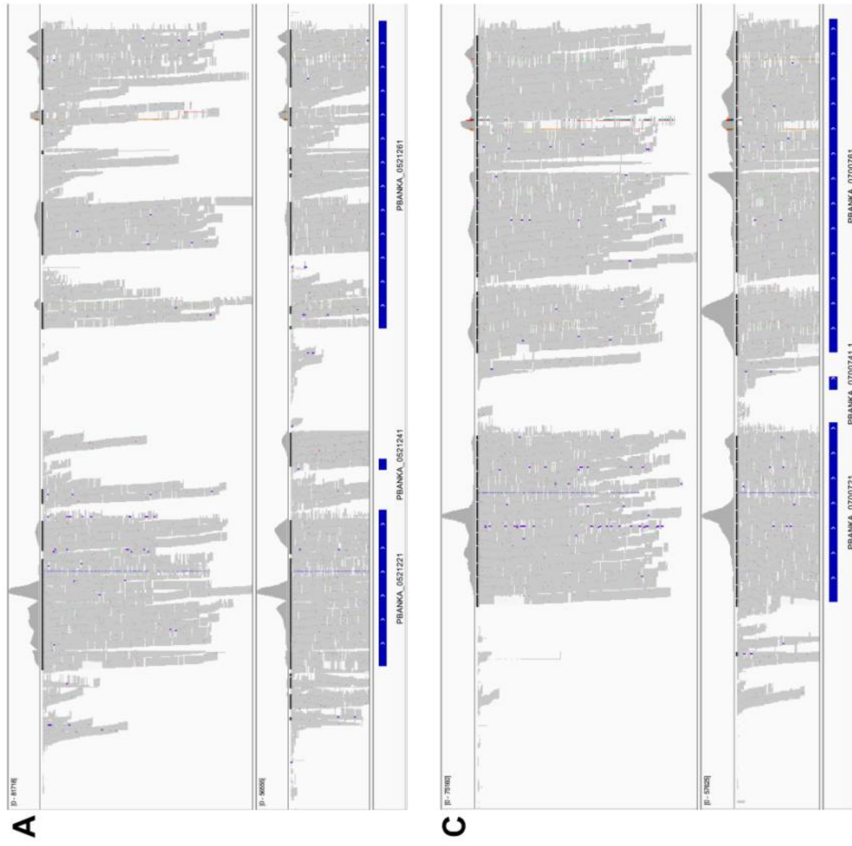
Our data provide a comprehensive perspective on the regulation of gene expression among three *Plasmodium* species and accompanying the development of *P. berghei* sporozoites

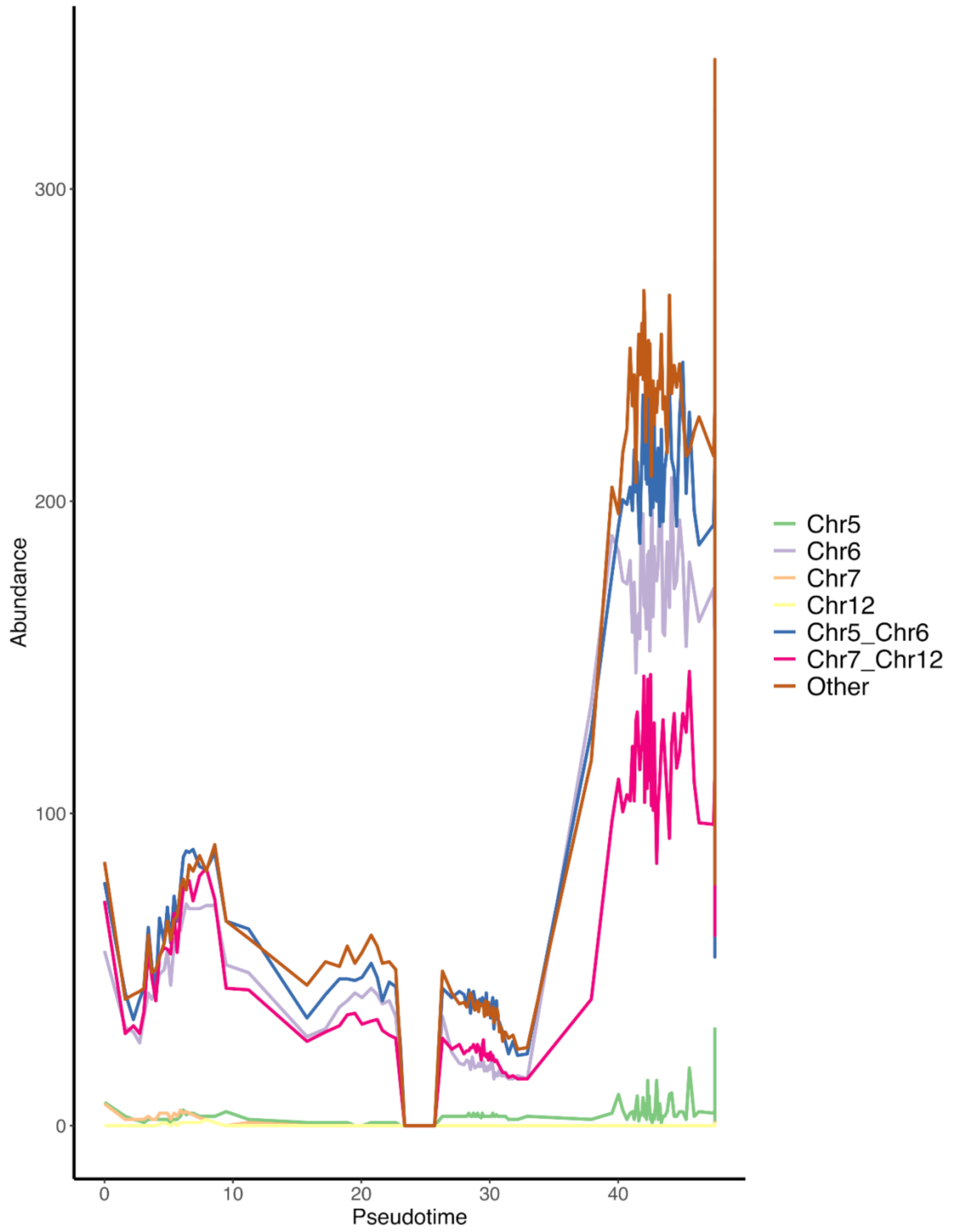
Figure 3.13. Integrative Genome Viewer (IGV) screenshots showing examples of reads derived from oocyst- (top track) and salivary gland sporozoites (bottom) mapped to the ribosomal RNA genes on chromosome 5 (A), 6 (B), 7 (C) and 12 (D).

Figure 3.14. Relative abundance of the different rRNAs during sporozoite development.

The graph shows the number of reads mapped i) uniquely to each chromosome rRNA genes (green - chromosome 5, purple - chromosome 6, orange - chromosome 7, yellow-chromosome 12), ii) specifically to a pair of chromosomes (blue - chromosomes 5 and 6, pink - chromosomes 7 and 12) or iii) mapping to three of more chromosomes (brown). Note that no reads from the ribosomal unit on chromosome 5 could be reliably detected (while 40% of the sequence is unique) and that, therefore, the reads mapped to both chromosomes 5 and 6 likely derived exclusively from chromosome 6. By contrast, the lack of reads mapped uniquely to chromosomes 7 or 12 is due to the high sequence similarity between these two sequences (less than 3% of their sequences are unique, although 55% of their sequences separate them from chromosomes 5 and 6).


B
PBANKA_0622921.1
PBANKA_0622941
PBANKA_0622961.1
D
PBANKA_1245821.1
PBANKA_1245841.1
PBANKA_1245861


A
PBANKA_0521221
PBANKA_0521241
PBANKA_0521261
C
PBANKA_0700721
PBANKA_0700741.1
PBANKA_0700761


Abundance
Pseudotime
300
200
100
0
0
10
20
30
40
Chr5
Chr6
Chr7
Chr12
Chr5_Chr6
Chr7_Chr12
Other

from midgut oocyst- to mature salivary gland sporozoites, and upon salivation. The single cell analyses reveal a high level of transcriptional heterogeneity among sporozoites that was masked in previous gene expression studies and suggest that the maturation of sporozoites is, at least partially, regulated by an intrinsic clock, which might tick at a slightly different rate in different individual parasites. In addition, the precise determination of the timing of expression of each *P. berghei* gene enable the identification of novel candidate genes that could underlie specific biological processes (e.g., oocyst egress or invasion of salivary gland cells) and further contribute to our understanding of the regulation of these functions in sporozoites. Finally, these data highlight the key role of transcriptional regulation in the quiescence and reactivation of salivary gland sporozoites: the genes underlying the global translational repression in mature parasites were specifically transcribed once the sporozoites reached the salivary glands and accompanied a general decrease in transcriptional activity, while, upon salivation, we observed a large increase in the transcription of ribosomal RNA genes consistent with a restarting of the entire transcriptional machinery. The data provided here also constitute a valuable resource for reverse genetic experiments to examine the role of novel genes in sporozoite biology, as well as a framework to identify and manipulate critical determinants of sporozoite development and transmission to mammalian hosts that may support the development of new strategies to malaria control.

Table 3.1. Sample summary and mapping results.

The table shows, for each sample, the parasite and An. stephensi strain, the collection site, the number of mosquitoes dissected, the time of collection (in days post-infection) as well as the number of read pairs generated (in millions), the percentage of those mapped to the *P. berghei* genome, the number of individual sporozoite transcriptomes obtained and the

average number of unique reads (with the min-max). The table is found on an excel (.xlsx) file in the following link https://github.com/Haikelnb/Dissertation_Chapter3_Tables/blob/main/Table_3_1.xlsx?raw=true

Table 3.2. Most expressed genes by species.

The table shows, the twenty most expressed genes for each *Plasmodium* species and the expression level of those genes in the other two species. The highlighted rows contain genes that are highly expressed in all three species. Genes that do not have an ortholog for any of species according to PlasmoDb are labeled as 'No ortholog'. The table is found on an excel (.xlsx) file in the following link https://github.com/Haikelnb/Dissertation_Chapter3_Tables/blob/main/Table_3_2.xlsx?raw=true

Table 3.3. Differentially expressed genes across species.

The table shows, out of 587 highly expressed (expressed in at least 20% of parasites from one species) orthologous genes, 157 that are differentially expressed (FDR = 0.1). The table is found on an excel (.xlsx) file in the following link https://github.com/Haikelnb/Dissertation_Chapter3_Tables/blob/main/Table_3_3.xlsx?raw=true

Table 3.4. Complete list of genes most differentially expressed according to the sporozoite developmental pseudotime.

The table shows, the entire list of genes detected in the scRNA-seq data and ranked based on the association between their expression level and the developmental pseudotimes. The table is found on an excel (.xlsx) file in the following link https://github.com/Haikelnb/Dissertation_Chapter3_Tables/blob/main/Table_3_4.xlsx?raw=true

Table 3.5. Thirty genes most differentially expressed according to the sporozoite developmental pseudotime, from oocyst to salivary gland sporozoites.

For each gene, the table indicates the gene name and annotation, the time of the peak of expression (Max Expr., in pseudotime units), whether the gene has been associated with a sporozoite phenotype in the literature (and the corresponding reference), and whether a phenotype is reported in the Rodent Malaria genetically modified Parasites database (RMgmDB). The genes are ranked based on the statistical significance of the association between expression and pseudotime (most significant on top). See Table 3.4 for the complete list of genes, the statistical significance, and the results of the association when salivated sporozoites are included in the analysis. The table is found on an excel (.xlsx) file in the following link

https://github.com/Haikelnb/Dissertation_Chapter3_Tables/blob/main/Table_3_5.xlsx?raw=true

Table 3.6. ***P. berghei*** **genes most expressed in salivated sporozoites.**

For each gene, the table lists the gene name and annotation, and mean expression value (mRNA/1,000). Note that this analysis does not exclude identical regions of ribosomal RNA genes and that reads that can be mapped to two loci are randomly assigned to one. For more specific results on rRNA gene expression, see Figure 3.12. The table is found on an excel (.xlsx) file in the following link https://github.com/Haikelnb/Dissertation_Chapter3_Tables/blob/main/Table_3_6.xlsx?raw=true

Chapter 4: Conclusions and Future Directions

Factors shaping the mosquito microbiota

It is clear that the microbial community of mosquitoes is critical for the vector's biology and a variety of mosquito factors, including mosquito habitat, species and genotype, or diet, can influence microbial composition. In Chapter 2, we showed that mosquito intrinsic factors (i.e., mosquito species and insecticide resistance genotype) and blood feeding status did not significantly contribute to the microbial variation among wild mosquitoes, while parasitic and viral infections showed statistically significant but marginal contribution [286]. However, the major contributor of the bacterial composition was the collection site, consistent with previous studies [159, 178, 179, 204, 205]. This broad parameter includes many distinct characteristics of mosquito habitat (e.g., larvae supporting water, vegetation for adult mosquito sugar source) and the exact contributions of each of these features will need to be further elucidated. To this end, we could for example, characterize the microbiota found in larvae-supporting bodies of water and sugar source vegetation of each mosquito collection site and test which habitat feature has a microbial composition that best correlates to that of the adult mosquitoes. If we find that the microbial composition of larval habitats is more similar to that of the adult mosquitoes, this would first, affirm that the adult *Anopheles* mosquito microbial community is primarily acquired at the aquatic stages (supported by recent studies [118, 119]), and second, inform current and future vector control approaches. For the latter implication, it could mean current larvicidal approaches need to emphasize consideration of the bacteria present in larval breeding sites. For example, organophosphate (OP) insecticides typically applied to larvae supporting

waters for mosquito control (e.g., Temephos [287]) could be undermined by OP-degrading bacteria [181] present in the waters. Furthermore, the impact the larval habitat's microbiota has on current microbiota dependent approaches (e.g., *Bacillus thuringiensis* dependent larvicides) would need to be investigated. Lastly, a potential profound influence of larval habitat microbial composition on adult mosquito microbiota could open new avenues for vector control leveraging the microbiota, for example, larvae acquired bacteria that interrupt the mosquito lifecycle (i.e., aquatic or terrestrial stages) or inhibit parasite transmission in the vector.

A more significant role of mosquito intrinsic factors in shaping the microbial composition than displayed in Chapter 2 cannot be ruled out without further investigation. To address any concerns of the close phylogenetic relationship of mosquito species identified, or insecticide resistance loci chosen and targeted that could minimize the relative contribution of intrinsic factors, future studies can be designed for mosquito collection in different geographical locations with a variety of mosquito species and/or multiple insecticide resistant genotypes present at high frequency. The methodology described in Chapter 2 is easily adaptable to facilitate such studies (i.e., substituting with PCR primers of choice).

Overall, our findings could have important implications for vector control approaches that leverage the microbiota. If the mosquito habitat is in fact the main determinant of the vector's microbial composition, this might require microbiota-dependent vector control methods to be location specific, for maximizing success. In other words, the design and implementation of novel approaches may need to follow the microbial community characterization of target vectors and testing in a geographical-dependent manner. For example, a recent study showed that the ability of *Serratia* to infect and exert deleterious

effects on *Aedes aegypti* depends on the native microbiota and is inversely correlated with the presence of specific bacteria [288]. These findings highlight the importance of microbe-microbe interactions for the outcomes of microbiota-dependent vector control strategies, and hence, indicate the need to design targeted approaches that consider variations of mosquito microbiota compositions between vector localities. Furthermore, surveying the microbiota of wild mosquitoes can be equally important for the application of insecticide-based approaches currently in use, that could interact with mosquito microbiota (e.g., OP insecticides vs. OP-degrading bacteria).

Transcriptional changes in *Plasmodium* sporozoite development

Chapter 3 highlights the power of single cell genomic technology to provide novel insights into sporozoite maturation and regulation by examining heterogeneity and regulation among individual cells, which are hidden in traditional bulk approaches. Our analyses show variation in the transcriptional regulation between salivary gland sporozoites of *P. falciparum*, *P. cynomolgi*, and *P. berghei* and extensive heterogeneity among *P. berghei* sporozoites found in the same mosquito anatomical site (i.e., midgut oocyst, hemolymph, and salivary gland) (Chapter 3). Furthermore, we identify genes that could be involved in critical sporozoite processes, including novel candidates potentially important for oocyst egress and invasion of the mosquito salivary gland, and regulators of salivary gland sporozoite quiescence and reactivation (Chapter 3).

The data described in Chapter 3 suggest that the anatomical location of parasites in the mosquito is not the sole determinant of sporozoite maturation, and that intrinsic factors (e.g., time since sporozoite budding and egress from mature oocysts) contribute to the regulation of sporozoite development. Alternatively, it is possible that the heterogeneity

displayed among sporozoites at the same anatomical site could derive from transcriptional differences among sporozoites collected from multiple mosquitoes (although these mosquitoes are the same age and collected from the same colony), and these differences could be the result of varied influences by individual mosquito components. To address this concern, for each anatomical site, we could apply the same study design described in Chapter 3 to investigate sporozoite transcriptional heterogeneity, but from individual mosquitoes instead of pools of mosquitoes (i.e., scRNA-Seq libraries prepared from an anatomical sites of a single mosquito). If the results show that the heterogeneity among sporozoites remains, this would confirm our interpretations and weaken the hypothesis that mosquito-specific factors are mainly responsible for the findings in Chapter 3. In the future, the observations presented in Chapter 3 need to be validated in other *Plasmodium* species. While the *P. berghei* model is exceptionally amenable for studying stages of the parasite in the mosquito and mammal (e.g., forward genetics), defining patterns of gene regulation in sporozoite development in human malaria parasites (e.g., *P. falciparum*) is indispensable for translational implication on malaria transmission.

Data generated in Chapter 3 also produced fascinating avenues to improve our current understanding of sporozoite biology, by providing excellent gene candidates for future functional studies. This includes *Plasmodium* genes previously described in other parasite developmental processes (e.g., Plasmepsin X in merozoite egress [271]), genes with predicted domains, or multiple genes that lack functional annotation (Chapter 3). In addition, the novel single cell characterization of the dynamic change in transcriptional profile from salivary gland to salivated sporozoite needs further investigation and could be facilitated with the following approaches: 1) identifying all components of the

transcriptional/translation machinery involved in translational repression and reactivation, 2) characterizing mRNA and rRNA transcripts upon salivation with traditional RNA-seq, 3) supplementing current *Plasmodium* genomic rRNA annotation (to correct possible misannotation). Addressing these largely unexplored questions in *Plasmodium* sporozoite biology could prove valuable for a couple of reasons. First, novel components and mechanisms identified in the translational repression/reactivation machinery could present potential targets for perturbing parasite transmission in the mosquito or mammalian host. Second, a better understanding of rRNA transcriptional regulation as *Plasmodium* parasites make the transition between the vector and the host (e.g., sporozoite salivation) could improve our current knowledge of *Plasmodium* rRNAs and functional differences between different types of rRNAs (A vs S form).

Despite differences in host species and fate once injected into the mammalian host, few phenotypic variations between *Plasmodium* species have been described. Understanding differences in the biology of parasites from different species can have profound implications on our ability to translate findings from one *Plasmodium* model to another (e.g., rodent to human malaria parasites). While this study presents a number of differentially expressed genes among salivary gland sporozoites of three *Plasmodium* species, genuine species differences need to be confirmed with more biological replicates (n>1). With regards to sporozoite maturation in the salivary gland of mosquitoes, more single cell studies are required to characterize potential heterogeneity among salivary gland sporozoite of the same species that could explain why only a handful of sporozoites that reach the salivary glands are injected into a mammalian host (i.e., are a only a subset of sporozoites fully mature/infectious?) and if the programming of sporozoites, for

differential outcomes/phenotypes, in the pre-erythrocytic stages (e.g., *P. vivax* hypnozoite dormancy and sporozoite evasion of host factors), occurs in the salivary glands.

Overall, the results from Chapter 3 essentially recapitulate findings that *Plasmodium* sporozoites found at different anatomical sites (i.e., oocysts, hemolymph, or salivary glands) of *Anopheles* mosquitoes, have largely different transcriptional profiles [230, 231]. However, the within-anatomical site sporozoite heterogeneity apparent in the data challenges the notion that all immature sporozoites are found in oocysts and that all mature sporozoites are found in the salivary glands [231]. Moreover, our results align with findings that some sporozoites from oocysts and the hemolymph, potentially the most mature sporozoites at those anatomical sites, can infect mammals [226-228]. These observations have important implication for our understanding of sporozoite biology and malaria control because they attempt to tackle the central question: how do *Plasmodium* sporozoites become infectious? On the path of trying to answer this question one could, for example, investigate how transcriptionally different or similar sporozoites that can induce infections in mammals without first infecting the mosquito salivary glands are compared to sporozoites that do not induce infection from oocysts or hemolymph, and/or, only induce mammalian infectious after infecting the salivary glands. For sporozoites that do successfully infect the salivary glands, we must better understand how sporozoite entry point of the glands, localization and motility within the glands, and overall load influence transmission [225]. Comprehensively, these studies could help identity sporozoite components that regulate sporozoite maturation with respect and regardless of their environment and inform novel malaria control strategies that attempt to arrest sporozoite development in the mosquito or mammalian host.

At the very least, the study in Chapter 3 can serve as a foundation to characterize and manipulate components of sporozoite development, to better understand parasite transmission. The approach from the study could be expanded to investigate how different factors influence sporozoite regulation and, for example, test whether the mosquito species, ambient temperature, time since feeding or the age of the mosquito impact sporozoite gene expression patterns.

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