THE HOST-FEEDING ECOLOGY OF MOSQUITO VECTORS IN THE *ANOPHELES PUNCTULATUS* (DIPTERA: CULICIDAE) SPECIES COMPLEX IN A MALARIA ENDEMIC PROVINCE OF PAPUA NEW GUINEA

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Entomology – Master of Science

2013

ABSTRACT

THE HOST-FEEDING ECOLOGY OF MOSQUITO VECTORS IN THE *ANOPHELES PUNCTULATUS* (DIPTERA: CULICIDAE) SPECIES COMPLEX IN A MALARIA ENDEMIC PROVINCE OF PAPUA NEW GUINEA

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The relative propensity to feed on humans of females of 5 species of *Anopheles* mosquitoes was studied in 4 malaria-endemic villages of Madang Province, Papua New Guinea. The vertical barrier screen, constructed of shade cloth configured to posts, was verified as a suitably unbiased sampling method as it captured both host seeking and blood fed individuals throughout the night More non-blood fed females were capture on the bush side of the screen earlier in the evening whereas more blood fed females were captured on the village side later in the evening. Host identification of blood meals by sequencing of the mitochondrial cytochrome B gene revealed that humans and domestic pigs were the most common and often only hosts, even though other potential vertebrate hosts were present in abundance. *Anopheles punctulatus* and *An. koliensis* were highly anthropophagous, *An. farauti* s.s, *An. longirostris*, and *An. farauti* (species 4) relatively less so, whilst *An. bancrofti* fed mostly on pigs. The implications of these findings for malaria transmission are discussed with reference to the human blood index.

Dedicated to my lovely son Mr. Jerome Keven, daughter Miss Jerolyn Keven, and my beautiful wife Mrs. Adela Ndroleu-Keven.

ACKNOWLEDGEMENTS

This thesis was made possible through the support of various people and organizations which I am indebted to acknowledge. The U.S Fulbright International Scholarship South Pacific Program and the U.S Fogarty Global Infectious Disease Research Training Program (GIDRTP) cosponsored my two-year Master's Degree program at Michigan State University, USA. The GIDRTP was a collaborative effort between Papua New Guinea Institute of Medical Research (PNG IMR) and Case Western Reserve University (CWRU) and subcontracted to the Department of Entomology, Michigan State University (MSU). This program supported global infectious disease research for young American and Papua New Guinean scientists. The PNG Institute of Medical Research (PNG IMR) played an important role in providing financial and logistical support during my field work in PNG during the summer of 2012. I would like to thank the staffs at the PNGIMR Medical Entomology Laboratory. Dr. Lisa Reimer who was the head of the entomology laboratory during that time provided invaluable guidance during my field work in PNG. The entomology field technicians worked tirelessly supervising the mosquito collections. The mosquito samples were brought to the laboratory in the US using Dr. Peter Zimmerman's CDC Import Permit. Dr. Zimmerman was the principle investigator for the Fogarty Training Grant based at the Center for International Health and Human Genetics, Case Western Reserve University. He was also a member of my Master's Thesis review committee. My laboratory work was carried out in Dr. Edward Walker's laboratory at the Department of Microbiology and Molecular Genetics at MSU. Dr. Walker was my major professor and served as the chairman of my thesis review committee. Special thanks to all the undergraduates, graduates and post-doctoral fellows in his laboratory who assisted in many ways to make my

training a successful one. Finally, my Master's program would have never been successful without the support of my family. My wife Mrs. Adela Ndroleu-Keven and two children Mr. Jerome Keven and Miss. Jerolyn Keven allowed me to pursue my MS degree at the expense of the presence of a husband and father for approximately two years. I am forever grateful.

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KEY TO ABBREVIATIONS

bp	base pairs
cm	centimeters
CV	Chikungunya virus
DDT	dichlorodiphenyltrichloroethane
DFV	Dengue fever virus
DNA	deoxyribonucleic acid
DNTP	deoxynucleotide triphosphate
EEE	eastern equine encephalitis
FMA	fluorescent microsphere assay
HBI	human blood index
IRB	Institutional Review Board
ITN	insecticide-treated nets
ITS2	internal transcribed spacer region 2
JE	Japanese encephalitis
kb	kilobases
km	kilometers
LDR	ligase detection reaction
LLIN	long-lasting insecticide treated nets
mf	microfilariae
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
Pf	*Plasmodium falciparum*

PM	peritrophic matrix
Pm	*Plasmodium malariae*
PNG	Papua New Guinea
PNG IMR	Papua New Guinea Institute of Medical Research
Po	*Plasmodium ovale*
Pv	*Plasmodium vivax*
rDNA	ribosomal deoxyribonucleic acid
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RRV	Ross river virus
RVV	Rift valley virus
SLE	St. Louis encephalitis
µL	microliters
VBD	vectorborne diseases
VEE	Venezuelan equine encephalitis
WB	*Wuchereria bancrofti*
WEE	western equine encephalitis
WNV	West Nile virus
YFV	Yellow fever virus

INTRODUCTION

This thesis is an account of a research work on the host-feeding ecology of the *Anopheles* vectors of Papua New Guinea (PNG). There are four chapters in this thesis. Chapter 1 is a broad review of topics and concepts that are relevant to this research project. It begins by briefly discussing the fundamental topics in medical entomology and ends by discussing in detail the mosquito vectors and the epidemiology of vector-borne diseases in PNG. The goal of this chapter is to provide the background on which this study was based. Chapter 2 provides the reasons for carrying out this research based on the review of previous work as discussed in chapter 1. Its states the scientific hypotheses and sets the objectives for testing these hypotheses. Chapter 3 is an account of the methods that was used in this study and how the data were recorded, stored and analyzed. The final chapter (chapter 4) presents the results of the work and discusses the significance of the findings.

CHAPTER 1: A REVIEW OF RELEVANT TOPICS AND CONCEPTS

1.1. OVERVIEW OF VECTOR-BORNE DISEASES

There are several ways in which an infectious pathogen can be transmitted to a human and cause illness. An airborne disease is one where the pathogen is dispersed into the air and is transmitted to a person through inhalation. Tuberculosis and anthrax are examples of airborne infectious diseases. A waterborne disease is one where the pathogen is dispersed into water and is transmitted to a person through drinking contaminated water. Typhoid, dysentery and cholera are examples of infectious waterborne diseases. A sexually transmitted disease is one where the pathogen is transmitted from an infected person to an uninfected person through sexual contact. Human immune virus, gonorrhea and syphilis are examples of sexually transmitted diseases. A vectorborne disease (VBD) is an infectious disease that is transmitted to a human through the bite of a hematophagous (blood-feeding) arthropod. A disease vector is the arthropod that transmits that disease. Malaria, filariasis, dengue and West Nile Virus are examples of many VBD. Unlike the others, vectorborne pathogens have complex biological systems that enable them to evolve, adapt, resurge, resile and be easily transmitted causing health and socioeconomic burdens in human populations. This is mostly because, unlike the two-factor system in the other infectious disease cycles which involve only two biological factors, a host and a pathogen, the vectorborne disease has three biological factors which involves a pathogen, its arthropod vector and a vertebrate host (figure 1). Each vertex of the triangle in figure 1 represents each of the three biological factors and each side of the triangle represents a distinctly complex level of biological interaction between the organisms at the corresponding vertices.

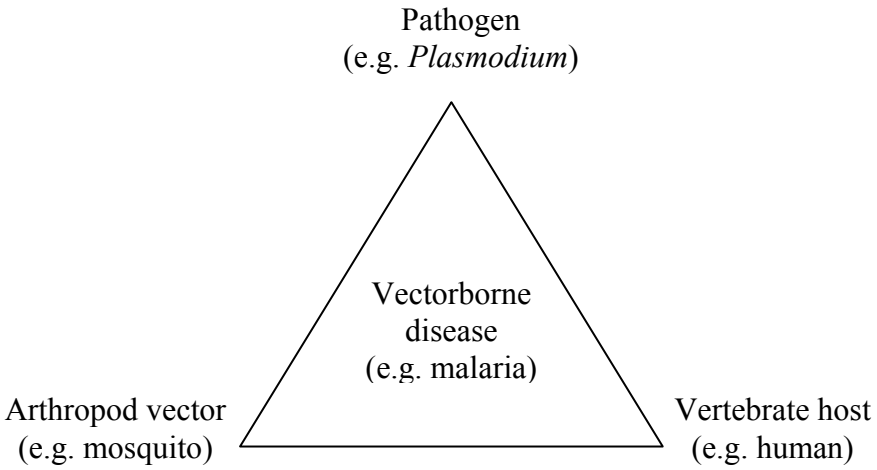

Figure 1. The three-factor vectorborne infectious disease system. Each vertex of the triangle represents each of the three biological factors that are part of the disease cycle.

VBD are caused by microscopic infectious pathogens that belong to any of four microbial classes: bacteria, viruses, protozoa and helminths. Bacteria are unicellular prokaryotic (lacks a membrane-bound nucleus) organisms that take on various shapes, are less than 2 μm long, and undergo asexual reproduction.[1] Viruses are non-living biological particles (<50 nm in diameter) that lack the ability to self-replicate (reproduce) but infect bacterial and animal cells and use the cell's genomic machinery to reproduce copies of themselves and eventually kill the cells.[1] Protozoa are unicellular eukaryotic (has true membrane-bound nucleus) organisms that are 10 to 52 μm long, highly motile, and move by means of flagellums.[1] Helminthes are parasitic worm-like eukaryotic organisms that lives in and feed on living hosts tissues where they receive nutrients and protection while causing diseases.[2] Helminthes are large enough to be seen under a light microscope and some adult stages can be seen with naked eyes. VBD that are caused by

bacteria are referred to as bacterial diseases, helminthes and protozoa are referred to as parasitic diseases, and viruses are referred to as arboviral diseases (short for arthropod-borne viruses) There are over 14 arboviral, 5 bacterial and 6 parasitic VBD (tables 1 and 2). Some of these diseases are caused by more than one pathogens of the same genus (e.g. malaria).

Most of the VBD have been around for many centuries. Since their discovery as the cause of diseases in human populations, there were significant human efforts against these diseases mostly by means of vector-based control programs. During the early and mid 19th century, diseases such as malaria, yellow fever, dengue, typhus, chagas disease, onchocerciasis, and bancroftian filariasis were successfully eliminated or controlled in some regions of the world.[3] By the 1960's most of these diseases were not considered public health problems outside Africa. These successes however, only lasted for a few years. There were signs of resurgence in previously controlled regions beginning with dengue and malaria in the 1970's. Despite early signs of disease resilience, warnings were largely ignored. By the 1990's most of the diseases have reemerged once again as major public health problems and this time more adaptable to control methods.[3] Not only have these diseases resurged, they are rapidly extending beyond their previously known geographical range. As we struggle to deal with the enormous force of the resurging VBD, new ones have emerged for the first time during the past 30 years and are becoming important public health problems. A comprehensive review on the resurging and emerging (infections that have newly appeared in the population) VBD have been published by Gratz and Gubler.[3-5] Most of these emerging diseases are notably zoonotic (diseases that are transmitted from an animal to a human by an arthropod vector) arboviral diseases.

Table 1. List of some common VBD, their regional distributions, causative agents and microbial class.

VBD	**Distribution**[¶]	**Pathogenic agent(s)**	**Class**	**Arthropod vectors**
Chikungunya	Africa, Asia, India	*Chikungunya virus*	virus	Mosquitoes
Dengue	Africa, South America, Asia, India, Pacific	*Dengue virus*	virus	Mosquitoes
West Nile	Global	*West Nile virus*	virus	Mosquitoes
Japanese encephalitis	Asia	*Japanese encephalitis virus*	virus	Mosquito
Eastern equine encephalitis	Americas	*Eastern equine encephalitis virus*	virus	Mosquitoes
Rift Valley fever	Africa	*Rift Valley fever virus*	virus	Mosquitoes
Ross River virus	Australia, Pacific Islands	*Ross River virus*	virus	Mosquitoes
Yellow fever	Africa, Americas	*Yellow fever virus*	virus	Mosquitoes
Plague	Africa, Americas, Asia	*Yersinia pestis*	bacterium	Fleas
Typhus	Africa, Asia, South America	*Rickettsia prowazekii*	bacterium	Fleas and louse
Trench fever	U.S.A, Europe	*Bartonella quintana*	bacterium	Louse
Lyme disease	Global	*Borrelia burgdorferi*	bacterium	Ticks
Malaria	Global	*Plasmodium**	protozoa	Mosquitoes
African trypanosomiasis	Africa	*Trypanosoma**	protozoa	Tsetse flies

Table 1 (cont'd)

VBD	Distribution[¶]	Pathogenic agent(s)	Class	Arthropod vectors
Chagas disease	Americas	*Trypanosoma cruzi*	protozoon	Kissing bugs
Leishmaniasis	Global	*Leishmania**	protozoa	Sand flies
Onchocerciasis	Africa, South America	*Onchocerca volvulus*	helminth	Black flies
Lymphatic filariasis	Africa, Americas, Asia, India, Pacific Islands	*Wuchereria bancrofti, Brugia malayi, Brugia timori*	helminths	Mosquitoes

* Several species in this genus are the pathogenic agents or vectors of the disease

[¶] Most of the information on disease distribution are taken from the WHO website and a review paper by Gratz.[4]

Table 2. List of some emerging VBD, their regional distributions, causative agents and microbial class.

VBD	Distribution[¶]	Pathogenic agent(s)	Class	Arthropod vector
Barmah Forest virus	Australia	*Barmah Forest Virus*	virus	Mosquitoes
Potasi virus	U.S.A	*Potosi virus*	virus	Mosquitoes
Rocio encephalitis	Brazil	*Rocio virus*	virus	Mosquitoes
Kyasanur forest disease	India	*Kyasanur forest disease virus*	virus	Ticks
Oropouche fever	South America	*Oropouche virus*	virus	Biting midges

[¶] Most of the information on disease distribution are taken from the WHO website and a review paper by Gratz.[4]

1.2. ARTHROPOD VECTORS

Arthropods are a group of invertebrate organisms that are characterized by having a hard chitinous exoskeleton, segmented body parts encased in the exoskeleton, and jointed appendages (legs). This is a large and diverse group of invertebrates but members of only a few taxa are medically important vectors. Arthropods constitute the taxonomic phylum Arthropoda.[1, 6] There are four extant subphylums within Arthropoda: Chelicerata (spiders, ticks, mites, scorpions, horseshoe crabs, and sea spiders), Myriapoda (centipedes and millipedes), Crustacea (shrimps, crabs, lobsters and their relatives), and Insecta (insects and their wingless relatives).[1] The chelicerates and insects are two important groups to the study of VBD because they include many important vector taxa.

1.2.1. The acarines (ticks and mites)

The chelicerates are distinguished from other subphylums by characteristically having four pairs of jointed legs and two major body segments: abdomen and cephalothorax (a fused head and thorax).[1] Chelicerata consist of three taxonomic classes: Arachnida (spiders, scorpions, ticks, and mites), Merostomata (horseshoe crabs), and Pycnogonida (sea spiders).[1] The Arachnida (consists of approximately 18 orders) is the class of chelicerates that is important to VBD study but, it is specifically members of the subclass Acarina (mites and ticks) that are important vectors of human (and animal) diseases.[6] The subclass Acarina consists of seven orders: Ixodida, Holothyrida, Mesostigmata, Prostigmata, Apilioacarida, Astigmata, and Oribatida. All of the seven orders are mites but Ixodida is commonly referred to as ticks.[7, 8]

The basic morphology, function and life cycle are the same for all acarines. The body is divided into two segments: capitulum and idiosoma. The capitulum bears the mouth parts: a pair of pedipalps, a pair of chelicerae, and a hypostome. The idiosoma is a combination of the head (eyes and brain), thorax (legs) and unsegmented abdomen (reproductive and digestive structures) all in one segment. The main morphological distinctions between a mite and a tick are size and presence of toothed hypostome. Adult ticks are larger (0.5-20 mm long) than mites (< 1 mm long) and mites lack toothed hypostome.[7, 8] Hypostome is the mouth part that thrusts through the wound, after the chelicerae had cut opened a host skin, to suck up blood or lymph. The toothed hypostome in ticks allow them to attach themselves to the host and feed for extended period of time.[8] Most acarines are parasites of vertebrates including mammals, reptiles, birds, and amphibians but some mites are free-living such as the house-dust mites. All acarines are fluid feeders including blood, lymph, and predigested skins or tissues. The parasitic behavior of acarines makes them efficient in transmitting diseases. Most mite-born diseases are zoonotic and result from human associations with animals.[8] Some mite-borne diseases are rickettsial pox, Argentine hemorrhagic fever, Korean hemorrhagic fever, and scrub typhus. Unlike mites, all ticks are obligate blood-feeding parasites of vertebrates.[7] This makes them the most important of acarine vectors and are second only to mosquitoes in the number of human and animal diseases vectored.[7] Some human tick-borne diseases are Lyme disease, babesiosis, Rocky Mountain spotted fever, human ehrlichiosis, Kyasanur Forest disease, and Crimean Congo hemorrhagic fever. The basic ontology of acarines includes seven stages: egg, prelarva, larva, protonymph,

deutonymph, tritonymph and adult.[8] A detailed review of medically important acarines including their taxonomy, form, function, life cycle, and role as vectors is presented elsewhere.[7, 8]

1.2.2. The insects

Insects are distinguished from other arthropods by characteristically having three pairs of jointed legs and three segmented body parts: head, thorax and segmented abdomen.[9] The head bears the brain and the feeding (mouthparts) and sensory (antenna, compound and simple eyes) apparatus. The thorax bears the locomotive (wings and legs) apparatus. Although not all insects have wings, they are the only arthropods with wings. The abdomen bears the reproductive organs. A complete description of the internal and external structure of an insect body is presented elsewhere.[9-11]

Insecta is ranked the first in species richness with over one million taxonomically described species compared to any other taxonomic class of invertebrates or vertebrates on earth.[9] Insects are found everywhere, occupying an astonishingly wide range of habitats both aquatic and terrestrial.[10, 11] They have developed complex and specialized anatomical, physiological, and behavioral features that enable them to respond to and successfully thrive in their environment. These features vary tremendously between species making insects anatomically, physiologically, and functionally diverse group of organisms on earth. This diversity allows them to spread and occupy every ecosystem making them ecologically important. Examples of their ecological roles include nutrient cycling, plant pests, plant propagation, pollination, and seed dispersion. They are

also important for maintenance of animal community structure through their roles as food source for other organisms, parasites of large animals, parasitoids of other insects, predators of smaller animals, and vectors of microorganisms.

From human perspectives, insects have a lot of beneficial roles. They produce honey, wax, lac, and silk.[12] They pollinate our crops, decompose our livestock's dung and serve as indicators of environmental pollution.[9, 12] In many cultures, insects serve as food source. While they have a lot of beneficial roles, insects can also be devastating to human. The most notable devastating role of insects is the transmission of infectious pathogens. Other than acarines (section 1.2.1), the rest of the arthropod vectors of human and animal diseases are insects. There are 32 orders of insects (table 3), each with a few to hundreds of thousands of species categorized under various families and genera. Of the 32 orders, only four (Siphonaptera, Phthiraptera, Hemiptera, and Diptera) have members that are hematophagous vectors of human and animal diseases.[13]

Table 3. List of the 32 orders of insects. Orders in bold font have members that are hematophagous vectors of human and animal diseases.

Hymenoptera (Ants, Bees, Wasps, and Sawflies)	Isoptera (Termites)
Diptera (**True Flies**)	Blattodea (Cockroaches)
Mecoptera (Scorpionflies and Hangingflies)	Phasmatodea (Walkingsticks and Leaf Insects)
Siphonaptera (**Fleas**)	Orthoptera (Grasshoppers, Crickets, and katydids)
Trichoptera (Caddisflies)	Mantophasmatodea (Gladiators)

Table 3 (cont'd)

Raphidioptera (Snakeflies)	Embioptera (Webspinners)
Coleoptera (Beetles)	Plecoptera (Stoneflies)
Lepidoptera (Butterflies and Moths)	Grylloblattodea (Rock Crawlers or Ice Bugs)
Neuroptera (Lacewings, Antlions, and Owlflies)	Dermaptera (Earwigs)
Megaloptera (Alderflies, Dobsonflies, Fishflies)	Zoraptera (Angel insects)
Strepsiptera (Twisted-wing parasites)	Odonata (Dragonflies and Damselflies)
Hemiptera (**True bugs**, Cicadas, Hoppers, and Aphids)	Ephemeroptera (Mayflies)
Thysanoptera (Thrips)	Thysanura (Silverfish and Firebrats)
Psocoptera (Barkflies)	Diplura (Bristletails)
Phthiraptera (**Lice**)	Collembola (Springtails)
Mantodea (Mantids)	Protura (Coneheads)

1.2.2.1. The Siphonaptera (fleas)

Adult fleas are tiny (1.5-3.3 mm long), wingless, hard-bodied, bilaterally compressed, dark-brown insects with many posteriorly directed spines all over its body.[9, 10] Another conspicuous feature is the presence of backward-projecting comb-like structures called ctenidia at the back of the head and cheeks.[9, 10] Fleas are highly specialized blood feeders and obligate ectoparasites of birds and mammals. The legs are modified for jumping onto their host and ctenidia and

posteriorly directed spines help them attach to their host. The mouthparts of adult fleas are adapted for piercing host skin and sucking up blood.[9, 14]

Fleas are holometabolous insects. This means they go through a complete life cycle with four developmental stages: egg, larva, pupa and adult.[9] Both males and females require blood to provide nutrition prior to mating. Females lay eggs singly either on the host or on host environments such as nests.[9, 14] Unlike the adults, legless larvae are free-living, have chewing mouthparts and feed on organic debris. A flea larva undergoes three instars. The third instar secrets silk and build cocoon for the pupa. Inside the silken cocoon, the pupa metamorphoses into the adult and emerges out of the cocoon to begin its parasitic and reproductive life.[9, 14]

Their parasitic and hematophagous behavior made them efficient vectors of human diseases. Plague, the cause of Black Death of the Middle Ages, is a zoonotic disease caused by a bacterium *Yesinia pestis* and is transmitted by infectious rodent fleas such as *Xenopsylla cheopis* and *X. brasiliensis*.[14] Murine typhus, carrion disease, trench fever tularemia and cat-scratch diseases are bacterial diseases transmitted from animals to humans by fleas.[14] Fleas also transmit pathogenic viruses and helminths.

1.2.2.2. The Phthiraptera (lice)

Adult lice are tiny (0.4-10 mm long), wingless, dorsoventrally flattened, usually elongate, pale beige to black insects.[9, 15] Almost all Phthirapterans are obligate ectoparasites of birds and

mammals. The tarsal claws are well developed and help them grasp their host hairs or feathers as a means of host attachment.[9, 15] There are 3200 known species of lice in 27 families worldwide. These families are further classified under two suborders: Mallophaga (chewing lice) and Anoplura (sucking lice). The chewing lice have chewing mouthparts. Their triangular head is broader than the thorax. Most chewing lice are parasites of birds. Chewing lice feed on various organic fragments from the host skins, feathers, and epidermal secretions. Some species are known to gnaw through skin and obtain blood. Unlike the chewing lice, all sucking lice are blood feeders. Their head is narrower than the thorax. They have piercing and sucking mouthparts were they use to pierce through their host skin and suck up blood.[9, 15]

Lice are hemimetabolous insects. This means that they do not have a pupal stage and the nymphs (larvae) resemble the adults. After mating and the eggs have been developed, a female lays 2 to 10 eggs per day. After laying the eggs, she then glues them one at a time, to the host hairs or feathers where they hatch in about 4-15 days. The newly emerged nymphs (1st instar larvae) undergo three larval stages in 3-8 days to become reproductive adults.[15]

The parasitic and hematophagous behavior of sucking lice made them efficient in transmitting human diseases. There are 15 families of sucking lice. Four families (Pediculidae, Phthiridae, Polyplacidae, and Hoplopleuridae) include species that are of direct or indirect medical importance to human. Murine typhus, which is transmitted by rat fleas, is also transmitted from rats to humans by rat lice *Polyplax spinulosa* and *Hoplopleura pacifica*.[15] Epidemic thypus,

trench fever and relapsing fever are bacterial diseases that are transmitted from person to person by body louse *Pediculus humanus humanus* and crab louse *Pthirus pubis*.[15, 16]

1.2.2.3. The Hemiptera (kissing bugs and bedbugs)

Hemiptera is the fifth largest order of insects with an estimated 50,000 to 80,000 species. It accounts for 10% of the total known insect species worldwide. There are many features that are used to identify hemipterans but a common feature is presence of a piercing and sucking beak (mouthparts). All members share a common mouthparts arrangement.[9] Based on the wing morphology the hemipterans are further grouped into two suborders: Heteroptera and Homoptera.[9, 11] The heteropterans, which include all true bugs, have a wing feature called hemelytra where the forewing is divided into a leathery part and a membranous part. The homopterans, which include aphids, leafhoppers, whiteflies and cicadas, have uniformed forewings (lack hemelytra). The suborder Heteroptera has approximately 40,000 species distributed under 54 aquatic and terrestrial families.[9, 11] Two heteropteran families Cimicidae (bed bugs) and Reduviidae (kissing bugs) have members that are important vectors of human diseases.[9, 17]

The family Cimicidae includes bedbugs, bat bugs and swallow bugs. Cimicids are usually reddish brown, dorsoventrally flattened, oval in shape and small (4-7 mm long) insect with highly reduced wings.[17, 18] Most cimicids are obligate hematophagous ectoparasites of their birds or mammalian hosts. Six species of bedbugs *Cimex lectularius* (common bedbug), *C.*

hemipterus, *C. columbarius*, *C. pipistrelli*, *C. dissimilis*, and *Oeciacus hirundinis* have been known to be associated with human.[17, 18] Bedbugs do not live on their host but hide in dark secluded parts of their host's environment such as crevices, cracks, holes, under beddings and carpets. They are nocturnal and come out to feed on their host during the night.[17] The females require blood for egg nourishments and lays in a batch 200-500 eggs.[17] Like all hemipterans, cimicids are hemimetabolous and their nymphs undergo five molts to become adults.[17] Although bedbugs are obligate blood feeders of humans, they have only been implicated as potential vectors of human disease including Chagas disease, Q fever, and Hepatitis B virus.[17, 18] A list of pathogens potentially transmitted by bedbugs has been reviewed by Delaunay et. al.[18] The bites of bedbugs can cause sores with serious allergic reactions.

Members of the family Reduviidae are predatory winged insects. General appearance includes subcylindrical head, large pronotum, long downward-pointing beak, and a distinctive triangular scutellum. The adults can be between 4-40 mm long.[17] This family has about 7000 known species distributed under 25 subfamilies. The subfamily Triatominae, commonly referred to as kissing bugs, has 130 described member species and most of them are blood-feeders.[17] Their piercing and sucking mouthpart is highly adapted for obtaining blood. Both sexes are hematophagous requiring blood for nutrition especially egg nourishment for females. Females lay eggs in batches with each batch having over a hundred eggs. As hemimetabolous insects, the nymphs undergo five stages before reaching reproductive adult stage. Their hematophagous behavior made them efficient vectors of *Trypanosoma cruzi* – a protozoon that cause Chagas

disease. Almost all species of triatomins are capable of transmitting Chagas diseases but those species that are closely associated with human cause great devastations. The most important vectors are *Rhodnius prolixus*, *Triatoma infestans*, *T. dimidiata*, and *T. brasiliensis*.[17]

1.2.2.4. The Diptera (tsetse flies, biting midges, sand flies, black flies, and mosquitoes)

Diptera, commonly referred to as the true flies, is the fourth largest order of insects with over 152,000 described species.[19] It accounts for 12.5% of the total known insect species worldwide. There are many features that are used to identify dipterans from other orders but a common feature shared by all members is presence of a single pair of membranous wings. The hindwings are reduced to a pair of knob-like structures called the halteres. All true flies are holometabolous. Detailed anatomical, physiological and behavioral description of true flies is presented elsewhere.[9-11]

Based on certain anatomical or morphological features, dipterans are generally grouped into two suborders: Brachycera and Nematocera. In brief, the adult brachycerans have a three-segmented antenna which is shorter than the thoracic length. The adult body is generally chunky or robust. The larvae are usually maggot-like.[9-11] There are about 120 families of Brachycera. Representative families include Asilidae (robber flies), Drosophilidae (fruit flies), Muscidae (house flies), Syrphidae (hover flies), Calliphoridae (green-bottle flies), and Glossinidae (tsetse flies). Unlike Brachycera, nematocerans have six-segmented and often plumose antenna that is longer than the thoracic length. The body is slender with long delicate legs. The larvae have well developed mandibulate mouthparts. There are over 25 families. Representative families include

Tipulidae (crane flies), Psychodidae (sand flies), Culicidae (mosquitoes), Ceratopogonidae (biting midges) and Simuliidae (black flies).[9-11]

There are five medically important hematophagous families of flies: Tsetse flies, biting midges, phlebotomine sand flies, black flies, and mosquitoes. Adult tsetse flies are small (5-10 mm long) light to dark brown winged insects. The tsetse family has only one representative genus *Glossina* with 23 species confined exclusively to Sub-Saharan Africa.[20] All species are obligate blood feeders of mammals including human. Both male and female tsetse flies take blood. Human diseases that are transmitted by tsetse flies include African trypanosomiasis (sleeping sickness) and filariasis. The most important vectors of these diseases include *G. tachinoides*, *G. fuscipes*, *G. palpalis*, *G. centralis* and *G. pallidipes*.[20] Details about the biology, of tsetse flies and their role as disease vectors are available elsewhere.[20-22]

Adult biting midges are small (1-4 mm long) light to dark brown winged insects. There are 103 genera but, only four (*Leptoconops*, *Austroconops*, *Forcipomyia*, and *Culicoides*) are actually blood feeders of vertebrates. Only the females are hematophagous. Human diseases transmitted by biting midges include Barmah forest virus, Oropouche virus, Rift Valley fever, Congo Virus and filariasis.[23] The most important vectors of these diseases are *Culicoides marxi*, *C. paraensis*, *C. austani*, and *C. grahami*. The biology of biting midges and their role as disease vectors are found elsewhere but have been reviewed extensively by Borkent.[23]

Adult sand flies are tiny (2.5-3.5 mm long), silvery grey to black with furry-looking scales all on their body.[24] There are over 1000 known species of phlebotomine sand flies distributed under 5 genera. Two of these genera *Lutzomyia* and *Phlebotomus* contain members that are important hematophagic vectors of human diseases. Only the female blood feed and vector diseases.[24] Human diseases transmitted by phlebotomine sand flies include leishmaniasis, bartonellosis, and vesicular stomatitis virus.[24] Characteristics like the relatively small size, inconspicuous behaviors, speciose but morphologically similar, difficulty finding larvae in field settings, and laboratory rearing difficulty made it hard to study this medically important insects.[24]

Adult black flies are black, sometimes yellow or orange in color, and are about the size of common house flies (~ 6 mm long). There are approximately 1,800 species distributed under 31 genera. Like sand flies and biting midges, only the females are hematophagous and feed on a wide variety of birds and mammals including human. Although black flies mostly transmit animal diseases, members of the genus *Simulium* transmit *Onchocerca volvulus*, a helminth that causes river blindness in human.[25] The biology of black flies and their role as disease vectors are found elsewhere but have been reviewed extensively by Adler.[25]

The fifth and the most medically important family of true flies is the Culicidae. Its numerous species coupled with the fact that the females of most species are obligate blood feeders made them the most important insect vectors. This family is comprehensively reviewed in section 1.3.

1.3. MOSQUITOES (DIPTERA: CULICIDAE)

1.3.1. Biology of mosquitoes

1.3.1.1. Taxonomy

There are three subfamilies of Culicidae: Culicinae, Toxorhynchitinae and Anophelinae. Characteristics of adult culicines include shorter maxillary palps than the proboscis, dark-scaled wing veins, and scaled abdomen. When at rest or feeding, the adult's body is horizontal to the resting surface. Culicinae consists of ten tribes each with varying number of genera (table 4). Aedeomyiini consist of 1 genus, Aedini (82 genera), Culicini (4 genera), Culisetini (1 genus), Ficalbiini (2 genera), Hodgesiini (1 genera), Mansoniini (2 genera), Orthopodomyniini (1 genus), Sabethini (14 genera), and Uranotaeniini (1 genus). Characteristics of adult anophelines include dark and pale spots of scales on the wings, maxillary palps are the same size as the proboscis, and unscaled abdomen. When at rest or feeding, the adult's body inclines at an angle (30-45°) to the resting surface. Anophelinae consists of only one tribe Anophelini (3 genera). The characteristics of Toxorhynchitinae are similar to the Culicinae except that Toxorhynchites do not blood feed and the larvae are predacious. Each genus in turn represents numerous species and species complexes. There are over 3500 known species distributed under approximately 109 genera of mosquitoes worldwide.

1.3.1.2. General anatomy of adult mosquitoes

Although there are distinct differences between species or different groups, all mosquitoes share some common characteristics. The adult stage is easily identified by having a long slender body that is composed of three well-defined segments: the head, thorax and abdomen. They head bears a pair of large compound eyes, a pair of branched, jointed and filamentous antennae, a long

piercing and sucking mouthparts (consisting of a pair of palps, and a sheathed proboscis) that is highly developed for taking nectar and blood. The head encases a number of vital internal organs including the brain, salivary glands, and part of the foregut. The thorax bears three pairs of long, delicate, seven-segmented legs and a pair of narrow scaly wings. It encases a number of vital internal organs. The abdomen bears the external reproductive organs and encases vital internal organs such as the mid and hind gut, malpighian tubules, and the internal reproductive organs. Figure 2 shows the general external anatomical feature of a mosquito. The internal anatomy of mosquitoes, which is generally similar to most other insects, is described elsewhere.[9]

Table 4. List of the mosquito tribes and their representative genera. The most medically important genera are in bold font.

Subfamily	**Tribe**	**Representative genera**
Anophelinae	Anophelini	***Anopheles****, Bironella, Chagasia*
Culicinae	Aedini*	***Aedes****, Haemagogus, Ochlerotatus, Psorophora, Armigeres, Stegomyia, Belkinius, Kompia, Bothaella, Collessius , Finlaya , Udaya , Scutomyia, Opifex, etc.*
	Culicini	***Culex****, Deinocerites, Galindomyia*
	Aedeomyiini	*Aedeomyia*
	Culisetini	*Culiseta*
	Ficalbiini	*Ficalbia, Mimomyia*
	Hodgesiini	*Hodgesia*
	Mansoniini	*Coquillettidia,* ***Mansonia***
	Orthopodomyniini	*Orthopodomyia*
	Sabethini	*Sabethes, Trichoprosopon, Wyeomyia, Isostomyia, Topomyia, Shannoniana, Johnbelkinia, Kimia, Limatus, Malaya, Maorigoeldia, Onirion, Runchomyia, Tripteroides,*
	Uranotaeniini	*Uranotaenia*
Toxorhynchitinae		*Toxorhynchites*

* There are 82 genera of Aedini but only 14 representative genera are listed.

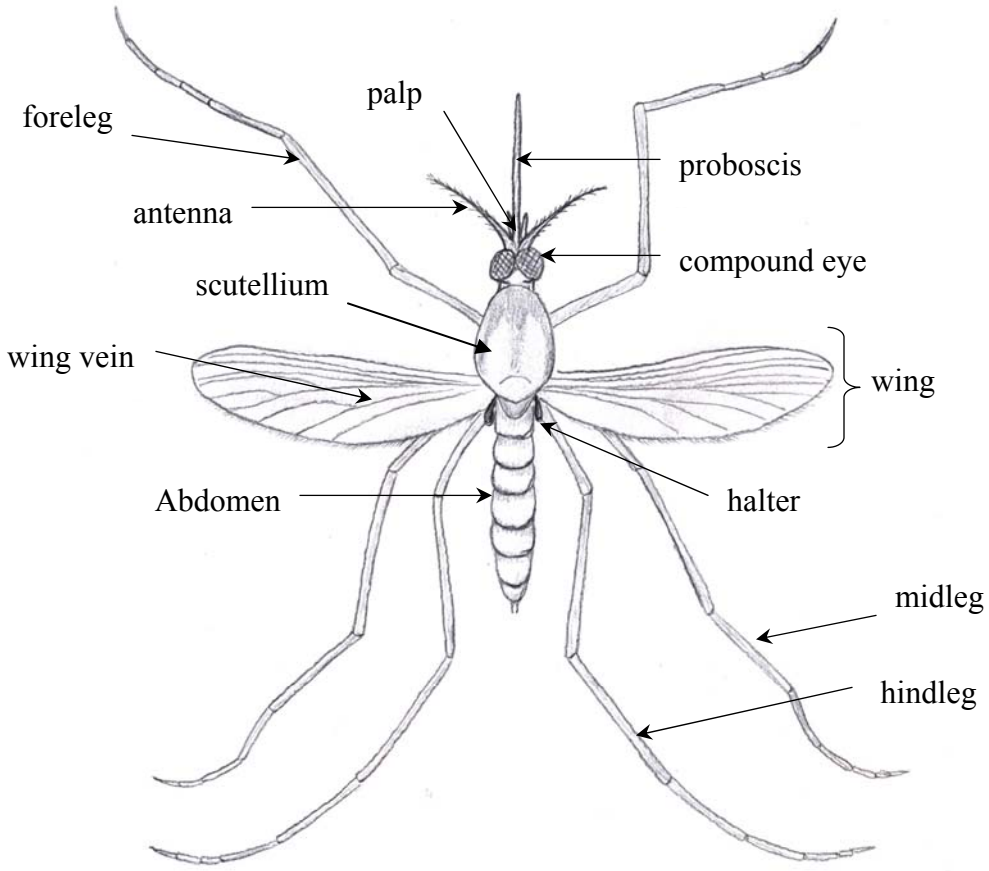

Figure 2. Dorsal view of the general external anatomical features of an adult mosquito.

1.3.1.3. Reproduction

Two to three days after emergence, an adult mosquito is ready to mate. For most species, reproduction in the wild usually involves a swarming behavior. The swarm usually consist of males from a single species but some can involve a number of different species.[26] Swarming usually occurs between dusk and dark. During the event, males form a large swarm that is usually 1-9 m above the ground at some specific swarming location guided by some environmental cues.[26] As the males fly about in the swarm, females would emerge from the nearby foliage and fly into the male swarm.[27] The harming sound of the males in the swarm is believed to attract females however, there is little evidence to support this suggestion.[26] Studies have shown strong evidence that a male mosquito recognizes females of its species by their wing beat using its auditory organ at the base of the antennae and fly to them to engage in mating attempts.[28, 29] If mating is successful, the pair remains attached while in flight for a few seconds during which copulation occurs and sperm is transferred into the female.[27] A female mosquito mates only once in her lifetime and stores the sperm she receives in a special sperm-storage organ called spermatheca where she releases some at a time to fertilized a batch of eggs. This means that fertilization does not occur during mating but oviposition.[30]

Subsequent activities after a female has been mated include obtaining a blood meal, seeking out a suitable larval habitat, and oviposition. Depending on its host preference or if opportunistic feeder hosts availability, a vertebrate host is visited and a blood meal is obtained. Females in the subfamily Toxorhynchitinae do not require a blood meal for egg production, a process known as

autogeny.[30] It takes 2-3 days after ingestion for the blood to be digested and the nutrients extracted for egg nourishments. When the mosquito is ready to oviposit, it must first identify a suitable larval habitat. Female mosquitoes choose sites that can support the growth and development of eggs and larvae. Mosquitoes base their decision on nutrient availability, presence of predators, and the physical and chemical conditions of the oviposition site.[30-32] Other factors include space and species competition. Different mosquito species prefer different oviposition sites.[33] During oviposition, eggs are released from the ovaries and pass down the common oviduct. As each egg passes by, a sperm enters it through a tube called the micropyle thereby fertilizing the egg before it is laid.[30] Females hover over water as they lay their eggs.[26] The process of blood feeding, habitat seeking, and oviposition can be repeated more than once throughout the life of a fertilized female.

1.3.1.4. Life stages

Mosquitoes are holometabolous insects. Throughout its life, a mosquito must go through an egg stage, a larval stage, a pupal stage and finally an adult stage. The first three are aquatic stages. A female mosquito can lay between 100-150 eggs in one batch. Depending on the species, the eggs can be laid singly (most *Anopheles* and *Aedes*) or as an egg raft (most *Culex* and *Mansonia*).[26] Egg raft is a cluster of eggs that floats about together. The proteinaceous egg chorion which is important for protecting the developing embryo is white and soft when it is first laid. The chorion later undergoes some protein structural rearrangement to harden.[34] Chorion tanning results from a biochemical process called melanization which involves the formation of melanin

pigment.[34] If an egg was not fertilized on its way out, it will not hatch. If it is successfully fertilized, a zygote is formed and embryonic development takes place inside the egg chorion. Most eggs hatch in about 2-3 days after being laid. Floodwater mosquitoes lay their eggs on moist substrates and do not hatch until next flooding.[26, 35] Eggs of mosquitoes in the temperate regions undergo diapause during winter and hatches during the spring and summer.[36] It is believed that a number of factors such as temperature stimulate egg hatching but most studies have shown that the concentration of dissolved oxygen is an important factor. Low concentration induces hatching.[37] The biological explanation for this is that water with low oxygen concentration indicates a high bacterial content which further indicates a large supply of food.[26]

The larval stage of a mosquito is the most critical of all the three aquatic stages because the quality of the adult mosquito depends on it. The reason it is critical is because the larval stage is the feeding stage of a mosquito. A larva spends most of its time simply feeding. Most of the adult's nutrients and energy reserves are acquired during the larval stage. Important qualities of an adult such as its size, reproductive fitness, vector capacity, longevity, and flight distance all depends on the success of the larva. There are four larval stages: first, second, third, and fourth instar. The first instar is the smallest larva that emerges after an egg hatches. A larva must undergo three molts to reach the fourth and final instar. Molting, which is a complex physiological process that involves shedding off of old cuticle, is part of the process of growth in insects and so the next instar comes with an increase in size because the previous instar has acquired more body-building materials through feeding. The time required for the next molt

depends on several factors including temperature, larval density, and food availability but, when all conditions are right, molting usually occurs in 2-3 days.

Unlike other aquatic insects that take oxygen by means of gills, mosquitoes breathe atmospheric oxygen. They breathe through spiracles or a tube-like structure called siphon.[26, 30] Thus, mosquito larvae spend most time on the surface of water to breath. Anophelines breathe through the spiracles located along the thorax and abdomen.[30] Because of the arrangements of the spiracles, anopheline larvae usually lie parallel to the water surface to allow their spiracles to be in contact with the air. Other species breathe through the siphon which is located at the tip of the abdomen.[30] Because of the siphon location, the larvae usually lie in a slightly vertical orientation with their heads down and the siphon on the water surface in contact with the air. Some species in the Mansonia and Coquillettidia have modified siphon for piercing tissues of aquatic plants for the purpose of obtaining oxygen.[26, 30]

Mosquito larvae mainly feed on microorganisms, particularly bacteria, fungus, algae and microcrustaceans.[26, 30] They also feed on detritus or plant particulate matters that are small enough for them to utilize. Feeding involves the use of a mouthpart called the mouthbrush that creates currents of flowing water toward their mouth and filtering out food particles that are then taken into the digestive tract.[26, 30] Some species of *Toxorhynchites* are predacious and feed on larvae of other mosquitoes and similar nektonic prey.[26, 30]

After all the hard work of energy and nutrient acquisition during the larval stages, the fourth instar finally pupates. The pupal stage is physiologically active but does not feed.[26, 30] It breaths through a funnel-shape breathing structure called the trumpet. At this stage the mosquito metamorphoses into an adult. The entire adult body structure is completely formed inside the pupal case.[26, 30] During eclosion, the pupa comes to the water surface where the dorsal surface of the pupal case splits and the completely formed adult emerges to begin its reproductive life.[26, 30] Adult mosquitoes have all its nutrients and most of its energy supply from the larval stage but they supplement the energy sources by feeding on sugary plant nectar and vertebrate blood.

1.3.1.5. Aquatic habitats of mosquitoes

Mosquito larvae are not found in large and deep bodies of water such as lakes. They are not found in fast flowing rivers. Mosquitoes however, colonize an astonishingly wide range of stagnant shallow bodies of fresh water. This includes ponds, streams, ditches, swamps, marshes, brackish marshes, temporary and permanent pools, rock holes, crab holes, lake margins, ground water-puddles, sewage ponds, road potholes, plant containers (leaves, fruits, husks, tree holes, etc.) and artificial containers (tyres, tin cans, clogged gutters, troughs, drums, etc.).[26, 38] The choice of habitats depends on a particular species or group. For example, *Anopheles* mosquitoes in general usually prefer shallow clear ground water puddles that are open to direct sunlight. Specific examples of such habitats include drainage, irrigation ditches, road potholes, flooded stream beds, lake margins, swamps and marshes.[38] *Culex* mosquitoes are non-habitat specific and colonize any kind of habitat. One can find a *Culex* mosquito anywhere and often co-

inhabiting the same habitats with other mosquitoes. *Aedes* mosquitoes are usually found in artificial containers, plant containers, tree holes, and shallow permanent ponds.[38] Some mosquitoes inhabit highly polluted water bodies. For example, *Aedes aegypti* and *Culex quinquefasciatus* larvae have been found inhabiting septic tanks.[39]

The study of larval habitats is important in many respects especially with regard to public health. This is because where there are suitable breeding sites there will be colonization by mosquito vectors, and where there are vectors, there will be potential for disease transmission. One way to look at this is the relationship between host preference and choice of habitats. An anthrophilic (prefers to feed on human) mosquito that prefers breeding sites that results from human activities or are usually in close proximity to human communities have higher transmission potential. In fact most of the anthropophilic vectors of malaria inhabit habitats that result from human activities such as agriculture, deforestation, and road constructions.[40-42]

1.3.2. Hematophagy and host-seeking behaviors in mosquitoes

1.3.2.1. Evolution of hematophagy

One might think that such highly specialized specialty as hematophagy should occur in a monophyletic group. When plotting the occurrence of hematophagic arthropods on the current arthropod phylogenetic relationships, Black and Boris showed that blood feeding occurred independently seventeen times in disparate insect taxa.[43] Nine of the taxa are Diptera families. The fact that hematophagy occurred independently suggests that each group took different evolutiona ry route to arrive at hematophagy. Little is known about the evolutionary routes but

some have suggested two major categories: prolonged close association with vertebrates and morphological pre-adaptation for piercing.[44] Mosquitoes are likely to fall into the second category. The ancestor of mosquitoes is believed to be an entomophagous insect with a pre-adapted piercing mouthpart which it uses to feed on the hemolymph of other insects. Its insect preys are believed to be associated in some ways with certain vertebrates. The presence of vertebrates around its insect preys may have lead to occasional accidental feeding on the vertebrate which eventually lead to hematophagy.[44] The transition from entomophagy to hematophagy in the ancestral mosquito requires modification of mouthparts to suit the new hosts. This explains why the extant mosquitoes have incredibly efficient blood sucking mouthparts.

1.3.2.2. Host-seeking behavior

In order for a mosquito to be in contact with a host and initiate the blood-feeding process, it must first locate its host. The process of locating a host requires the mosquitoes to develop specialized sensory receptors. Like any other behavioral events, these sensory receptors detect appropriate stimuli from the environment and translate them into nerve impulses. The impulses are carried by sensory neurons to the central nervous system (CNS). The CNS then processes the information it receives and directs motor neurons to regulate the contraction of specific muscle cells that eventually elicit specific host-seeking behavioral response.[45] Flight towards a host location is a behavioral response that results from regulation of the contraction of the wing muscles by the motor neurons. Female mosquitoes locate their hosts mostly by means of olfactory cues. Auditory, visual, thermal and mechanical cues are also utilized but to a lesser degree.[45]

Since mosquitoes must locate their host from a long distance they have to depend very much on olfactory events. The antennae and to a lesser degree, maxillary palps are covered with numerous sensilla (receptor cells) that detect volatile chemicals (stimuli) in the air.[45] Mosquitoes have learned to associate certain chemical odorants in the air to the presence of a host. Most of these are byproducts of host metabolisms from body emanations. Carbondioxide (CO_2) from expired breath of vertebrates is the most universal and reliable odorant mosquitoes use to detect the presence of their host. Based on field and laboratory behavioral assay, some volatiles that work alone or as synergists to CO_2 or one another include L-lactic acid, acetone, 1-octen-3-ol, ammonia, aliphatic carboxylic acids, 2-oxypentanoic acid, methyl-2-hexenoic acid, and 7-octenoic acid.[46-50] These odorants emanates from vertebrate hosts into the air and are carried down wind in a manner similar to a smoke pluming out of a chimney. Mosquitoes detect the odors from far distance and fly upwind following the odor plume toward the source of the odors.[45] The levels (concentrations) of these volatiles usually decrease down plume. By sensing the fluctuations in the levels of these volatiles, they can differentiate odors that indicate a host source from normal levels present in the air.[51] As a mosquito gets closer to the source of the emanations, visual, auditory and thermal cues become involved to help them narrow in on their host.[52] Once they have landed on their host, a different set of behavioral response kicks in preparing the mosquitoes to begin blood feeding.

1.3.2.3. Blood-feeding mechanism

The mouthparts of all insects are composed of four basic components: labrum, mandibles, maxillae, and labium. The labrum is a large chitinous flap anterior to other mouth parts and serves as a lid preventing food from escaping posteriorly when feeding. Sitting behind the labrum are two opposable sclerotized mandibles with sharp dentations for crushing and slicing food. Posterior to the mandibles are two sclerotized blade-like maxillae that manipulates food pierces from the mandibles into the mouth cavity. The maxillae also have large lateral palps covered with chemoreceptors, thermoreceptors, and mechanoreceptors. Attached posterior to the maxillae and extending forward below all the mouthparts is labium, a chitinous flap that serve as another lid to prevent food falling off when feeding.[43] This description is true for chewing mouthparts as in grasshoppers but are modified differently in different insect groups to suit their dietary requirements. Mosquitoes have evolved piercing-sucking type mouthparts.

All piercing-sucking mouthparts consist of structures for puncturing host skin, penetration and anchorage, a food canal, a saliva canal, and a sheath-like covering.[53] In mosquitoes, they are combined into a narrow elongate structure called the proboscis. There are two parts to the proboscis: stylet and labium.[43, 53] The mandibles, labrum hypopharynx and maxillae each forms a narrow elongate sheath that are glued together to form the tube-like stylet with a food channel and a saliva channel (fig. 3A). The tip of the stylet is modified to puncture and penetrate the host skin. The labium forms a narrow elongate sheath that serves as the covering for the stylet.[43, 53]

After a female mosquito has landed on a host it begins the feeding process. It first probes the skin surface with the tip of the labium. After a while the labium stops probing around and remained stationary at a particular spot. During this time the stylet emerges from inside the labium and pierces its way through the skin until it reaches a certain depth.[54] As the stylet makes its way into the tissue, the labium becomes increasingly kinked (fig. 3B). Gordon and Lumsden showed that the stylet does not remain rigid, acting like a hypodermic needle, instead the tip of the stylet is actively flexible, bending and turning until an arteriole or venule is encountered (fig. 3B).[54, 55] Blood is taken up the food canal of the stylet by means of a pumping mechanism of the cibarial and pharyngeal muscles attached to the pharynx.[30] The muscles expand and contract during blood feeding creating pressure difference inside the pharyngeal chamber. The pressure difference inside the chamber causes fluid (blood) to move up the food canal and into the digestive tract.[30, 56] Mosquitoes release saliva during the feeding process to facilitate the uptake of blood. The salivary glands (fig. 4) secrete bioactive compounds that act as blood-coagulation inhibitors, platelet aggregation inhibitors, anti-histamine, vasodilators, and bacteriolytic enzymes, all of which assist with the efficiency of blood uptake.[30, 55, 57] Saliva is released into the host wound through the saliva canal of the stylet (fig. 3A).

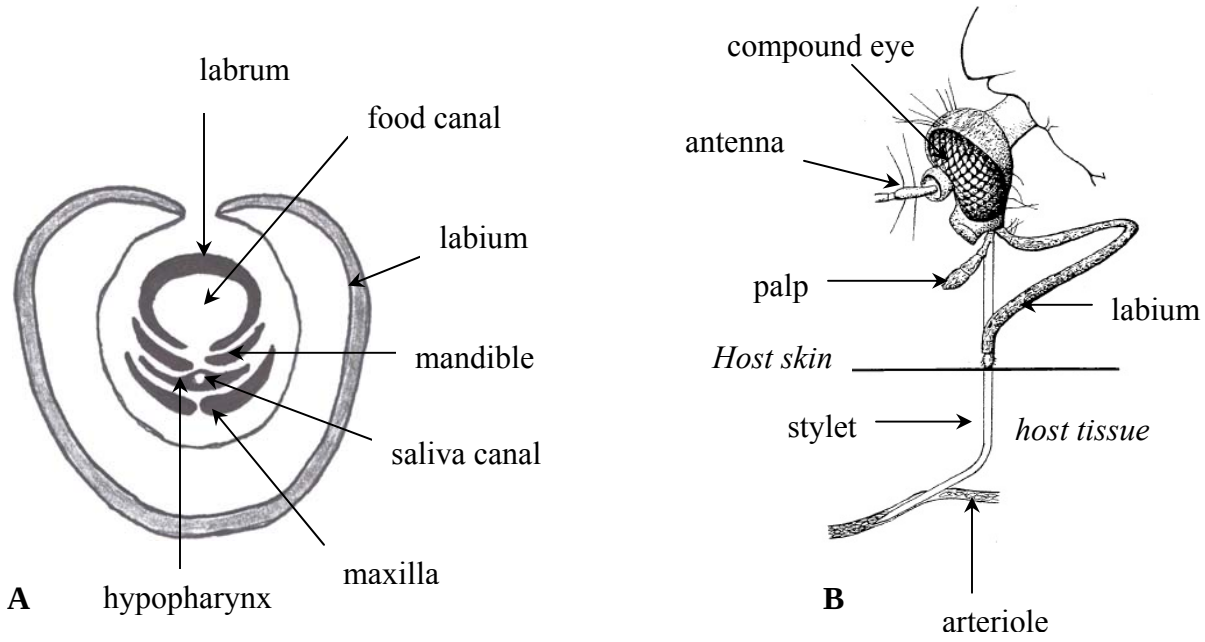

Figure 3. Mouthparts of a mosquito. A: cross-section of a female mosquito proboscis; B: a female mosquito in the act of piercing host skin and sucking up blood. (fig B adapted from Gordon and Lumsden).[54]

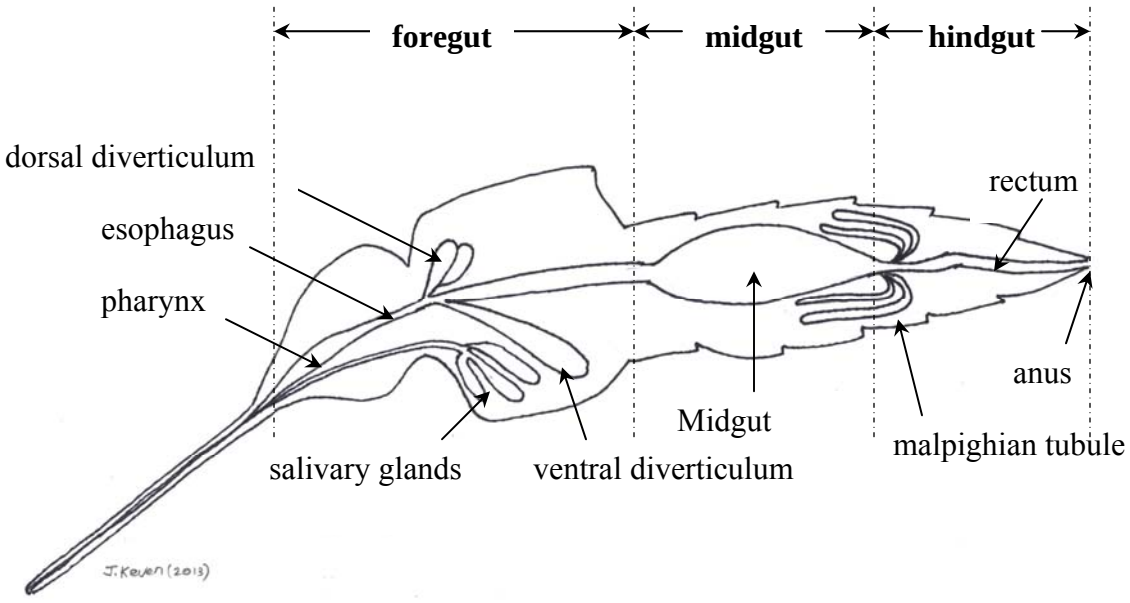

Figure 4. The alimentary canal of a female mosquito.

The alimentary canal of a mosquito extends from the pharynx to the anus and consists of three major regions: foregut (including pharynx and esophagus), midgut and hindgut (Fig. 4).[58, 59] Midgut is the most important region of the alimentary canal because it is here that active digestion and absorption occur. The midgut epithelium is a monolayer of adjacent digestive, absorptive, and receptors cells that rests on a basal lamina.[58, 59] The primary function of the foregut is to transport the ingested blood into the midgut. As the blood enters the midgut lumen, the epithelial cells secrete a chitinous material that forms a semipermeable extracellular lining called the peritrophic matrix (PM) over the epithelial cells, separating the blood from the cells.[60] The PM protects the epithelial cells from abrasion, toxic compounds and pathogens from the blood while allowing digestion to take place inside the lumen.[60] Feeding is stopped by signals from stretch receptors in the midgut to the brain when the midgut is full. Digestive enzymes are secreted by the epithelial cells into the midgut lumen to digest the blood material into nutrients that are then absorbed by the epithelial cells. The waste products of digestion are passed into the rectum of the hindgut where water is absorbed before offloading through the anus.[58]

1.3.3. Pathogen acquisition, incubation, and transmission in mosquitoes

The obligatory nature of hematophagy in mosquitoes is so reliable that some disease-causing pathogens have taken advantage of it to facilitate their own life cycle. The life cycle of the mosquito-borne pathogens occur partly in the mosquito and partly in the vertebrate host. The process of blood feeding becomes the means by which vector-borne pathogens are acquired from an infected host, incubated in the mosquito and then transmitted to an uninfected host during subsequent feeding. Mosquitoes transmit a number of viral, protozoan, and helminthic diseases

(table 1 and 2). Interestingly no bacterial disease has been found that is transmitted by a mosquito. The life cycle of three most common mosquito-borne pathogens *Plasmodium*, *Wuchereria bancrofti*, and *Dengue virus* are briefly discussed to help us appreciate the importance of blood-feeding in pathogen acquisition and transmission.

1.3.3.1. Plasmodium (Malaria)

Plasmodium is a genus of protozoa that belongs to the family Plasmodiidae in the order Haemospororida.[2] These are motile unicellular eukaryotic organisms that parasite on the blood of vertebrate hosts. Four species, *P. ovale*, *P. malariae*, *P. vivax*, and *P. falciparum* cause human malaria with *P. falciparum* being the deadliest of the four pathogens.[2] Female mosquitoes in the genus *Anopheles* are the vectors of human malaria.

Sexual development of *Plasmodium* occurs in the mosquito vector (fig. 5). When a female *Anopheles* vector obtains a blood meal from an infected person, it ingests together with the blood, the gametocytes into its midgut. There are two forms of gametocytes: microgametocytes and macrogametocytes. Both must be ingested by the mosquito for sexual development to occur. Stimulated by increased pH and drop in temperature from ~37 °C in human to ambient temperature in the mosquito midgut, the macrogametocytes and microgametocytes undergo gametogenesis.[61, 62] Gametogenesis occur immediately after entering the midgut lumen where each macrogametocyte transforms into a single non-motile spherical female gamete while each of the microgametocyte exflagellates into eight motile male gametes.[61, 63] A flagellated microgamete swims around until it finds a female macrogamete and fertilizes it forming a diploid

cell called the zygote. The process of gametogenesis to formation of an zygote occurs within one hour of the blood uptake by a mosquito.[61] A short while later the zygote transforms into an elongated motile form called the ookinete.[61-63]

Approximately twenty hours after blood meal, invasive mature ookinetes traverse the midgut epithelium and attach themselves to the outer surface (the hemocoel side) of the basal lamina of the midgut.[62] There the ookinetes transform into an immobile stage called the oocysts. Each oocyst undergoes sporogony, a cell division process where the parasite undergoes multiple rounds of endomitosis which result in the formation of thousands of sporozoites.[61] Sporogony can take 10-14 days before the oocyst ruptures releasing the sporozoites.[2] The sporozoites swim through the hemocoel of the mosquito's body cavity and inhabit the salivary glands of the mosquito. The infective sporozoites are released with the saliva of an infective mosquito into a human host when the mosquito obtains its subsequent blood meals. [61-63]

Inside the human host, the sporozoites infect liver cells and form schizonts. Each schizont forms thousands of merozoites.[2] Rupturing of a schizont releases thousands of merozoites from the liver into the blood stream. Inside the blood stream each merozoite infects an erythrocyte (red blood cell). The infected erythrocyte undergoes schizogony forming a schizont.[2] Each schizont then undergoes merogony where thousands of merozoites are formed. These merozoites are then released into the blood stream where they infect the red blood cells forming schizonts thereby, repeating the erythrocytic cycle.[2] During the erythrocytic cycle, some schizonts differentiate into

haploid gametocytes and circulate in the blood waiting for a chance to be ingested by a mosquito.[2]

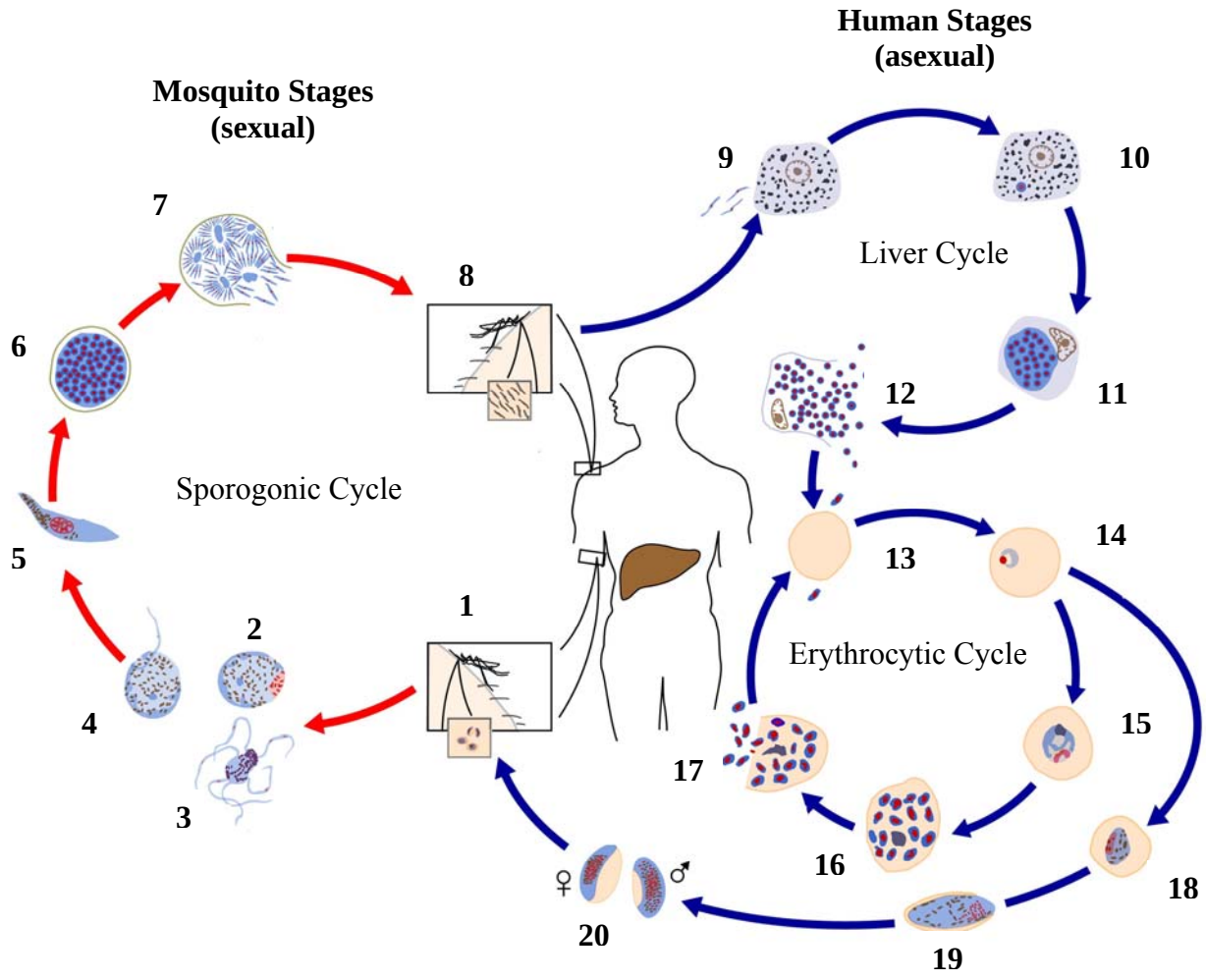

Figure 5. The life cycle of *Plasmodium* parasites in the *Anopheles* vector and human host. Stage 1: a mosquito taking a blood meal and ingesting gametocytes; 2: macrogametocyte; 3: exflagellated microgametocyte; 4: fertilization; 5: ookinete; 6: oocyst; 7: ruptured oocyst with the release of sporozoites; 8: an infected mosquito taking a blood meal and injecting sporozoites into the human host; 9: sporozoites infecting human liver cell; 10: an infected liver cell; 11: schizont; 12: ruptured schizont with the release of merozoites; 13: merozoite-infected erythrocyte; 14: immature trophozoite; 15: mature trophozoite; 16: schizont; 17: ruptured schizont with the release of merozoites; 18,19,20: different gametocyte stages. (Adapted from the Center for Disease Control, Public Health Image Library identification number #3405. website: *http://phil.cdc.gov/phil/details.asp?pid=3405*). For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

1.3.3.2. Wuchereria bancrofti (bancroftian filariasis)

Wuchereria bancrofti (WB) is a parasitic nematode (round worm) that infects human. It belongs to the family Onchocercidae in the order Spirurida.[2] WB is the causative agent of lymphatic filariasis, one of the most tragic, horrifying and debilitating diseases on earth. As a VBD, the life cycle of WB occurs partly in the mosquito and partly in the human host. Unlike *Plasmodium*, the sexual stages of WB occur in the human host and the asexual stages occur in the mosquito vector (fig. 6). WB is transmitted by a number of mosquito species mostly from the genera *Mansonia*, *Culex, Aedes*, and *Anopheles*.[2, 64]

A microfilaria is the tiny juvenile stage of WB that develops from an egg and is often referred to as advanced embryo.[2] There are three variants of WB that are recognized by the periodic presence of their microfilariae (mf) in the peripheral blood of humans. These are nocturnally periodic, nocturnal subperiodic and diurnal subperiodic.[64] The mf of nocturnally periodic form sequester in some body tissues during the day and enter the peripheral blood during the night between 2200-0300 hours.[64] The mf of the other two forms are present in the peripheral blood 24 hours a day with peak densities between 1800-2000 hours.[64] The periodicity of these variants is believed to be an adaptation to the host-seeking and biting times of the primary vectors.[64]

When a female mosquito takes a blood meal from an infected person it ingests some mf that circulate in the peripheral blood. Approximately two hours after entering the midgut, the mf penetrate the midgut epithelial cells and migrate through the hemocoel to the thoracic muscles of

the mosquito.[64] Inside the muscle, the mf develop into first stage larvae (L1). Approximately eight days later, the L1 worms develop into short, sausage-shaped second stage larvae (L2). Four days after becoming L2, the worms finally molt to the third and final stage (L3).[2, 64] Both L2 and L3 feed on mosquito host's tissues. L3 worms are elongated, slender and relatively larger than the previous stages.[2]

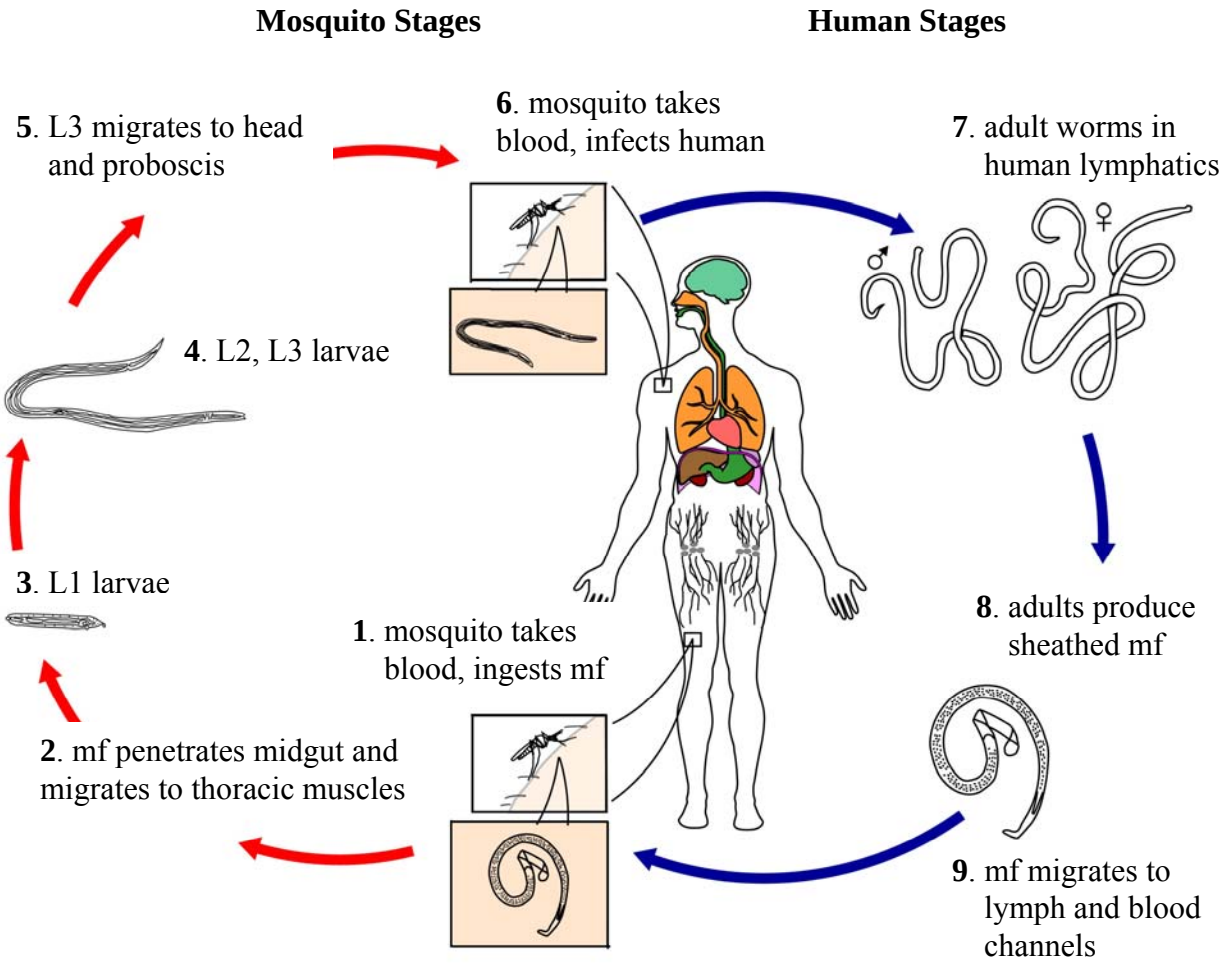

Figure 6. The life cycle of *Wuchereria bancrofti* in the mosquito vector and human host. (Adapted from the Center for Disease Control, Public Health Image Library identification number #3425. website: *http://phil.cdc.gov/phil/details.asp?pid=3425*)

As the infective stage, the L3 worms migrate from the thoracic muscles to the mouthparts (usually the labium) ready to be transferred into a human host.[2, 64] Unlike *Plasmodium*, filarial worms are relatively too large to pass through the food or saliva canals of the mosquito proboscis. Instead, they literally escape from the labium while the mosquito is feeding and enter the human body through the wounds made by the mosquito.[2, 64] Inside the human host the L3 larvae migrate through the lymphatic vessels and settle in the lymph nodes and glands. There they mature, differentiate as sexual (male and female) adults where they mate and produce thousands of mf. The mf sequester in some human tissues and periodically enter the blood stream where they wait for the chance to be picked up by a mosquito.[2]

1.3.3.3. Dengue virus (Dengue fever)

Dengue virus is a member of the genus *Flavivirus* in the family Flaviviridae. These are single stranded, positive sense, ribonucleic acid (RNA) viruses with genome size around 11 kilobases (kb).[65] Dengue virus is the causative agent for dengue fever and dengue hemorrhagic fever. Dengue is transmitted by *Aedes* mosquitoes, primarily *Ae. aegypti*. The process of acquisition, infection, replication and transmission of the virus by a mosquito vector is generally similar to other arboviral pathogens.[66] Upon ingestion of a viremic blood meal from an infected person, the viruses must overcome the midgut-infection barriers and establish infection in the midgut epithelial cells.[67] As a virus, it does not replicate through cell division, instead the virions use the midgut epithelial cell's genome to make multiple copies of their own genome thus, replicating. When the newly produced viruses have matured, they are released from the infected

midgut cells into the hemocoel of the mosquito where they infect other secondary tissues.[67] Some end up in the lumen of the mosquito's salivary glands where they are transferred to a new human host during next blood feeding.[67]

1.3.4. Medically important mosquitoes

Out of approximately 109 known mosquito genera on earth, four are regarded as the most medically important group. These are *Mansonia*, *Culex*, *Aedes*, and *Anopheles*. This statement does not mean that medically important mosquitoes are restricted to these four genera. It implies that most of the vectors are members of these four groups.

Mosquitoes in the genera *Mansonia* are important vectors of brugian filariasis, a filarial disease that is common in South Asia (including India). Important *Mansonia* vectors in South Asia are *Mn. annulata*, *Mn. annulifera*, *Mn. uniformis*, *Mn. bonneae*, and *Mn. dives*.[68] *Mn. uniformis* and *Mn. titillans* are subsidiary vectors of bancroftian filariasis in New Guinea and Tropical America respectively.[68]

Culex mosquitoes mainly transmit zoonotic arboviral diseases. These diseases include Japanese Encephalitis (JE), St. Louis encephalitis (SLE), Western equine encephalitis (WEE), West Nile fever (WNV), Ross river fever (RRV), Rift valley fever (RVV), Eastern equine encephalitis (EEE) and Venezuelan equine encephalitis (VEE). Some *Culex* mosquitoes vector filariasis. Important *Culex* vectors include *Cx. gelidus* (JE), *Cx. molestus* (WB), *Cx. tritaeniorrhynchus* (JE), *Cx. quinquefasciatus* (SLE, WB, WNV), *Cx. pipiens* (WB, RVV, WNV), *Cx. annulirostris*

(RRV), *Cx. tarsalis* (SLE, WEE), *Cx. nigripalpus* (SLE), and *Cx. portesi* (VEE).[68-71] *Aedes* mosquitoes transmit filariasis and some of the zoonotic arboviruses but are famous for transmitting yellow fever virus (YFV), dengue fever virus (DFV) and chikungunya (CV). This is because YFV and DFV are the two most deadly arboviral diseases and CV is an epidemically important emerging infectious disease. Some important *Aedes* vectors are *Ae. aegypti* (YFV, DVF, CV), *Ae. albopictus* (DFV, CV), *Ae. polynesiensis* (DFV, WB), *Ae. africanus* (YFV), *Ae. simpsoni* (YFV), *Ae. harinasutai* (WB), *Ae. cooki* (WB), *Ae. taeniorhynchus* (VEE, EEE), *Ae. sollicitans* (VEE, EEE), and *Ae. camptorhynchus* (RRV).[68, 70 72]

Anopheles mosquitoes transmit protozoan and helminthic diseases but are generally incompetent vectors of arboviruses. They are the only genus responsible for the transmission of human malaria. In Africa where the burden of malaria is the greatest, the primary vectors in this region are *An. gambiae*, *An. arabiensis*, *An. melas*, *An. merus* and *An. funestus*.[68] All four species are also the primary vectors of WB in Africa. In Asia (including Malaysian region), *An. dirus*, *An. minimus*, *An. maculatus*, *An. sinensis*, and *An. nigerrimus* are some of the primary vectors for both malaria and WB.[68] In North and Central America, the primary malaria vectors include *An. albimanus*, *An. quadrimaculatus*, *An. aquasalis*, and *An. darlingi*.[68] South America have *An. pseudopunctipennis*, *An. cruzii*, *An. aqualsalis*, *An. darlingi*, and *An. albimanus* as the main vectors.[68] *An. stephensi*, *An. culicifacies*, and *An. fluviatilis* are some important vectors in India.[68] In the Australasian region which includes Australia, Solomon Islands and the Island of

New Guinea, the main *Anopheles* vectors for malaria and WB are those of the *Anopheles punctulatus* group.[68] This group is reviewed in detail in section 1.4.

1.4. THE *ANOPHELES PUNCTULATUS* SPECIES COMPLEX

1.4.1. Taxonomy and species composition

1.4.1.1. The morphological species

Two most notable taxonomists who began the early work on this group of mosquitoes are Donitz and Laveran.[73, 74] Working on specimens collected from various locations on the Island of New Guinea, Donitz described a type form as having a proboscis which was dark on the basal half and pale or white on the apical half with a small dark area at the tip. He named the species *Anopheles punctulatus*.[73] Laveran was working on specimens from Vanuatu. He noticed that his specimens were morphologically different from *An. punctulatus* Donitz by having a completely dark proboscis with a small white patch at the extreme apex. He named the form *Anopheles farauti*.[74] During the same time Dutch workers in the Moluccas (Indonesia) collected specimens that were morphologically similar to *Anopheles farauti*. Swellengrebel and Graaf named the form *Anopheles moluccensis* .[75]

Anopheles specimens collected by military and civilian entomologists from Vanuatu, Solomon Island, Australia and Papua New Guinea (PNG) during the early 1900's were recognized as *An. punctulatus* Donitz or *An. farauti* Laveran. However, as more collections were made, they began to notice specimens that show variable degree of pale scaling on the proboscis but were considered as intermediate forms. When working with specimens from the Solomon Island,

Owen noticed that a small dark spot that is present on the costal margin of the wings of *An. farauti* Laveran and *An. punctulatus* Donitz was consistently absent in the intermediate forms.[76] He named the form *An. koliensis*.[76]

By the 1940's there were conflicting descriptions regarding the taxonomy and nomenclature of this group of *Anopheles*. To deal with the problem, Rozeboom and Knight and other prominent entomologists of that time collected specimens from all over the region from Vanuatu, Solomon Island, Australia, Island of New Guinea and far west to the Moluccas and systematically studied the morphological forms.[77] They concluded that *An. punctulatus* Donitz, *An. farauti* Laveran, and *An. koliensis* Owen were systematically different species and *An. moluccensis* Swellengrebel and Graaf is a synonym for *An. farauti* Laveran.[77] They also described for the first time two rare member of the group which they named *An. rennellensis* and *An. clowi*.[77]

1.4.1.2. Cryptic species: Cross-mating approach

Several biologists did not accept the view of Rozeboom and Knight that *An. koliensis* Owen is a distinct species.[78, 79] To support the view that *An. koliensis* is a hybrid of *An. farauti* and *An. punctulatus* they needed to do a cross-mating experiment. Using the induced mating technique for *Anopheles* developed by Baker and others, Bryan carried out a cross-mating experiment to deal with the controversy.[80, 81] The mosquito populations he worked with were offsprings of females collected from various locations in PNG and Australia.[81] Bryan's result showed that *An. koliensis* Owen is not a hybrid. An interesting observation was noted in the same experiment.

The *An. farauti* Laveran was found to consist of two morphologically indistinguishable but reproductively incompatible species. They were designated as *An. farauti* No. 1 and *An. farauti* No. 2.[81, 82] Similar cross-mating experiment by Mahon and Miethke discovered a fourth cryptic species designated *An. farauti* No. 3.[83]

1.4.1.3. Cryptic species: Molecular approach

Bryan's cross-mating experiment was evidence that morphological characteristics are not sufficient to resolve the taxonomy of *An. punctulatus* complex. Charlwood was probably the first person to apply molecular technique to distinguish between cryptic species in the *An. punctulatus* complex.[84] He applied allozyme analysis (protein electrophoresis), using phosphoglucomutase as the marker, to determine between *A. farauti* No. 1 and No. 2 in his work on host feeding behavior.[84] Resolving the taxonomy of the complex using molecular technique really began with the work of Foley and others.[85] They also used protein electrophoretic technique using cellulose acetate allozyme as the molecular marker. Working with *An. punctulatus* s.l specimens from nineteen sites in PNG and laboratory reared *An. farauti* No. 1, No. 2 and No. 3, they discovered three additional molecular forms of *An. farauti* Laveran. These were designated as *An. farauti* No. 4, *An. farauti* No. 5 and *An. farauti* No. 6.[85] Extension of their allozyme analysis work in Solomon Island and the Western Province of PNG revealed two more molecular forms: *An. farauti* No. 7 and *Anopheles* species near *punctulatus* (abbreviated *An*. sp. near *punctulatus*).[86, 87] The later species is morphologically indistinguishable from *An. punctulatus* Donitz.

After the 12 species of the *An. punctulatus* complex have been identified, Schmidt et al. (2001; 2003) performed detailed morphological study on the adult, larvae and pupae of the members of the farauti group.[88, 89] They succeeded in describing and renaming only four of the species (table 5).[88, 89] Although close examination by Benet and others show evidence of more than one genotypes for the *An. koliensis*, there is a general consensus that much of the taxonomy of *An. punctulatus* species complex has been resolved.[90] Their phylogenetic relationship has also been determined.[91, 92] Table 5 lists the 12 members of the complex.

Table 5. Members of the *An. punctulatus* species complex.

An. punctulatus s.s Donitz	*An. farauti* No. 4
An. koliensis Owen	*An. farauti* No. 5
An. farauti s.s Laveran (formerly *An. farauti* No.1)	*An. farauti* No. 6
An. hinesorum Schmidt (formerly *An. farauti* No. 2)	*An*. sp. near *punctulatus*
An. torresiensis Schmidt (formerly *An. farauti* No. 3)	*An. klowi* Rozeboom and Knight
An. irenicus Schmidt (formerly *An. farauti* No. 7)	*An. rennellensis* Rozeboom and Knight

1.4.1.4. Species diagnostics

The morphological characters described by Schmidt are difficult to work with. Even *An. farauti* No. 4, No. 5 and No. 6 cannot be identified morphologically and *An. koliensis* Owen are often mistaken for *An. punctulatus* Donitz or *An. farauti* s.l and vice versa. This posed an important problem when dealing with large numbers of field samples for epidemiological studies. The

allozyme method can discriminate between the species but requires fresh or frozen samples which are often difficult to achieve in the field. Also, large numbers of loci are required for statistical significance and electrophoretic standards must be used.[86, 87, 93] To deal with this problem, a number of new DNA-based methods were developed.[94-97] Two of these methods are frequently used in epidemiological and vector biology studies.

Beebe and Saul developed a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method that detects all species except *An. rennellensis* and *An. klowi*.[96] This method is based on the PCR amplification of the ribosomal deoxyribonucleic acid (rDNA) transcriptional unit's internal transcribed spacer region II (*ITS2*), and digestion of the amplicons with *Msp*I restriction endonuclease into short DNA fragments. The amplified *ITS2* sequence is 750 base pairs (bp) long and is highly variable among the species.[96] The band pattern of the short DNA fragments visualized on a gel electrophoresis serves as the marker for identifying each of the species.[96] This method is cheap and robust and has significantly improved the epidemiological and biological studies of these important vectors.

Recently, Henry-Halldin and colleagues developed an advanced high throughput multiplex assay.[97] The method involves a PCR step, a ligase detection reaction (LDR) step, and a fluorescent microsphere assay (FMA) step. The PCR amplifies the *ITS2* locus of the rDNA using the same primers described by Beebe and Saul in their PCR-RFLP method. The amplicons are used in the LDR step where species-specific probes labeled with synthetic fluorescent dyes are

used to target species-specific polymorphism of the *ITS2* sequence. The LDR products are then used in the FMA step which reveals the species based on fluorescent signals.

1.4.2. Geographic distribution of the *Anopheles* mosquitoes of PNG

Description of the distribution of the *An. punctulatus* complex requires a geoclimatic description of PNG. Extending from the midland border of PNG and Irian Jaya in the west and running southeasterly across the country to Central province at the southeastern tip of PNG are massive mountain ranges which rise beyond 2400 m high. These ranges split the country into northern and southern plains (fig. 7). The northern plain (NP) consists mainly of lowland river valleys and flood plains of major rivers with several smaller scattered mountain ranges. The climate of the NP is continually hot and wet. The southern plain consists of two geoclimatic regions. These are a continually hot and wet lowlands (SL) that extends south from the foot of the mountain range, and further south is an open savannah monsoonal plains (SP).[98] Areas below 1300 m are considered lowlands. This includes the NP, SL and SP regions (fig. 7). Areas above 1300 m are considered highlands region (HR) (fig. 7).[98] The climate of HR is mostly wet with mild to cold temperature. Scattered over the north and eastern seas of PNG is a group of islands that constitute the Bismarck Archipelago. Further east of PNG is the Guadalcanal of Solomon Islands and south is Australia. The northern tip of Australia is an open monsoonal savannah region.[98]

The distribution of the *An. punctulatus* complex differs greatly (table 6). *An. farauti s.s* is found predominantly in coastal areas.[98] It is abundant in areas within 2 km from the coast and diminishes beyond this boundary. *An. hinesorum* is probably the most successful of all members

of the complex with regard to its dispersion. It is extensively distributed in both the coastal and inland lowland areas with a limited distribution in the highlands.[98] Although *An. farauti* No. 4 can be found occasionally on the coast, it is more restricted to the inland lowland areas of the NP.[98] *An. farauti* No. 5 and No. 6 are found only in the highlands region but with a sparse or limited distribution. *An. torresiensis* and *An. irenicus* have not been found in PNG. *An. koliensis* is widespread in the hot and wet climate of the northern plains of PNG but its distribution in the south is limited to the SL region.[98] *An. punctulatus* s.s is predominantly an inland species. Its distribution on the coast is limited in the NP.[98] *An*. sp. near *punctulatus* and *An. klowi* have limited and sparse distributions respectively and are found in the inland lowland areas of the NP and SL regions. *An. klowi* and *An. rennellensis* are rare species. The former has a sparse distribution in the inland areas of the NP but information on the later species is lacking.

An. bancrofti and *An. longirostris* are non members of the punctulatus complex. *An. bancrofti* is predominantly found in the inland lowlands of the NP.[99] They are occasionally found along the coast of the NP. *An. longirostris* is widespread throughout coastal and lowland areas of PNG.[99] However, both species have low densities compared to other species in the same populations.[100]

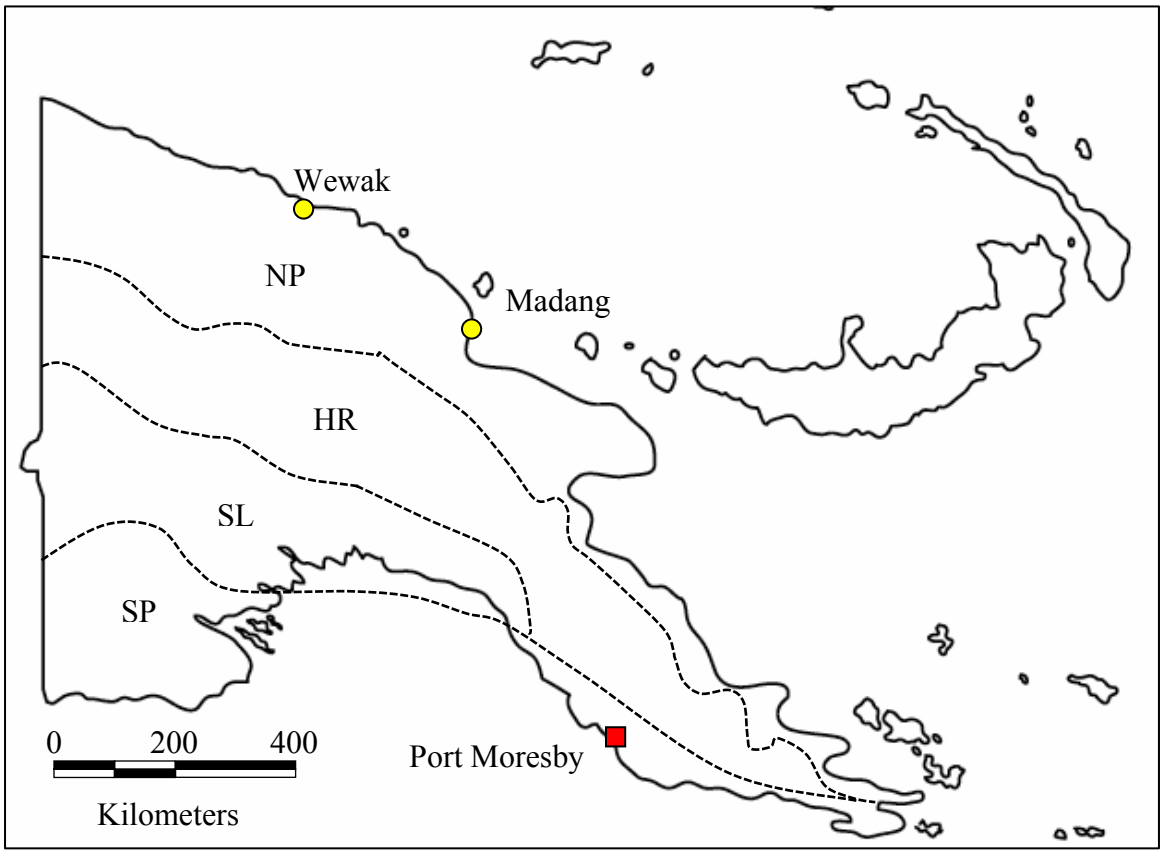

Figure 7. Generalized map of PNG showing the major geoclimatic regions separated by the dotted lines.

Table 6. Geographic distribution of the members of the *An. punctulatus* species complex (from Beebe et. al).[98]

	Papua New Guinea							**Solomon Island**		**Australia**	
	NP (hot and wet)		SL (hot and wet)		SP (monsoonal)		HR (mild)	GC		NA (monsoonal)	
Species	Coastal	Inland	Coastal	Inland	Coastal	Inland		Coastal	Inland	Coastal	Inland
An. farauti s.s	***		***		***	*		***		***	
An. hinesorum	*	**	***	***	**	***	**	***	***	**	**
An. torresiensis										**	***
An. farauti No. 4	*	**									
An. farauti No. 5							*				
An. farauti No. 6							**				
An. irenicus								***	***		
An. koliensis	***	**	**	**			*				
An. punctulatus s.s	**	***		***			*	*	**		
An. sp. near *punctulatus*		**		**							
An. klowi		*									

***, Extensive distribution; **, Limited distribution; *, sparse distribution
Major geoclimatic regions: NP, northern plain; SL, southern lowland; SP, southern plain; HR, highlands region; GC, Guadalcanal; NA, northern Australia

1.4.3. Ecology of the *Anopheles* vectors of PNG

Of the 12 species comprising the *An. punctulatus* complex, six are considered the major disease vectors. These are *An. farauti* s.s, *An. hinesorum*, *An. farauti* No. 4, *An. farauti No. 6, An. punctulatus*, and *An. koliensis.*[90, 102-104] The other six species are considered minor vectors. Two species outside of the *An. punctulatus* complex, *An. longirostris* and *An. bancrofti*, are also considered important vectors in some lowland areas of PNG.[102]

1.4.3.1. Larval habitats

The larvae of *An. farauti* s.s are usually found in brackish water habitats.[98, 105] This species has been shown to be salt-water tolerant.[106] But it can also breed in freshwater habitats near the coast such as permanent swamps and temporary pools.[105] Information on the larval habitats of *An. hinesorum* and *An. farauti* No. 6 is lacking. However, based on their geographical distribution, *An. hinesorum* is probably non-specific in its habitat preference and breeds in any suitable freshwater bodies while *An. farauti* No. 6 is highly specific.[98] Larvae of *An. farauti* No. 4 have been collected from shallow temporary water puddles on the ground along the flood plains of the Ramu River in the NP (PNG IMR unpublished data). However, more work is needed to characterize the larval habitats of this species. *An. punctulatus* s.s prefers shallow water bodies that are open to direct sunlight. This species is quick to colonize transient water puddles shortly after they are formed, especially those that are formed as a result of human activities such as logging, mining, road constructions, or gardening.[105] They also breed in sunlit water formed by natural means such as stagnant puddles along the edges of rivers. *An. koliensis*

usually breeds in shallow temporary pools in grasslands and in pools around the edges of forests.[105] *An. bancrofti* prefers breeding in large water bodies such as large ponds and swamps while information is lacking for *An. longirostris* breeding habitats.[99]

1.4.3.2. Host preference

A number of studies have been performed that tried to determine the host preference of the major disease vectors of PNG based on the human blood index (HBI).[84, 107-110] HBI is the ratio of the number of human-fed mosquitoes to total blood mosquitoes. However, these studies were conducted before robust species-diagnostic techniques were made available and were based on the three morphological species (*An. farauti* s.l, *An. punctulatus*, and *An. koliensis*). Although their results show varying levels of HBI between mosquito populations of the same species, the HBI of most populations is higher than the blood indexes for the other hosts. The authors ascribed the variation in HBI between the mosquito populations to the availability of alternative host species. Even though all species have higher HBI compared to the blood index for other hosts, some vector species have more affinity for humans than others. *An. punctulatus* is more anthropophilic than *An. koliensis* and *An. farauti* s.l.[108] However, there are conflicting results for *An. koliensis* and *An. farauti* s.l.[111] This leads some authors to conclude that the major malaria vectors of PNG are more anthropophagic but feeding on humans is reduced dramatically by the presence of alternative hosts.[101] Others ascribed the HBI variations to other factors such as availability of bednets and claim that dogs are the preferred hosts for the three vectors compared to human.[112] *An. longirostris* and *An. bancrofti* are considered zoophilic.[84, 99] Interestingly,

some populations of *An. longirostris* show high prevalence of sporozoites infections.[102] Thus, more work needs to be done on the host preference of PNG vectors.

1.4.3.3. Host biting cycles

The biting cycles of the three morphological species have been observed for populations in the Madang area. *An. farauti* s.l tends to bite during the early hours of the evening with peak biting time occurring before midnight.[105] *An. koliensis* and *An. punctulatus* tend to bite after midnight with peak activity occurring in the early hours of the morning.[105] This information is from studies in the early 1980's when the molecular species-diagnostic methods were unavailable. A more recent study confirmed the biting cycle of *An. punctulatus* as reported previously.[90] However, there were three genotypic variants of *An. koliensis* that had different biting cycles. One variant had a late biting cycle similar to the *An. punctulatus*. The other two had an early biting cycle between 6pm and midnight but their peak biting time differs.[90] *An. farauti* s.s had a uniform biting activity from evening to morning. *An. hinesorum*, and *An. farauti* No. 4 tended to bite earlier in the evening but each species showed a slightly different peak biting times.[90] Such variations in the biting cycles could potentially undermine the outcome of LLIN campaign (see section 1.5.2) as mosquitoes are biting during the periods when people are not under their bednets. In many rural parts of PNG, some people go to sleep very late in the night and some get up in the early hours of the morning to prepare for the activities of the new day. Active transmission can occur during those periods when people are awake.

1.5. MOSQUITO-BORNE DISEASES IN PNG

1.5.1. Epidemiology and transmission

PNG is strikingly diverse in its geography, ecology, and human biology. Except the southern savannah plain which shows monsoonal variation, most parts of the lowland PNG is continually hot and wet with rainfall ranging from 1300 to 7000 mm annually with some rains throughout the year.[101] Because PNG is situated close to the equator, temperature is generally stable throughout the year and differs only with respect to altitude. Temperature range from 30°C at sea level and drops with increasing altitude to as low as 0°C beyond 2400 m above sea level. The entire landscape of PNG consists of a patchwork of many different ecological zones clumped within a 450000 km^2. These ecological zones are represented in the sea by coral reefs, offshore atolls and islands. On the coast are swamps, marshes, mangroves, lakes, grasslands, and plains. Further inland are dense rainforests, mountains, ranges, plateaus and valleys. Cutting across this land divide are fast-flowing rivers with flood plains and deltas that open into the sea. Not only are these ecological zones inhabited by species-rich communities of flora and fauna that form complex ecosystems, but human populations of exceptionally diverse cultural and linguistic groups. Human populations are concentrated in the highland areas above 1300 m and in the coastal plains below 600 m with sparse distribution in the intermediate zones.[101] This environmental and cultural diversity is reflected in the complex variation in the vector ecology (section 1.4.3) and disease epidemiology (section 1.5.1).

The most common mosquito-borne diseases in PNG are malaria and filariasis. Dengue fever has been shown to be prevalent in some parts of PNG however, its epidemiology is less understood

compared to malaria and bancroftian filariasis.[113] Only recently in 2012, cases of chikungunya were reported in some parts of PNG. However, little is known about its epidemiology.[114]

1.5.1.1. Malaria

Malaria endemicity and transmission varies geographically throughout PNG. Transmission is intense and perennially stable in most of the hot and wet lowland areas below 1300 m. As a result, these regions are holoendemic. In this endemicity, all age groups are equally exposed but children are more susceptible to infection and clinical complications than adults. Transmission is seasonal and less intense in the southern monsoonal plain (fig.7).[101] As a result, this region is mesoendemic.[101] In this endemicity, infection is tolerated well in adults than children. In the highlands above 1600 m, temperature is too low for transmission to occur. At the intermediate altitudes (>1300 m and <1600 m) where bulk of the highland populations inhabit, malaria transmission is unstable and prone to epidemics.[101, 115] This region is hypoendemic with occasional severe outbreaks clinically affecting all age groups.[101] Most of the islands are mesoendemic with unstable less intense transmission.[101] Within each of these relatively large geographic regions there is fine scale spatial heterogeneity of malaria endemicity at the village or even household cluster level.[116, 117] Malaria in the coastal and island areas is mostly transmitted by *An. farauti* s.s, *An. hinesorum*, and *An. koliensis*.[90, 98, 102] In the inland lowlands *An. punctulatus*, *An. hinesorum*, *An. koliensis* and *An. farauti* No. 4 are responsible for most of the

transmission.[98, 102, 118] Transmission in the highlands is mostly perpetrated by *An. hinesorum* and *An. farauti* No. 6.[98, 102, 118]

Four of the five human malaria species, *P. falciparum* (Pf), *P. vivax* (Pv), *P. ovale* (Po) and *P. malariae* (Pm), are present in PNG.[101, 119] Pf and Pv are widely distributed in PNG. These two species are the main causes of morbidity and hospital admissions in PNG.[101, 119, 120] Pf is generally the most predominant species followed by Pv. Recent survey in a holoendemic lowland population of PNG reveals as high as 55% and 35.7% blood-stage infection prevalence (based on molecular assays) by Pf and Pv respectively.[117] In the intermediate altitudes low-level transmission of Pv occurs but epidemic outbreaks is more associated with Pf.[115] An investigation of malaria outbreak in the highlands of PNG in 2005 reveals that of the 29% infection prevalence, Pf accounted for 59% and Pv 34% of all identified infections.[121] Although recent studies show some evidence for association of severe malaria with Pv infections, most studies in PNG show results that conform to the central dogma in malariology which states that Pf is clinically severe and life-threatening while Pv tends to be mild.[122-124] Furthermore, the frequency of Pv-associated severe malaria observed in PNG is very low compared to Pf-associated severe malaria in the same study populations.[122, 124] Morbidity is high among children than adults in the holoendemic regions.[101] Interestingly, while most of the features of severe falciparum malaria in PNG children are comparable to other areas of the world that have comparable endemicity, mortality rate is very low (>1%) in PNG.[124, 125] Some authors believe

that this observation is due to the acquisition of cross-species protective immunity from greater exposure to Pv in early childhood.[101, 124]

Unlike Pf and Pv, Pm has a patchy distribution with human blood-stage infection rate of 13% in some PNG populations.[101, 117] However, its health and epidemiological significance in PNG is not known due to limited studies on this species.[101] Po is detected occasionally with human blood-stage infection rate below 4.8%.[117] Like Pm, its epidemiological significance is not understood due to limited studies.[101, 119, 120] Sympatric combinations of the four *Plasmodium* species in a human population as well as mixed-infections in an individual are common phenomena in PNG, making it a prime location to study *Plasmodium* parasites interactions.[115, 117, 119, 120]

1.5.1.2. Bancroftian filariasis

Bancroftian filariasis, one of the world's most debilitating diseases, is an important public health problem in PNG. Studies since 1980's show 16 out of 20 provinces to be endemic with local prevalence of microfilariae infection rate ranging from 10% to 92%.[126, 127] Studies in the late 1990's and early 2000's showed that approximately one million residents of PNG were infected and an additional three million people estimated to be at risk of infection.[126, 128] Clinical manifestations (body disfigurements) in the form of lymphedema of the leg and hydrocele were also prevalent in PNG.[126, 129] Although *Culex* and *Aedes* species that transmit bancroftian

filariasis in other parts of the world are present abundantly in PNG, transmission in PNG is perpetrated by the same *Anopheles* vectors that transmit malaria.[103, 126, 130]

The epidemiological significance of this disease in PNG is reflected in the PNG's participation in the Global Program to Eliminate Lymphatic Filariasis (GPELP) which began in 2000. The goal of this global project is to eliminate lymphatic filariasis by the year 2020 using a strategy of preventative chemotherapy.[131] In this case, it involves mass administration of ivermectin, diethylcarbamazine and albendazole over a period of five years to reduce the reservoir of microfilariae in the blood to a level that is insufficient to maintain transmission by the mosquito vector. Although studies show a decline in the prevalence of human infections, cases of clinical manifestations, and transmission rate after the implementation of the GPELP in PNG, the goal of the program is yet to be achieved in PNG.[131, 132] The program was implemented as pilot projects in focal areas of PNG but country-wide intervention is yet to be implemented.[131, 133] Thus, bancroftian filariasis is still a major public health problem in PNG.

1.5.2. Vector-based disease control in PNG

Many parts of the world are depending on vector control methods and in PNG insecticide-based vector control is recommended by public health authorities. Although large scale DDT-based indoor residual spraying was administered in PNG during 1950's - 1980's, currently pyrethroid-based residual spray is practiced only locally in epidemic prone sites and controlled industrial sites such as mining.[134, 135] The use of insecticide-treated bednets (ITN) was recommended for large scale vector control interventions in PNG since 1987.[134] However, coverage remained low

and patchy in most parts of PNG.[134] It was not until 2004 when large scale distribution campaign began.[134] The program was funded by the Global Fund and administered jointly by the PNG National Department of Health, Rotary Against Malaria and provincial and district health authorities.[134, 136] Over two million long-lasting insecticide-treated nets (LLIN) have been freely distributed throughout PNG between 2004 and 2009.[136] Unfortunately, the goal of the LLIN campaign to reduce the burden of malaria by achieving 80% ownership and usage of LLIN was not achieved. Ownership and usage were 64.6% and 39.5% respectively.[136] However, these figures show an increase in LLIN ownership and usage compared to pre-LLIN campaign where they were below 10%.[136] Hetzel and others listed the factors that prevented the LLIN campaign from achieving 80% ownership and usage.[136]

A number of studies before and during the LLIN campaign have been published. In both Madang and Sepik provinces where transmission is perennially intense, malaria infection prevalence prior to the LLIN campaign ranged from 20-86% (based on molecular diagnostic techniques).[116, 120] Entomological inoculation rate in Madang ranged from 9-526 infective bites per person per year.[104] The mf infection prevalence pre-LLIN campaign in the East Sepik ranged from 30-68% and the annual infective biting rate ranged from 15-836 infective bites per person per year.[129, 137,103] Although there is no data directly comparing malaria infection prevalence during the LLIN campaign to pre-LLIN campaign, one study show that infection prevalence decreases with increasing LLIN coverage in the East Sepik.[117] However, malaria transmission data during

LLIN campaign is lacking. There is evidence that WB infection rate in both human and mosquitoes declined during the LLIN campaign period. However, this observation is attributed to the effects of mass drug administration campaign instead of LLIN campaign.[132] Studies assessing the impact on the epidemiology and transmission post-LLIN campaign are ongoing.

CHAPTER 2: RESEARCH RATIONALES, OBJECTIVES AND HYPOTHESES

2.1. WHY DO WE NEED TO KNOW MORE ABOUT THE HOST-FEEDING ECOLOGY OF MOSQUITO VECTORS?

Maintenance of vector-borne pathogens in natural cycles depends on the interactions of competent arthropod vectors and their primary vertebrate hosts. Blood feeding by a mosquito vector on human hosts is an important interaction as it is through this process that pathogens are acquired or transmitted between both organisms. Although pathogen exchange occurs during the feeding process, the vector status and disease transmission dynamics depend on the mosquito's host species preferences and feeding patterns.

2.1.1. Factors affecting the host species choice and feeding patterns

Non-random host selection pattern can be explained by physiological, biological, and ecological factors that determines a mosquito's host preference.[138, 139] Nutritional reward and corresponding fitness (number of offsprings produced after obtaining a blood meal) from feeding on different hosts is one important physiological factor that determines host preference by mosquitoes. Mosquitoes tend to feed more on blood sources that are nutritive.[140] They may also prefer blood that is easily digested without expending a lot of energy.[141] When the nutritional value of blood from different host sources is similar, choices may be made based on energy expenditure.[141] Vertebrate hosts also mount immune responses to the saliva of arthropod vectors.[142] This physiological response can impede mosquito's blood feeding success and consequently, affect its reproductive success.[142] Hence, variation in the immunocompetence

among vertebrate host species may result in the preference for low-response hosts by mosquitoes.

Biological factors that influence host selection by mosquitoes include host body-size, anti-mosquito behavior, and body odors. A number of studies have shown that some mosquitoes prefer vertebrate hosts that have a larger body size. Assertions were made that preference for larger hosts may be related to increased probability of contact due to larger body surface area.[143, 144] However, these studies focused on intraspecific differential feeding where comparisons were made between individuals of the same host species.[143, 144] Although evidence is yet to be provided for interspecific differential feeding, it is possible that mosquitoes may prefer host species that have larger body size than smaller species. Hence, variation in the body-size among host species may result in the preference for relatively larger host species. Some host species exhibit anti-mosquito behaviors against mosquitoes that try to feed on them.[145, 146] Such behaviors reduce the probability of contact and the mosquitoes' feeding success.[138, 146] Variation in the level of defensive behaviors among host species may result in the preference for low-response species. Some mosquitoes are more attracted to hosts that emanate certain body orders.[147-149] For example, *An. gambiae* was more attracted to human odor than that of a cow.[147, 149] There may be many reasons for such choice but one explanation could be that certain chemical volatiles in the odors are associated with the quality of blood preferred by the mosquito.

Although the physiological and biological factors described above may cause mosquitoes to prefer a certain host, ecological factors such as the availability and distribution of the preferred as well as alternative hosts may affect the way they actually feed. If there is spatial heterogeneity in the distribution of the preferred host species, the advantage of searching and feeding on that host is traded off against the amount of energy expended in search of the desired hosts.[138] If there is temporal heterogeneity in the availability of the preferred host species, the advantage of waiting to feed on the desired host is traded off against the risk of death before feeding.[138] Likewise, reduction in the availability of desired hosts due to other factors such as bednets or screened houses may results in the diversion of feeding on human to domestic animals. Thus, whether a mosquito species or a vector population exhibits a specialist (feeding only on a few specific hosts) or generalist (feeding on a wide range of hosts) behavior depends on their history of optimal foraging. Generalism should evolve in environments where the chances of feeding on optimal host are very low and the cost of waiting or searching for the host is higher than the benefits. Specialism on the other hand should arise in environments where the chances of feeding on optimal hosts are high.[138]

2.1.2. Epidemiological significance of host-feeding pattern

Even if a mosquito species (or a population of mosquitoes) is competent in supporting the development of pathogens to the infective stage and is abundant around human communities, whether it is regarded as a primary or secondary vector of human disease depends largely on the degree of anthropophagy (tendency to feed more on human blood) it displays.[150] Anthropophilic mosquitoes tend to be anthropophagous compared to zoophilic mosquitoes. As a result most

anthropophilic mosquitoes are primary vectors.[151, 152] However, the degree of anthropophagy depends largely on whether a species or a mosquito population display a generalist or specialist host feeding behavior.

Anthropophilic mosquitoes that highly specialize on human blood are serious vectors as the presence of other hosts will not have an impact on them. Human must depend on vector control strategies such as insecticide spray and environmental management to reduce the number of mosquitoes in the environment. On the other hand, the vector status of anthropophilic mosquitoes that are generalist in their feeding habits may depend on the present state of the environment. For example, when alternative domestic hosts are abundantly available, blood feeding is distributed among the hosts and the frequency of anthropophagy is reduced.[138] The frequency of human feeds may be reduced when vector control methods such as zooprophylaxis and bednets diverts the vectors to feed on alternative hosts or reduces the availability of human hosts.[153] Even generalist zoophilic mosquitoes may occasionally display an anthropophagous behavior when conditions (such as reduction in the number or availability of their primary host) favors a switch in their feeding pattern.[151] In general, spatial and temporal heterogeneity in the distribution and availability of alternative hosts may affect the feeding patterns of generalist feeders and ultimately influence disease transmission dynamics.[138]

2.2. WHY DO WE NEED TO KNOW MORE ABOUT THE HOST-FEEDING ECOLOGY OF PNG VECTORS?

Current knowledge of the host feeding ecology of the anopheline vectors of PNG based on past studies is briefly discussed in section 1.4.3.2. However, the information that is available is insufficient to characterize the host feeding ecology of the local PNG vectors for a number of reasons. Firstly, the information is based on past studies (the most recent of all was conducted in 1988) when the species status of the *An. punctulatus* species complex was not completely characterized and the species-diagnostic methods were unavailable. As a result these studies were mostly based on the three morphological species *An. farauti* s.l, *An. koliensis*, and *An. punctulatus*. It is now known that *An. farauti* s.l is a sub-complex and consists of seven reproductively isolated species that are morphologically identical and are distinguishable only through molecular methods (1.4.1.3 and 1.4.1.4). Overlapping morphological characteristics between the three species results in field samples identified wrongly. *An. koliensis* has frequently been wrongly identified as *An. punctulatus* or *An. farauti* s.l and vice versa (PNGIMR entomologists, personal communications).

The consideration of members of the farauti sub-complex as one species and the frequent misidentification of one species for the other based on morphology can have serious consequences. The variations in the HBI observed between different populations of the same species (see section 1.4.3.2) may have been due to the confounding effects of multiple species that were considered as one. For example, *An. farauti* s.s and *An. hinesorum* coexists in coastal areas. They may occupy different ecological niches but because they were considered one species, their host-feeding data were pooled and hence, produced bias results. Similarly, the misidentification of one species for another can bias the results.

Secondly, most of the host-feeding ecology studies were conducted in selected populations, mostly in convenient villages near Madang town.[84, 108] It is improper to generalize the host feeding behavior of a vector species based on limited informations obtained from only a few similar populations. This argument is made because of the great ecological heterogeneity between vector populations even between those that are only a few kilometers apart.[111] It would be appropriate to characterize the host feeding pattern of a vector species at the population level.

Thirdly, almost all of the studies on host feeding ecology conducted in PNG focused on interspecific host preference by mosquito vectors. Information on intraspecific host preference is lacking. Intraspecific host preference refers to the tendency to feed more on a particular group of individuals than other members within the same species. For example some mosquitoes feed more frequently on gametocyte-infected individuals than people that do not carry gametocytes.[154] Given it epidemiological significance, information on such discriminatory feeding behavior is needed for PNG vectors.[154]

Fourthly, unbiased estimates, such as the HBI, of the host feeding preference depend on the methods used to collect engorged mosquitoes.[155] Although there are a number of methods that are used to collect engorged mosquitoes, two common methods used in PNG were indoor and outdoor resting collections.[84, 108] Collections of large numbers of engorged endophilic mosquitoes that rest for prolonged periods inside houses are relatively easy and straight forward.

However, such collection is biased towards human-fed mosquitoes and ignores the portion that has fed on other hosts. Consequently, analyses based solely on such collections tend to bias the results. On the other hand, outdoor collection of engorged mosquitoes can be challenging as exophilic mosquitoes disperse widely and tend to utilize a wide range of resting sites. This challenge is magnified in tropical environments like PNG and when the number of blood-fed mosquitoes is limited as it can be laborious and time consuming to search all potential resting sites.[84, 108, 155]

Finally, we do not know enough to determine whether a vector species or a population of mosquitoes in PNG is generalist or specialist in its host feeding behavior. The importance of these behaviors is described in section 2.1.2. In light of the ongoing mass distribution of LLIN (see section 1.5.2) and the potential for implementation of zooprophylactic method in PNG, such feeding behavior must be determined.

2.3. RESEARCH OBJECTIVES AND HYPOTHESES

Section 2.2 discusses some of the gaps in the current knowledge of the host feeding ecology of the anopheline vectors of PNG and provides justifications for why more work needs to be done in this important area of vector ecology. This study was therefore proposed to address some of these needs by targeting two broad objectives. The first objective was to determine the efficacy of a novel mosquito sampling technique: barrier screens. This sampling technique was developed to address the limitations faced by outdoor resting collection of engorged exophilic mosquitoes (see section 2.2). It has not been tested elsewhere except once in PNG and Solomon Island.[156] However, its efficacy with regard to host preference studies is yet to be evaluated. This objective

is based on the hypothesis that barrier screen is more robust and unbias in sampling blood-fed mosquitoes in PNG settings compared to the indoor and outdoor resting collection methods. The second objective of this study was to determine the host feeding preference of the anopheline vectors in different populations and compare the findings with the findings of past studies. This objective was based on the hypothesis that all species of the anopheline vectors (both primary and secondary vectors) in PNG are generalist feeders but their degree of anthropophagy differs.

CHAPTER 3: METHODOLOGY

3.1. STUDY LOCATIONS

Field study was conducted during the period between June and August of 2012 in five rural tropical villages in the Madang Province of PNG. Four villages Matukar (S 04° 53.788'; E 145° 47.147'), Mirap (S 04° 45.3'; E 145° 40.0'), Wasab (S 04° 53.3'; E 145° 45.5') and Dimer (S 04° 47.8'; E 145° 37.4') are located in the Sumkar District. Mirap and Matukar are situated right on the coastline, approximately 21 km from each other. Both villages share similar environmental features that are typical of tropical coastal areas (fig. 8). They represent the coastal populations. Wasab and Dimer are located approximately 3 to 4 km directly inland from Matukar and Mirap respectively. Both villages are slightly elevated on hilly areas and share similar environmental features that are typical of tropical rainforests (fig. 9). They represent the inland populations. The fifth village Kokofine (S 05° 41.9'; E 145° 28.9') is located in the Usino-Bundi District. This village is located further inland. It is situated on the lowland flood plain of the Ramu River. The vegetation is mostly grassland and swamps (fig. 10). All five villages are holoendemic with intense malaria transmission all year round.

Figure 8. Photos showing the vegetation type and typical village setting of the two coastal villages. A: Coast of Mirap village; B: coastal swamp in Mirap; C: Betelnut palm plantation; D: A hamlet in Matukar. (Photographs taken by the author)

Figure 9. Photos showing the vegetation type and typical village setting of the two inland villages. A: Rainforest canopies in Dimer; B: A hamlet in Wasab. (Photographs taken by the author)

Figure 10. Photos showing the vegetation type and typical village setting of Kokofine. A: Grassland on the road to Kokofine; B: A hamlet in Kokofine. (Photographs taken by the author)

3.2. MOSQUITO SAMPLING

Mosquitoes were sampled using the novel barrier screen technique described by Burkot and others.[156] A 2 meters (m) high and 20 m long green polyethylene shade-cloth (70% shading) was erected vertically on wooden poles about 20 centimeters (cm) above the ground (fig. 11A). The screen was constructed at the edge of village hamlets between the hamlets and potential resting or oviposition sites in the environment. The screen was positioned perpendicular to the suspected flight paths, intercepting both host-seeking mosquitoes that are coming into the village and the blood-fed mosquitoes that are exiting the village. Mosquitoes were collected as they rest temporarily on the shade-cloth on their way into or out of the village (fig. 11C).

Mosquitoes were collected all through the night from 6:00 pm to 6:00 am. There were four collectors per barrier screen. Two collectors worked from 6:00 pm to 12:00 am before they were replaced by the other two who worked from midnight to 6:00 am. The collectors were sited approximately 30 m from the barrier screen and visited the screen every 15 minutes to collect the resting mosquitoes. Mosquitoes were manually searched with the aid of a flash light and

collected using a mouth aspirator. Mosquitoes were placed in collection cups pre-labeled according to the hour and the side of fence (i.e. side facing the bush or the village) that the mosquitoes were collected.

Anopheles mosquitoes that were collected during the night were killed by exposing them to chloroform fumes. Only the females were identified into their morphological species with the aid of a light microscope. Males were discarded. Mosquito were identified as *An. punctulatus* Donitz, *An. farauti* Laveran, or *An. koliensis* Owen using the morphological criteria described in section 1.4.1.1. *An. longirostris* Brug and *An. bancrofti* Giles were also morphologically identified based on published criteria.[157] Mosquitoes were then stored singly in a 1.5 milliliters (mL) microcentrifuge tubes (Fisher Scientific, Catalog Number 05-408-129) pre-packed with silica gels (desiccant) and cotton. Each mosquito was given a unique sample identification number which was written on the tube it was stored. All informations about the mosquito (e.g. morphological species, engorgement status, the village it was collected, which side of fence it was collected, hour of collection, and date it was collected) were written next to the mosquito identification number on a field form. With the aid of a light microscope, a mosquito was categorized as unfed, half-fed (if $\leq$ 50% of its abdomen was filled with fresh blood), or fully-fed (if > 50% of its abdomen was filled with fresh blood). Mosquitoes were brought to the laboratories and stored at room temperature. The data on the field forms were transferred to an excel spreadsheet on a computer.

Figure 11. Photos showing the barrier screen sampling method. A: a barrier screen that was just constructed perpendicular to the suspected flight path of mosquitoes at the edge of Matukar village; B: a barrier screen with a hamlet in the background in Wasab; C: a mosquito collector collecting mosquitoes using a mouth aspirator aided by a flashlight in Kokofine; D: an entomology field technician identifying the mosquitoes into their morphological species. (Photographs taken by the author)

3.3. EXTRACTION OF GENOMIC DNA FROM MOSQUITOES AND HOST-BLOOD TISSUES

Blood-fed mosquitoes were bisected before DNA was extracted. Each mosquito was removed from its storage tube with a forcep and placed on a microscope slide. Using a scalpel blade, each mosquito was cut into two segments (abdomen and head-thorax) by cutting along the region where the abdomen and the thorax meet. The two segments were transferred to separate tubes which were then labeled according to the mosquito sample number and the corresponding

segment. The tubes were stored in a -20 °C freezer. All bisections were done using sterile techniques under a fume hood. A new sterile microscope slide and sterile scalpel blade was used for each mosquito. The forcep was dipped into DNAaway solution (ThermoScientific, Catalog Number 7010-SDG) and wiped dry before it was used on a new mosquito. Gloves were worn during the bisection process.

Genomic DNA was extracted from blood-fed abdomens of *Anopheles* mosquitoes using DNeasy Blood & Tissue Kit (Qiagen, Catalog Number 69506). Each abdomen was placed in a pre-labeled 2.0 mL microcentrifuge tube (Eppendorf, Catalog Number 022363352) followed by a 5 millimeters (mm) stainless steel bead (Qiagen, Catalog Number 69989) and 180 microliters (µL) of phosphate buffer solution (50 mM potassium phosphate and 150 mM sodium chloride, pH 7.2). Samples were homogenized at 15 hertz for 1.5 minutes on an electric homogenizer (Qiagen Tissue Lyser II, Catalog Number 85300). The homogenates were transferred to a new set of pre-labeled 1.5 mL microcentrifuge tubes followed by 20 µL of proteinase K enzyme and 200 µL of lysis buffer AL. After briefly mixing the content of the tubes on a vortex to achieve a homogeneous mixture, the tubes were incubated at 56 °C for one hour on a heat block or a water bath. After the incubation, 200 µL of 100% ethanol was added to each tube and the solutions were mixed thoroughly on the vortex. A corresponding number of spin columns were labeled and each column was placed on a 2 mL collection tube. Solutions were transferred into their appropriate spin columns and were centrifuged for one minute at 8000 rotations per minute (rpm). The used collection tubes were discarded and the spin columns where transferred to a second set of collection tubes. DNA's were washed by adding 500 µL of wash buffer AW1 into each spin column and then centrifuged for one minute at 8000 rpm. The spin columns were

transferred to a third set of collection tubes, 500 µL of wash buffer AW2 was added to each tube, and the tubes were centrifuged for 3 minutes at 14000 rpm. The spin columns were transferred to a new set of pre-labeled 1.5 mL microcentrifuge tube and 100 µL of elution buffer AE was added to each tube. DNA's were collected in the microcentrifuge tubes by centrifuging for one minute at 8000 rpm. Spin columns were discarded and the DNA's were stored at -80 °C.

3.4. *ANOPHELES* SPECIES DIAGNOSTICS

3.4.1. ITS2 PCR amplification

Members of the *An. punctulatus* species complex were identified using the PCR-RFLP method discussed in section 1.4.1.4. PCR amplification of the 750 bp *ITS2* gene locus was performed by adding 2 µL of extracted DNA to a reaction mixture (25 µL final volume) containing 67 mM Tris-HCl, pH 8.8, 6.7 mM $MgSO_4$, 16.6 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, 100 mM of each of the four deoxynucleotide triphosphate (dNTP), 2.5 units of thermostable DNA polymerase, and 10 µM of forward (ITS2A; 5'-TGT GAA CTG CAG GAC ACA T-3') and reverse (ITS2B; 5'- TAT GCT TAA ATT CAG GGG GT-3') primers. The reaction was carried out on a thermal cycler using a program that involved an initial denaturation at 95 °C for 2 minutes, followed by 35 cycles of 95 °C for 30 seconds (denaturation), 55 °C for 30 seconds (annealing), and 72 °C for 1 minute (extension), followed by a final extension at 72 °C for 4 minutes.

3.4.2. PCR product digestion and visualization

Each of the ITS2 PCR products was further digested using a restriction endonuclease. Ten µL aliquot of the PCR product was added to 7.75 µL water, 2.0 µL 1X NE Buffer 4 (New England

Biolabs, Catalog Number B7004S) and 0.25 µL *Msp*I restriction enzyme (20000 units/mL; New England Biolabs, Catalog number R0106L) in a 0.2 mL PCR tube (Bio-Rad, Catalog Number TWI 0201) to a final volume of 20 µL. Samples were digested at 37 °C for 3 hours in a water bath. Eight µL of each of the digested products was mixed with 3 µL of 6X gel loading dye (New England Biolabs, Catalog Number B7021S) and were ran at 100 volts for 30 minutes on a 2% agarose gel (Bioline, Catalog Number BIO-41025) containing 0.5 micrograms per mL of ethidium bromide. The gel was visualized at 312 nm on an ultraviolet transilluminator (Bio-Rad, Catalog Number 170-8195). Mosquitoes were identified to their species based on the banding patterns as shown in figure 12.

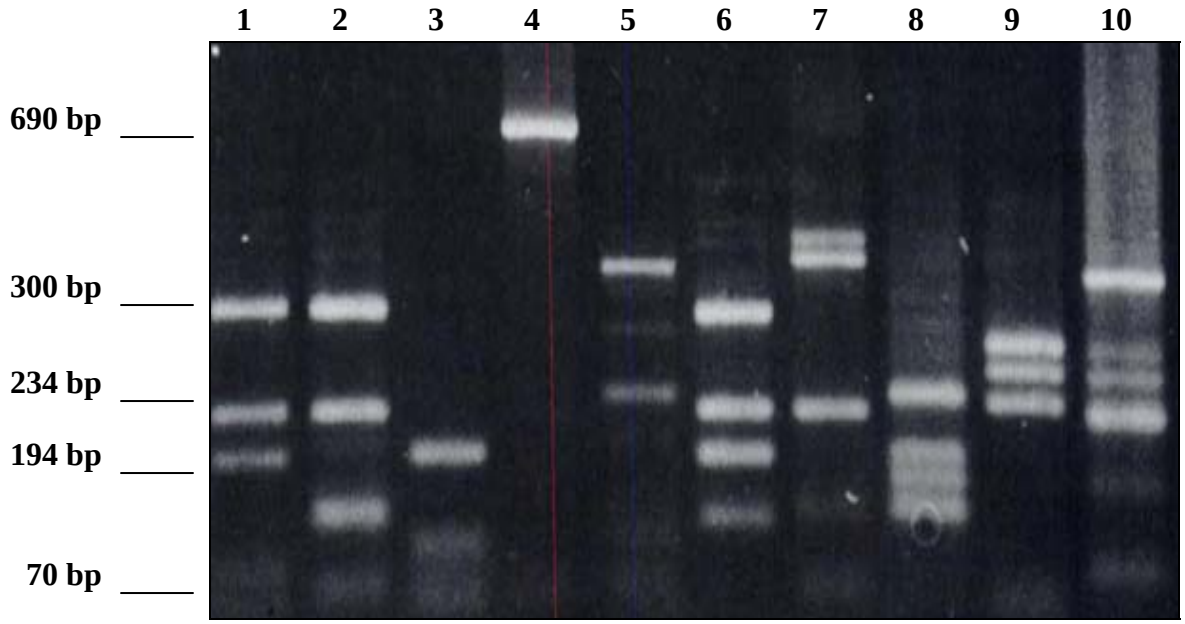

Figure 12. A gel photo showing the RFLP banding patterns for each of 10 members of the *An. punctulatus* species complex. Lanes 1: *An. farauti* s.s; 2: *An. hinesorum*; 3: *An. torresiensis*; 4: *An. farauti* No. 4; 5: *An. farauti* No. 5; 6: *An. farauti* No. 6; 7: *An. irenicus*; 8: *An. koliensis*; 9: *An. punctulatus*; 10: *An*. sp. near *punctulatus*. (Adapted from Beebe et. al)[96]

3.5. BLOOD MEAL ANALYSIS

3.5.1. Cytochrome B PCR

The first step in the identification of the host blood source was a PCR amplification of a 344 bp region of the mitochondrial cytochrome B gene of the vertebrate hosts using a primer pair described by Hamer and others.[158] The PCR amplification was performed using ready-made PCR reagents (Failsafe PCR System; Epicentre Biotechnologies). A 50 µL final reaction volume was prepared by adding 21.30 µL of water, 23 µL of 2X Premix E (Epicentre Biotechnologies, Catalog Number FSP995E), 1.6 µL of each of the 10 µM forward (BM1; 5′-CCC CTC AGA ATG ATA TTT GTC CTC A-3′) and reverse (BM2; 5′-CCA TCC AAC ATC TCA GCA TGA TGA AA-3′) primers, 0.5 µL of thermostable DNA polymerase (Epicentre Biotechnologies, Catalog Number FS9901K), and 2 µL of the extracted DNA to a 0.2 mL PCR tube. The PCR reaction was carried out on a thermal cycler using a program that involved an initial denaturation at 95 °C for 30 seconds, followed by 36 cycles consisting of 95 °C for 30 seconds (denaturation), 60 °C for 50 seconds (annealing), and 72 °C for 40 seconds (extension), followed by a final extension at 72 °C for 5 minutes. Amplified products were then ran on an agarose gel and visualized on an ultraviolet transilluminator as described in section 3.2.2.2.

3.5.2. DNA purification, sequencing and gene bank search

Samples that yielded a PCR-positive result were further purified using QIAquick PCR Purification kit (Qiagen, Catalog Number 28106). Forty µL of each of the PCR products was thoroughly mixed with 200 µL of binding buffer PB in a 1.5 mL microcentrifuge tube and then transferred into appropriate spin columns. After centrifuging at 10400 rpm for one minute, the liquid flow-through was discarded and the spin columns were replaced on the same collection

tubes. Seven hundred µL of wash buffer PE was added to each of the spin columns and then centrifuged at the same condition as above. After discarding the liquid flow-through, the spin columns were replaced on the collection tubes and were centrifuged again at the same condition as above. The spin columns were then transferred onto appropriate pre-labeled 1.5 mL microcentrifuge tubes and 30 µL of water was added to each spin column. A final centrifugation was performed at the same condition as above. The spin columns were discarded and the microcentrifuge tubes containing the purified DNA were stored at -80 °C.

The purified amplicons were further sequenced. An 11 µL final reaction volume was prepared for each of the samples by adding 10 µL of water, 1 µL of the 10 µM forward primer (BM1), and 2 µL of the purified DNA (10-40 ng/ µL) into a 0.2 mL PCR tube. Nucleotide sequences for each of the samples were obtained by direct sequencing method (ABI Prism 3700 DNA Analyzer; Applied Biosystems) following the manufacturer's protocol. Samples that yielded good quality sequences were subjected to BLAST search in GenBank (http://www.ncbi.nlm.nih.gov/blast/) and returns that showed ≥ 99% sequence match were accepted as the likely vertebrate host that the mosquito had fed on.

3.6. STATISTICAL ANALYSES

The field and laboratory data were recorded on a Microsoft Excel spreadsheet and saved as comma separated value (CSV) file. The dataset was read into R statistical software (R-Software version 3.0.1) and analyzed statistically. The data were read into R as factor data-type and analyzed as categorical data. Chi-squared tests of proportions were performed to test for statistical difference between groups.

CHAPTER 4: RESULTS AND DISCUSSIONS

4.1. RESULTS

4.1.1. Species composition

A total of 3628 female anophelines were caught in the five villages during the sampling period. Of these, 2971 (81.9%) were morphologically identified as *An. farauti* s.l, 374 (10.3%) as *An. punctulatus*, 180 (5.0%) as *An. longirostris*, 84 (2.3%) as *An. bancrofti*, and 19 (0.5%) as *An. koliensis*. The species composition and density vary within and among the populations (fig. 13). *An. farauti* s.l constitutes 99.3%, 75.3% and 88.4% of the total mosquitoes collected in Kokofine, Matukar, and Mirap respectively. Molecular analysis of a subset of mosquitoes identified as *An. farauti* s.l in each of the three villages (Matukar: n = 55; Mirap: n = 521; Kokofine: n = 508) revealed that *An. farauti* s.s is the predominant species in the two coastal villages (Matukar: 94.5%; Mirap: 98.3%) while *An. farauti* No. 4 is the predominant species in Kokofine (99.4%). Although, other *An. farauti* species were present in the three villages, their densities were very low (< 2%). In the two inland villages, morphological identification revealed that *An. punctulatus* constituted a large portion (81%) of the total anophelines collected in Dimer (fig.13). Both *An. punctulatus* and *An. longirostris* were abundantly collected in Wasab constituting 51.4% and 42.3% respectively (fig. 13) of the total anophelines collected in Wasab. A subset (n = 92) of the mosquitoes morphologically identified *An. punctulatus* were confirmed by molecular analysis to check the reliability of morphological identification. The result showed 100% accuracy. Species not identified as the predominant species in any village were collected in relatively low numbers below 13% (fig. 13).

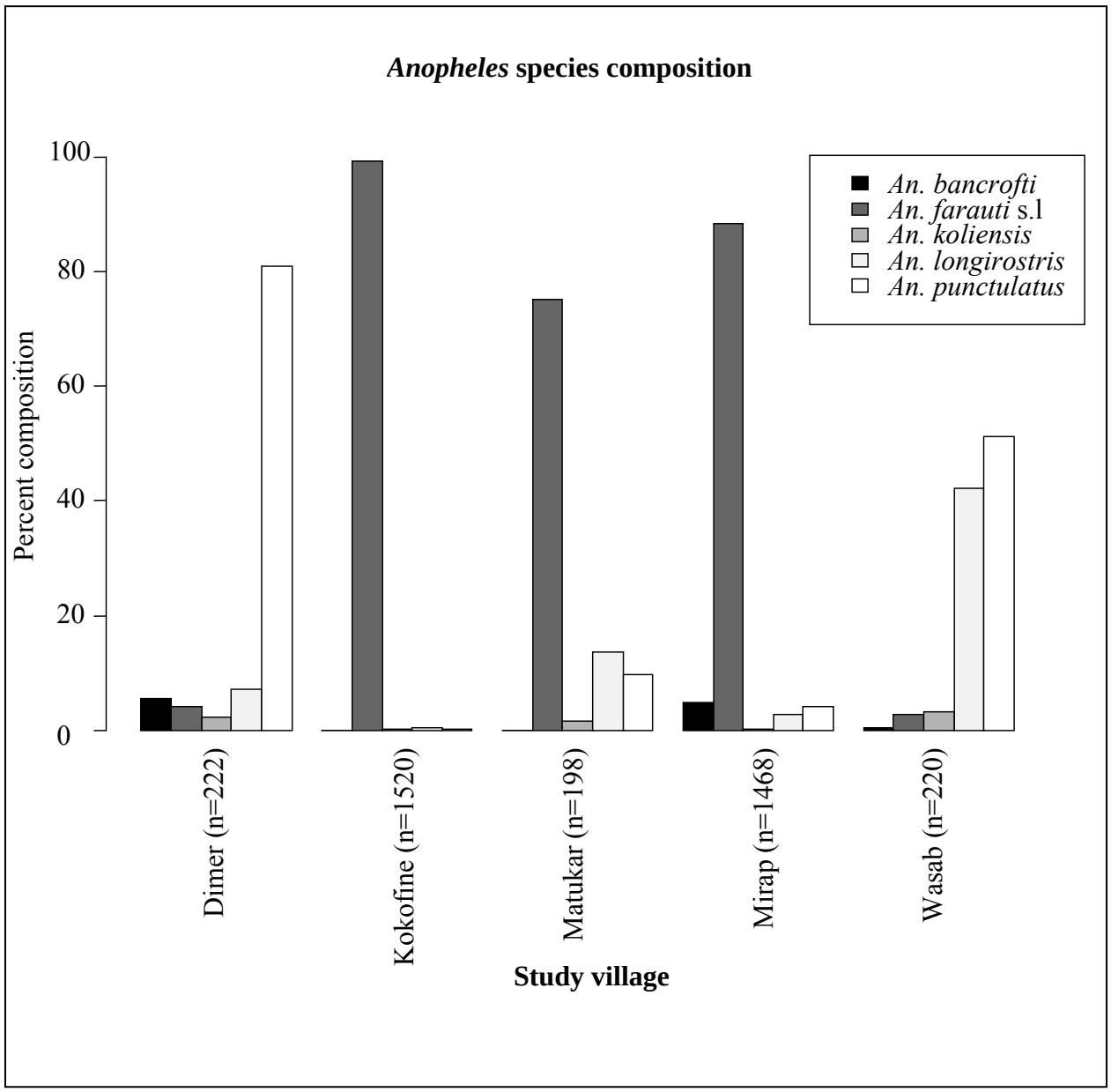

Figure 13. Bar graph showing percent proportion of the five morphologically identified *Anopheles* species sampled in each of the study villages over the sampling period.

Table 7. Tabular comparison of the density of blood-fed and unfed resting mosquitoes and mean number of mosquitoes caught per night per 20 m barrier screen in the five study villages.

Study village	Mean number of mosquitoes caught per night (total)	Blood-fed% (n)	Unfed% (n)
Dimer	19 (222)	21 (46)	79 (176)
Kokofine	380 (1520)	32 (486)	68 (1034)
Matukar	28 (198)	32 (64)	68 (134)
Mirap	82 (1468)	38 (562)	62 (906)
Wasab	16 (220)	37 (82)	63 (138)

4.1.2. The barrier screen sampling method

Mosquitoes were collected for 12 nights in Dimer, 4 in Kokofine, 7 in Matukar, 18 in Mirap and 14 in Wasab. The mean number of anophelines (all species combined) collected per night ranged from 16 in Wasab to 380 in Kokofine (table 7). To show the reliability of the barrier screen as a method to sample blood-fed mosquitoes, a Poisson distribution function was developed for each village. The expected number λ (i.e. mean) of blood-fed anophelines caught per night is 3.4 for Dimer, 121 for Kokofine, 9.1 for Matukar, 31.2 for Mirap, and 5.9 for Wasab. The probability of collecting any number (y) of blood fed mosquitoes in one night in any of the villages can be obtained by substituting the variable y in the general probability function $prob(y) = (\lambda^{y} e^{-\lambda})/(y!)$. The probability distribution for each of the villages is shown in figure 14a, 14b and 14c.

Table 8. Tabular comparison of the number of blood-fed versus unfed mosquitoes caught on each side of the 20 m long barrier screen in each of the study villages.

		Blood-fed		Unfed		Combined	
Study village	**species**	**Bush% (n)**	**Village % (n)**	**Bush% (n)**	**Village % (n)**	**Bush% (n)**	**Village % (n)**
Dimer	AP	22 (8)	78 (28)	73 (105)	27 (39)	63 (113)	37 (67)
Kokofine	AF4	26 (128)	74 (358)	54 (556)	46 (467)	45 (684)	55 (825)
Matukar	AF1	34 (20)	66 (38)	44 (40)	56 (51)	40 (60)	60 (89)
Mirap	AF1	14 (76)	86 (449)	47 (360)	53 (413)	34 (436)	66 (862)
Wasab	AP	40 (19)	60 (28)	38 (25)	62 (41)	39 (44)	61 (69)
Wasab	AL	50 (13)	50 (13)	42 (28)	58 (39)	44 (41)	56 (52)

AF1: *An. farauti* s.s, AF4: *An. farauti* No. 4, AP: *An. punctulatus*, AL: *An. longirostris*.

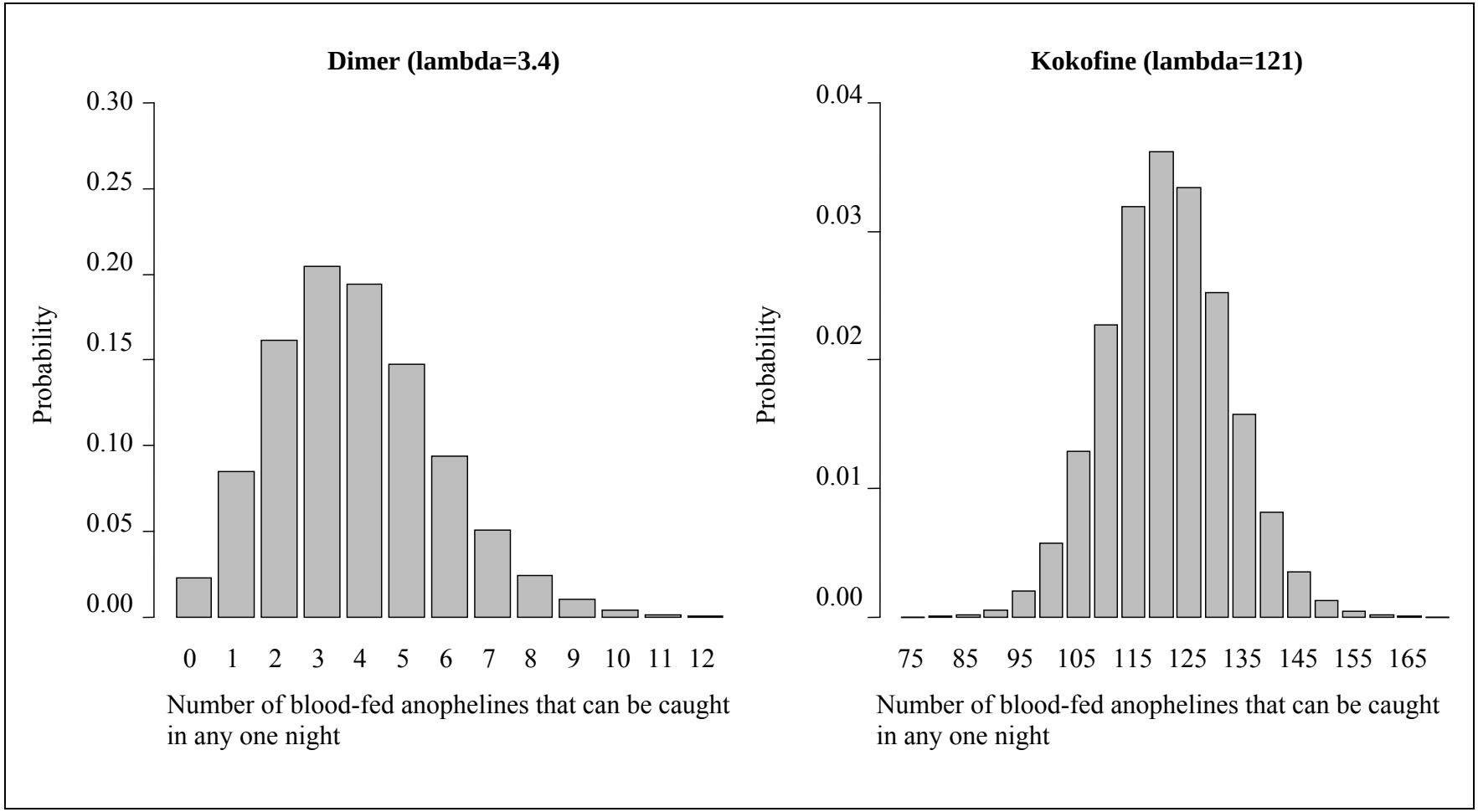

Figure 14a. Poisson probability distribution showing the probability of collecting any number of blood-fed anophelines in any given night in Dimer and Kokofine.

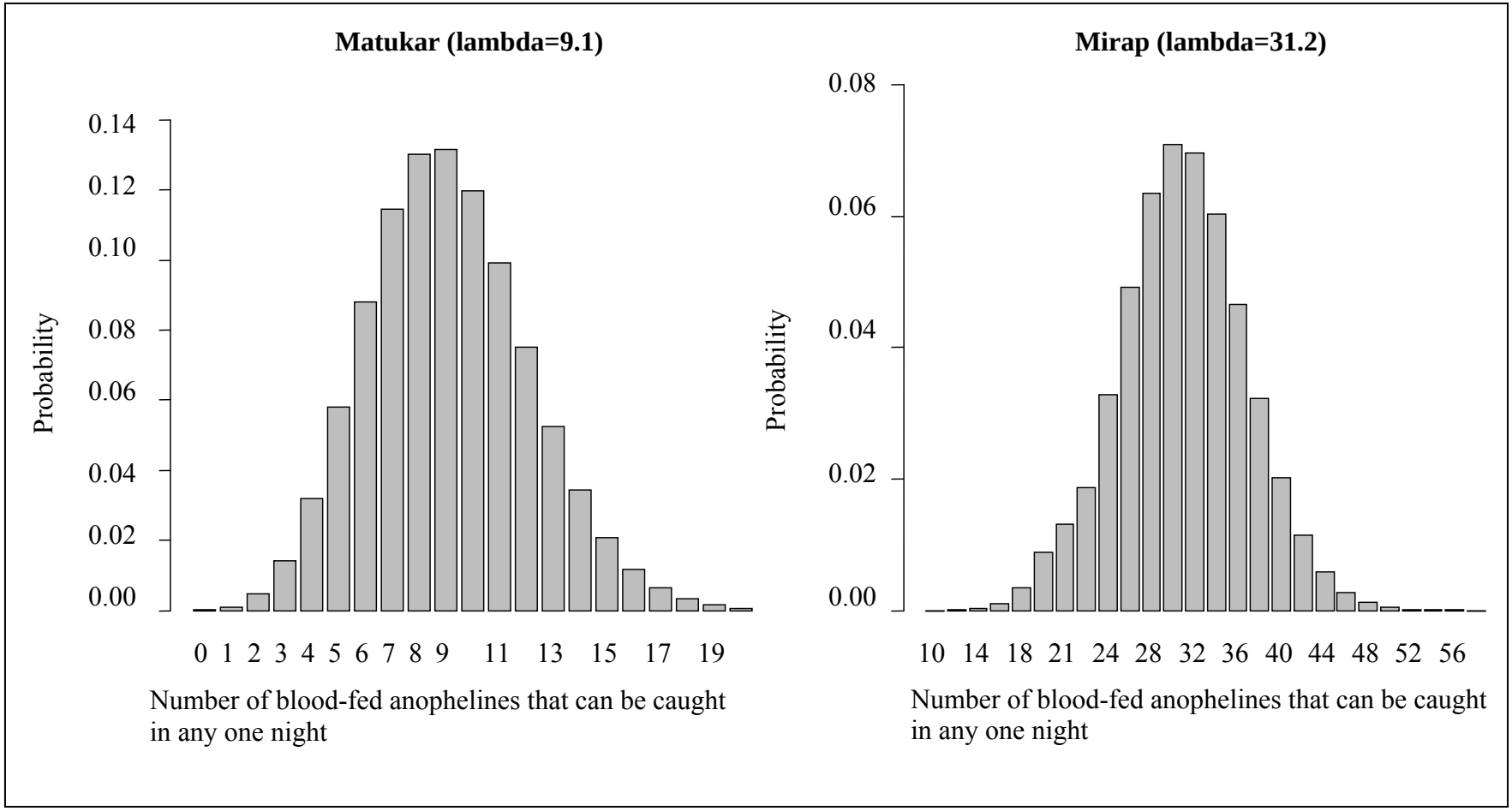

Figure 14b. Poisson probability distribution showing the probability of collecting any number of blood-fed anophelines in any given night in Matukar and Mirap.

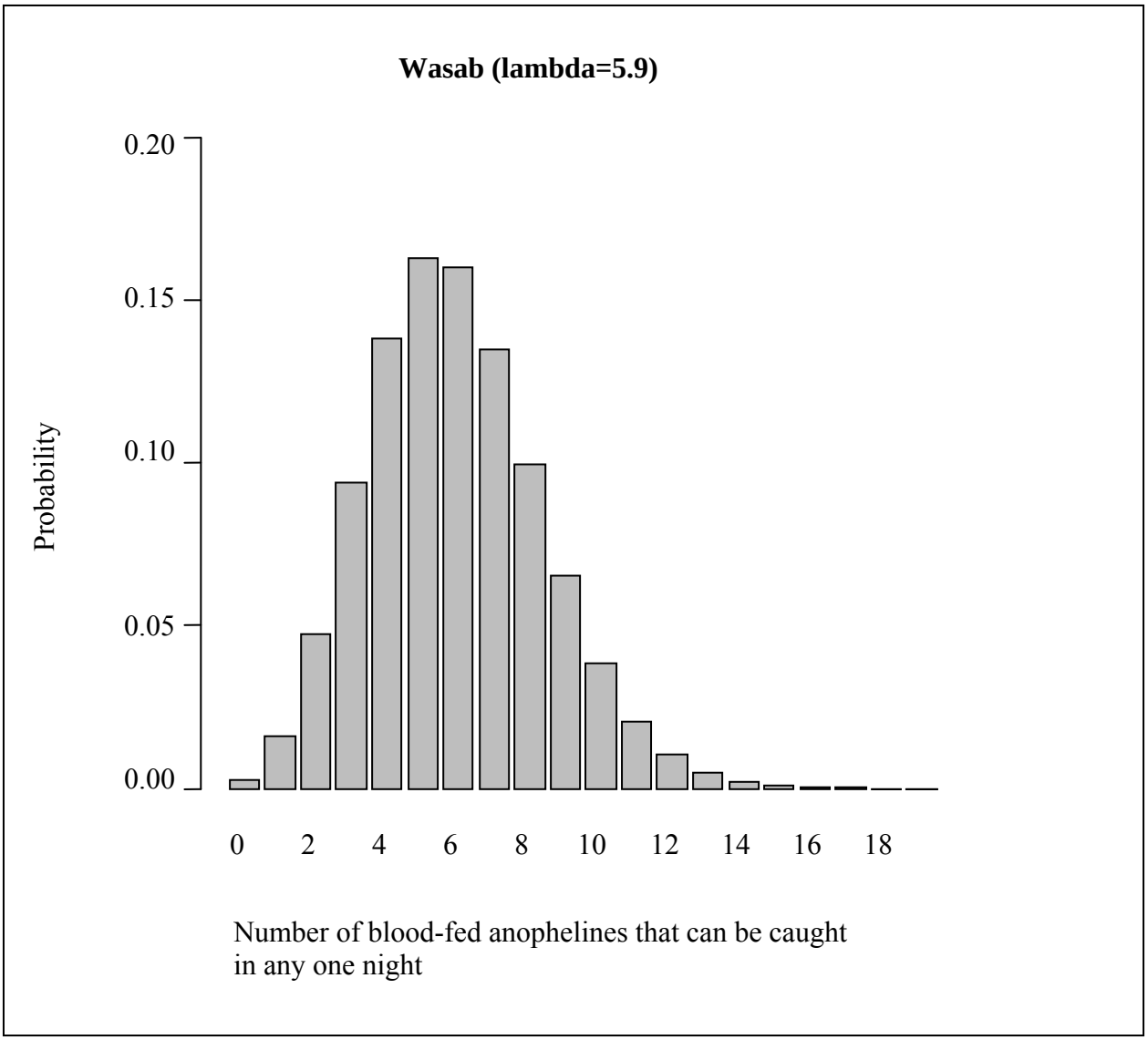

Figure 14c. Poisson probability distribution showing the probability of collecting any number of blood-fed anophelines in any given night in Wasab.

The proportions of mosquitoes caught resting on either sides of the barrier screen (designated as "bush side" and "village side") were compared. Due to low sample sizes in some species, only the predominant species were considered. The number of mosquitoes caught resting on the village side is greater than the bush side for all villages except Dimer (table 8). A test for homogeneity of proportion based on a 2x6 contingency table showed that this observation did not occur due to chance ($\chi^2 = 76.8$, df = 5, p-value < 0.0001) but there is a tendency for mosquitoes to rest more on the village than bush side. The numbers of blood-fed mosquitoes were consistently higher on the village side of the barrier screen compared to the bush side which had more unfed than fed mosquitoes across the five villages (table 8). A 2x2 contingency table was constructed and Pearson's continuity-adjusted chi-squared test of proportions was performed to test for statistical difference in the proportion of blood-fed mosquitoes collected on each side of the fence in each village. Three villages Dimer ($\chi^2 = 29.5$, df = 1, p-value < 0.0001), Kokofine ($\chi^2 = 103$, df = 1, p-value < 0.0001), and Mirap ($\chi^2 = 142.9$, df = 1, p-value < 0.0001) turned out to be statistically significant, suggesting that blood-fed mosquitoes had a higher tendency to rest on the village side of the barrier screen.

To show the potential of the barrier screen method as a tool to evaluate mosquito movement, graphs showing the mean number of mosquitoes caught hourly were constructed. Again only the predominant species were considered. The peak resting times for the mosquitoes differ among villages (fig. 15a, 15b and 15c). *An. punctulatus* in Dimer and *An. farauti* s.s in Matukar have similar temporal resting pattern (fig. 15a and 15b). Their peak resting time occurred between 12:00 am and 1:00 am and then drastically declined before slightly peaking again between 5:00

am and 6:00 am. *An. farauti* No. 4 in Kokofine had a distinct resting pattern (fig. 15a). The peak resting time occurred between 9:00 pm and 11:00 pm and then steadily declined right through the morning hours. The resting pattern for *An. farauti* s.s in Mirap (fig. 15b) resembled the pattern for *An. farauti* No. 4 in Kokofine but its peak resting time occur earlier (7:00 pm to 9:00 pm) than *An. farauti* No. 4. *An. longirostris* in Wasab (fig. 15c) had two peak resting times. One is between 8:00 pm and 9:00 pm and the other is between 10:00 pm and 11:00 pm. More *An. longirostris* were collected in the early hours of the night from 6 pm to 12 am. *An. punctulatus* in Wasab (fig. 15c) had three peak resting times at 8:00 pm – 9:00 pm, 10:00 pm – 11:00 pm and 1:00 am – 2:00 am.

Due to low numbers of mosquitoes caught in the other study villages, only the graphs of Kokofine and Mirap were further resolved by comparing the movement of unfed and blood-fed mosquitoes (fig. 16). In Kokofine, the temporal resting pattern of blood-fed *An. farauti* No. 4 is generally the same as for the unfed mosquitoes. However, the peak resting time for the blood-fed mosquitoes occurred an hour after the peak resting time of the unfed mosquitoes (fig. 16). Although the number of unfed mosquitoes steadily declined after the first peak resting time, the number of blood-fed mosquitoes increased again between 12:00 am to 3:00 am resulting in a second peak resting time. The temporal resting patterns for the blood-fed and unfed *An. farauti* s.s in Mirap were not the same (fig. 16). The unfed mosquitoes had a peak resting time early in the evening (7:00 pm to 9:00 pm) and then drastically declined until midnight before leveling off for the rest of the morning hours. The blood-fed mosquitoes did not show a clear peak resting time but the numbers of blood-fed mosquitoes caught resting on the barrier screen moderately increased every hour until midnight where it leveled off through the remaining hours (fig. 16).

The notion that mosquitoes caught resting on the bush side of the fence are mostly host-seeking mosquitoes entering the village while those on the village side are mostly blood-fed and unsuccessful host-seeking mosquitoes exiting the village was tested for *An. farauti* No. 4 and *An. farauti* s.s in Kokofine and Mirap respectively. Other species were ignored due to low sample sizes. A chi-square test of proportion based on a 2x4 contingency table was constructed for the 2 fence sides and 4 time periods (early evening: 6 pm – 9 pm, late evening: early morning: 9 pm – 12 am, morning: 12 am – 3 am, and period 4: 3 am – 6 am). The result turned out to be statistically significant for both villages (Kokofine: $\chi^2 = 32.69$, df = 3, p-value < 0.0001; Mirap: $\chi^2 = 26.13$, df = 3, p-value < 0.0001). In Kokofine, the number of mosquitoes on the bush side was almost equal to the number on the village side in the first two periods but more mosquitoes were caught on the village side compared to the bush in the last two periods (fig. 17). In Mirap, the number of mosquitoes caught on the village side is greater than the bush side across the four time periods. However, while the number of village mosquitoes was generally the same across all periods, the number of bush mosquitoes steadily declined across the four periods (fig. 17). Both trends suggest that more host-seeking mosquitoes enter the village during the evening and leave the village later in the night as blood-fed or as mosquitoes that were unsuccessful in finding a host.

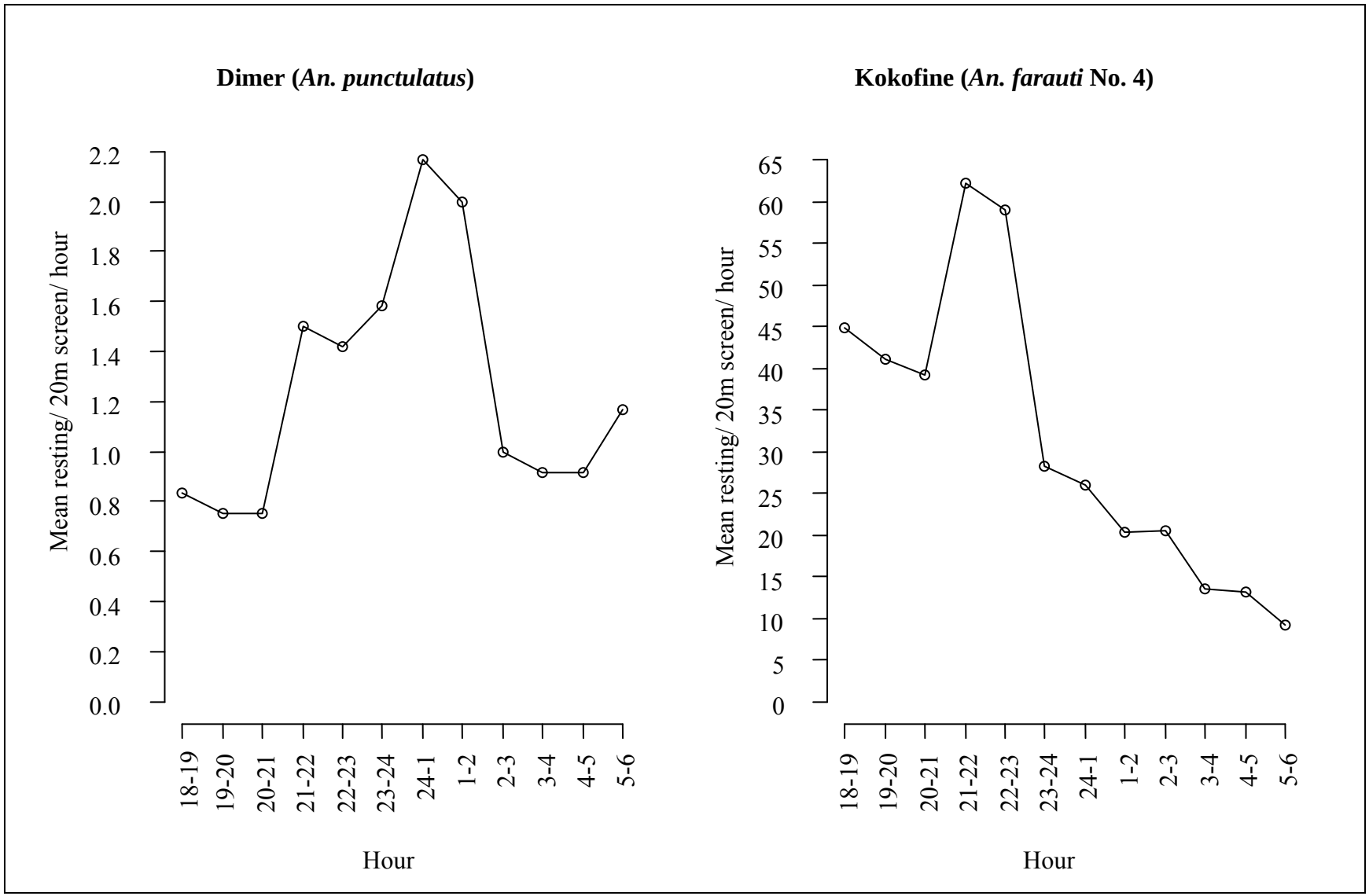

Figure 15a. Line graphs showing the mean densities of female *Anopheles* mosquitoes collected hourly from 8 pm to 6 am per night in Dimer and Kokofine.

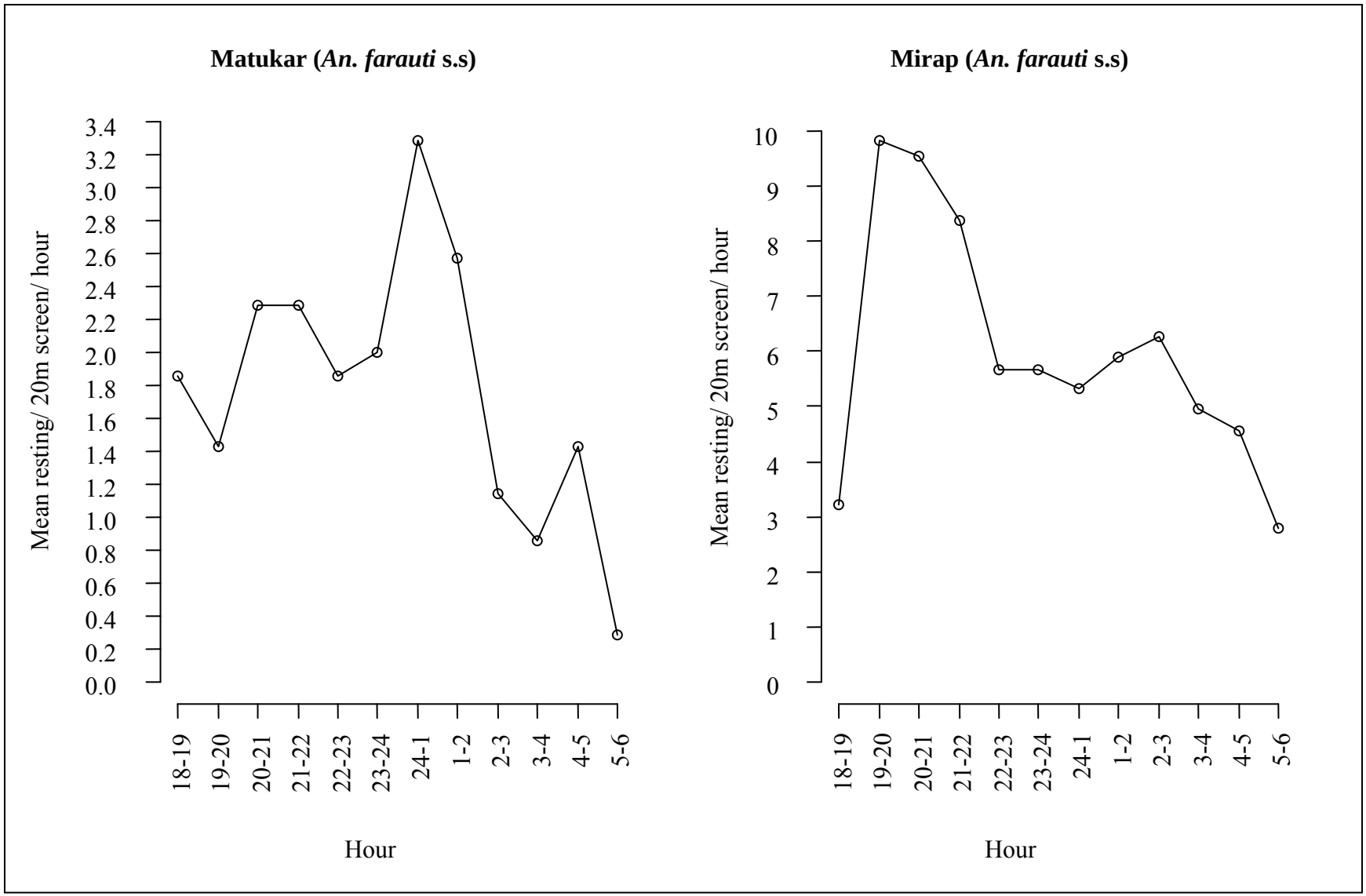

Figure 15b. Line graphs showing the mean densities of female *Anopheles* mosquitoes collected hourly from 8 pm to 6 am per night in Matukar and Mirap.

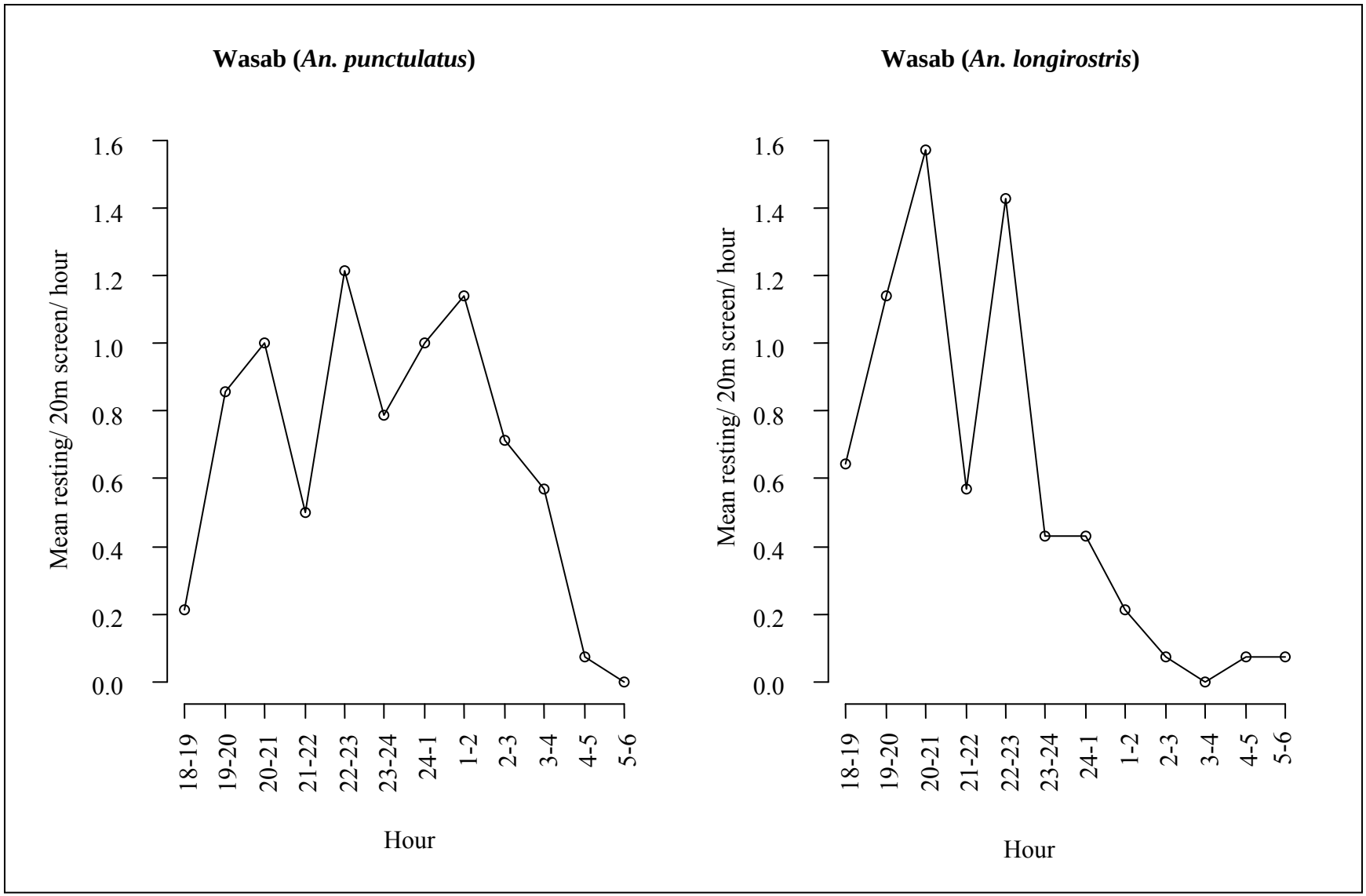

Figure 15c. Line graphs showing the mean densities of female *Anopheles* mosquitoes collected hourly from 8 pm to 6 am per night in Wasab.

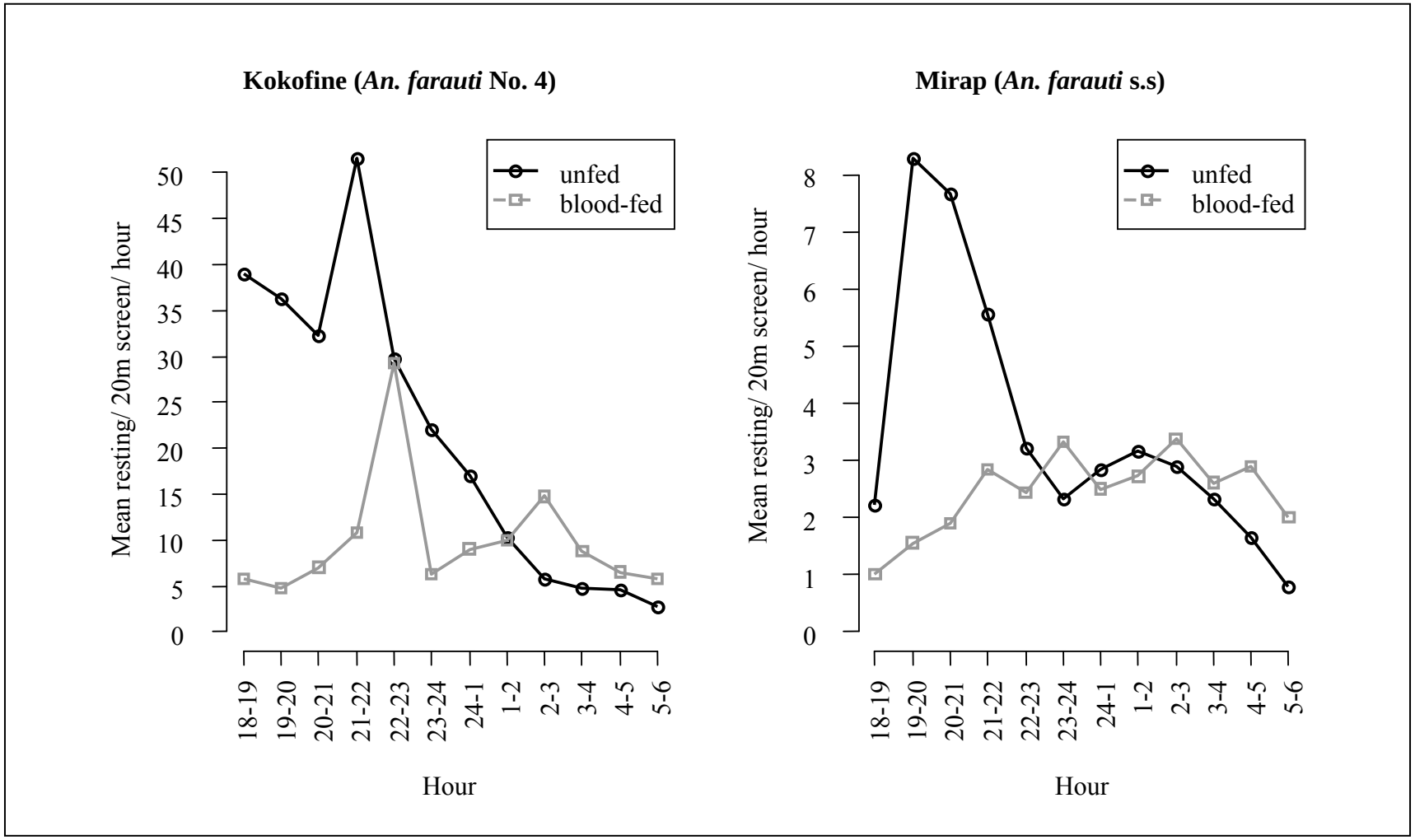

Figure 16. Line graphs showing the mean densities of blood-fed and unfed female *Anopheles* mosquitoes collected hourly from 8 pm to 6 am in Kokofine and Mirap.

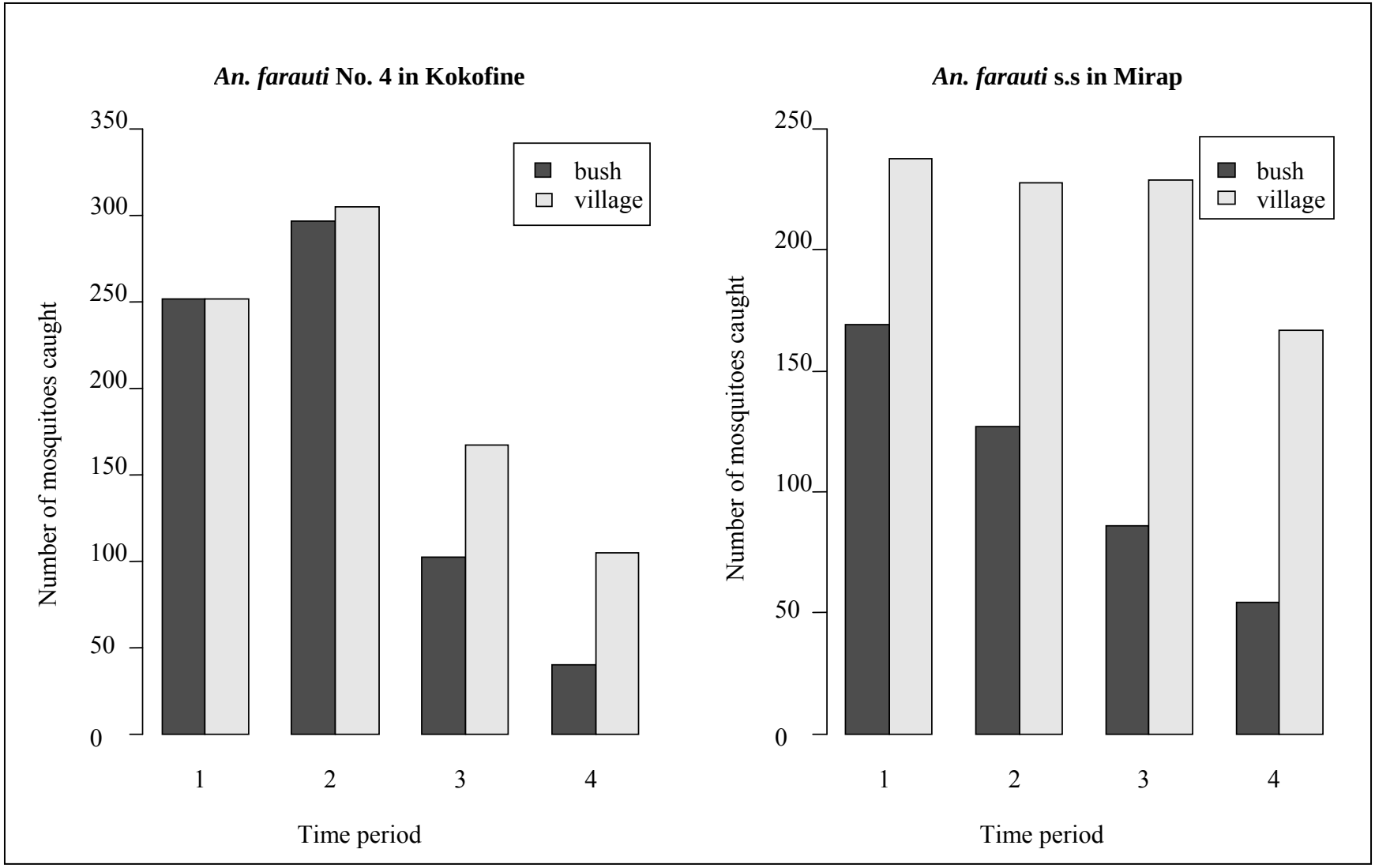

Figure 17. Bar graph showing the number of mosquitoes caught on the bush versus village side of the barrier screen across four time periods in two study villages. Period 1: 6 pm – 9 pm, 2: 9 pm – 12 am, 3: 12 am – 3am, 4: 3am – 6 am)

4.1.3. The host-feeding preference

Genomic DNA was extracted from 98% (n=1213) of the total (n=1240) blood fed mosquitoes from all villages combined. Identification of these mosquitoes either by morphology or molecular assay revealed 22 (1.8%) *An. bancrofti*, 39 (3.2%) *An. longirostris*, 92 (7.6%) *An. punctulatus*, 22 (1.8%) *An. Koliensis*, 567 (46.7%) *An. farauti* s.s, 5 (0.4%) *An. hinesorum*, and 466 (38.4%) *An. farauti* No. 4. The distribution of each of these species according to their village of origin is presented in table 9. Of the 1213 blood-fed mosquitoes extracted, 84% (n=1018) were successfully identified for for their host-blood source. The other portion (16%) either failed to amplify at the PCR stage or had a bad sequence quality.

The vertebrate host species that were observably present in the vicinity of the barrier screen in all five study villages during the sampling period include humans, pigs, dogs, chickens, cats and ducks. Of the 1018 anophelines that were identified for their host source, 68% (n=692) fed on humans, 31.9% (n=325) fed on pigs and 0.1% (n=1, species = *An. farauti* No. 4, village = Kokofine) fed on a cat. No mosquito was positive for dog, chicken, or duck. The number of mosquitoes that fed on humans or pigs for each *Anopheles* species in each village is presented in table 10.

The level of anthropophily for each *Anopheles* species was measured based on their relative HBI. When mosquitoes from all villages (populations) were pooled by species, *An. punctulatus* is ranked the most anthropophilic followed, in the order from the highest to the lowest, by *An. koliensis*, *An. farauti* s.s, *An. longirostris*, *An. farauti* No. 4, and *An. bancrofti* (table 11). The HBI of *An. hinesorum* was not calculated due to low sample size (n=3 blood-feds). *An. bancrofti*

had a HBI below 50%, suggesting a prefernce for pig over human (table 11). The rest of the species have HBI's greater than 50% indicating a preference for human over pig. A chi-square test of homogeneity of proportions based on a 2x6 contingency table (2 vertebrate hosts and 6 mosquito species) was peformed to statistically test for differences in their degree of anthropophily. The result turned out to be statistically different ($\chi^2 = 53.7$, df = 5, p-value < 0.0001) indicating that although human was the preferred host compared to pig for most species, they statistically differ in their degree of anthropophily. Pairwise comparison for all species with HBI's above 50 percent was performed. Of 10 pairwise comparisons, 5 (*An. farauti* s.s versus *An. farauti* No. 4, *An. farauti* s.s versus *An. punctulatus*, *An. farauti* No. 4 versus *An. punctulatus*, *An. farauti* No. 4 versus *An. koliensis*, and *An. punctulatus* versus *An. longirostris*) were statistically different (p-values <0.05). Due to heterogeneity in species composition among villages, comparison of host preference for each species among different villages was not tested.

Table 9. Distribution of blood-fed anopheline species according to their village of origin.

Village	**AF1 (%)**	**AF2 (%)**	**AF4 (%)**	**AP (%)**	**AK (%)**	**AL (%)**	**AB (%)**
Dimer	2 (0.4)	0 (0)	0 (0)	34 (37)	4 (18.2)	4 (10.3)	1
Kokofine	0 (0)	0 (0)	466 (100)	0 (0)	3 (13.6)	0 (0)	0
Matukar	53 (9.3)	2 (40)	0 (0)	4 (4.4)	0 (0)	2 (5.1)	0
Mirap	512 (90.3)	3 (60)	0 (0)	7 (7.6)	7 (31.8)	8 (20.5)	20
Wasab	0 (0)	0 (0)	0 (0)	47 (51)	8 (36.4)	25 (64.1)	1
Total	567	5	466	92	22	39	22

AF1: *An. farauti* s.s, AF4: *An. farauti* No. 4, AP: *An. punctulatus*, AK: *An. koliensis*, AL: *An. longirostris*, AB: *An. bancrofti*.

Table 10. Numbers that fed on humans or pigs for each *Anopheles* species in each village.

	Dimer		Kokofine		Matukar		Mirap		Wasab		
Species	human	pig	human	pig	human	pig	human	pig	human	pig	**Total**
AF1	2	0	-	-	38	2	303	116	-	-	461
AF2	-	-	-	-	1	0	2	0	-	-	3
AF4	-	-	234	169	-	-	-	-	-	-	403
AP	22	4	-	-	2	2	5	1	40	4	80
AK	2	1	2	1	-	-	6	0	7	1	20
AL	3	0	-	-	0	1	4	3	14	8	33
AB	1	0	-	-	-	-	4	12	0	0	17

Table 11. The HBI by village and by species (all villages combined). Missing value (-) means that the sample size is inadequate (< 15).

Species	**Dimer**	**Kokofine**	**Matukar**	**Mirap**	**Wasab**	**By species**
AF1	-	-	0.95	0.72	-	0.74
AF4	-	0.58	-	-	-	0.58
AP	0.85	-	-	-	0.91	0.86
AK	-	-	-	-	-	0.85
AL	-	-	-	-	0.64	0.64
AB	-	-	-	0.25	-	0.29

4.2. DISCUSSION

4.2.1. The barrier screen method

Indoor resting collection can be a biased sampling method. Most of the mosquitoes that rest indoors are endophilic mosquitoes that have fed on human hosts. When estimates of important ecological parameters such as the HBI are based solely or in large part on samples from indoor resting collections, there is a potential for bias towards human-fed mosquitoes, resulting in higher HBI. Thus, conclusions about the host preference or anthropophily are incorrectly made. The outdoor resting collection method is time-consuming and laborious. Often, the numbers of mosquitoes collected are very low and are not worth the effort. This problem is attributed to the fact that mosquitoes disperse widely throughout the environment and thick vegetations make mosquito-search difficult. The barrier screen method was developed to substitute these two traditional sampling methods. This method is based on the idea that newly blood-fed female exophilic mosquitoes temporarily rest a number of times after short-distance flights on their way out of the village. Any surface that intercepts their flight path can serve as a temporary resting site for the heavy-laden blood-fed female mosquitoes. Mosquitoes can then be collected as they temporarily rest. In this way a large enough number of blood-fed mosquitoes can be collected before they disperse widely into thick vegetations. Unlike the indoor resting collection method which is bias to human fed mosquitoes, mosquitoes collected on barrier screen should be fair to all hosts.

This study showed that barrier screen is a very effective method for sampling exophilic blood-fed mosquitoes. This claim is based on the mean number of blood-fed anopheline mosquitoes caught per night in each of the study villages. A mean of 121 blood-fed *Anopheles* mosquitoes

caught in a single night in Kokofine is astonishing. In villages like Kokofine and Mirap where mosquitoes are abundant, it only takes a few nights (time effective) with less efforts and expenses to sample a large enough number of blood-fed mosquitoes. Even in villages like Dimer or Wasab where mosquito population size is low, mean numbers of 3.4 and 5.9 blood-fed *Anopheles* mosquitoes caught in a single night are good sample sizes. Especially when compared to results from past studies where only 27 blood-fed anophelines were collected over 128 days of outdoor resting collection effort.[112] In all villages, the probability of obtaining no (n=0) blood-fed mosquito is almost zero, which means that there will always be some blood-fed mosquitoes caught over a number of sampling trials.

The barrier screen method is also useful in evaluating the behavior of blood-fed and host-seeking mosquitoes. For example, more blood-fed mosquitoes were caught resting on the village side of the barrier screen compared to the bush side. A plausible explanation for this observation is related to the direction of movement of blood-fed mosquitoes. After a mosquito had successfully obtained blood, it normally exits the village and flies towards oviposition sites out into the environment. The village side of the fence is where mosquitoes exiting the village are most likely to rest on and be caught. In contrast, one would think that because host-seeking mosquitoes normally fly into the village to find a host, more unfed mosquitoes should be caught resting on the bush than village sides of the barrier screen. However, although more unfed than fed mosquitoes were caught on the bush side, more unfed mosquitoes were caught on the village compared to the bush side. A plausible explanation for this observation is that host-seeking mosquitoes do not rest as much as blood-fed or unsuccessful mosquitoes but actively fly around searching for their hosts. Thus, a large number of mosquitoes entering the village as host-seekers

do not rest on the bush side of the barrier screen but rest more on the village side as blood-fed or unsuccessful mosquitoes exiting the village.

Barrier screen method can also be used to evaluate the temporal pattern of mosquito movement. For example, in Mirap and Kokofine the number of unfed host-seeking mosquitoes peaks in the evening and continue to decline throughout the night (fig. 17). In Kokofine, the temporal pattern of movement for blood-fed *An. farauti* No. 4 is the same as host-seeking but the peak resting time occurs an hour after the peak resting time for the host-seeking mosquitoes. In comparison, the temporal pattern of blood-fed *An. farauti* s.s in Mirap is not the same as the host-seeking. The number of blood-fed (fig. 16) or unsuccessful mosquitoes (fig. 17) caught is generally the same throughout the night. The most probable explanation for the observed difference between the two villages is that in Kokofine, the hosts are spatially easily accessible compared to Mirap. Because the hosts are easily accessible it takes less time to search for hosts and feeding occurs immediately after entry into village followed by immediate exit after feeding. In Mirap the hosts are probably more dispersed and therefore mosquitoes requires more time to linger in the village until a host is encountered resulting in untimely exiting pattern.

The barrier screen method gives researchers some control over the way they sample mosquitoes. For example, when only the blood-fed mosquitoes are required for a particular study, the unfed mosquitoes can be left alone. When unfed mosquitoes are not collected, the chance of collecting a larger number of blood-fed mosquitoes is increased. This is because unfed mosquitoes are host-seeking mosquitoes. If they are not collected, they will return as blood-fed mosquitoes sometime

later during the night and be caught. If they fail to find a host, they might return again in the following nights and might be caught as blood-feds.

4.2.2. The host-preference

This study showed that regardless of the presence of other hosts such as dogs, cats, chickens and ducks, *Anopheles* mosquitoes fed primarily on two host species: humans and pigs. *An. bancrofti* was the only species that preferred pigs over humans making it the poorest vector among all the species considered in this study. The rest of the *Anopheles* species preferred humans over pigs. However, their level of anthropophily varies. The HBI of *An. punctulatus* (86%) and *An. koliensis* (85%) are statistically indifferent and are the highest compared to the rest of the species Thus, both are equally the most important vectors among all *Anopheles* species considered in this study. *An. farauti* s.s is the second most anthropophagous mosquito with a HBI of 74%. It is also an important vector after *An. punctulatus* and *An. koliensis*. *An. farauti* No. 4 and *An. longirostris* have statistically similar HBI (58% and 64% respectively). Both species are ranked third in their level of anthropophily.

Anopheles mosquitoes of PNG are all potential vectors of malaria and filariasis in PNG however, their vector status were ranked based on their parasite rates. The HBI of *An. punctulatus*, *An. koliensis* and *An. farauti* s.s found in this study is similar to the findings of a previous study in which all were ranked the most anthropophilic.[108] The relatively high sporozoite rates found in these species relative to the other *Anopheles* species is probably because of their high anthropophilic behavior.[90, 104] This study is the first to report the HBI of *An. farauti* No. 4. The fact that it's HBI is very low and that one fed on cat suggests that this species is a generalist

feeder. The proportions that fed on humans will depend on the proportions of the human hosts relative to the other potential hosts. Thus, its vector status will depend on the local condition. Previous studies have mixed reports about the vector status of *An. longirostris* because of inconsistent parasite rates found in different populations of this species.[102] A plausible explanation for the inconsistency in the parasite rates of *An. longirostris* is that this species is a generalist feeder. Like *An. farauti* No. 4, its vector status depends on the local condition. *An. bancrofti* is considered a poor vector in PNG because of its consistently low sporozoite rates.[102] Again this is probably because of its low affinity for human hosts as shown in this study.

Humans, dogs and pigs are usually found living together in many parts of rural PNG. It is not surprising that from previous studies these three were the most preferred hosts. The fact that in this study, no *Anopheles* mosquito was found to have fed on dog is interesting because previous studies have shown that the proportion of mosquitoes that fed on dogs were often higher than that for pig or human.[84, 108] One plausible explanation is that although dogs were present, the proportion of dogs to humans or pigs were very low at the time of this study so that the probability of collecting a mosquito that had fed on dog was low.

4.2.3. Conclusions and recommendations

Comparing with the outdoor resting collection, the barrier screen method has been shown to be time and cost effective with high probability of collecting large enough number of samples for meaningful analyses. Comparing with the indoor resting collection method, the barrier screen seems to be unbias but fair to all hosts. Its roles can be expanded from sampling blood-fed and

host-seeking mosquitoes to evaluating mosquito movement into and out of the village. It can also be used to study the temporal hosts feeding pattern. Furthermore, unlike the other two methods, this method allows field researchers to have control over the way they want to sample mosquitoes. Therefore, the barrier screen method should be considered when sampling mosquitoes. It can be used alone or along with other methods.

Regardless of the presence of other hosts, pigs and humans are the only preferred hosts for the *Anopheles* mosquitoes collected in this study. *An. bancrofti* is the only species that preferred pigs over humans and is therefore considered zoophilic. The other species preferred humans over pigs however, their level of anthropophily varied. *An. punctulatus* and *An. koliensis* were equally the most anthropophagous of all species followed by *An. farauti* s.s, *An. longirostris*, *An. farauti* No. 4. The first three species are considered the most important vectors because of their high affinity for humans. The other three are considered generalists feeders and their vector status depends on the local conditions of hosts distributions. Due to insufficient number of blood-fed samples and the great heterogeneity in species composition among the five populations, comparison of the host-feeding preference of each of the vector species in different populations were not made. Future similar studies should incorporate a census of the number of the different hosts and bednet ownerships and usage to test for their effect on host-selection by the mosquito vectors. Although the *Anopheles* species described in this study are anthropophilic, they do not strictly specialize on humans. A good portion of each of the species fed on pigs. This is an important finding as zooprophylaxis depends on the ability of vectors to feed on alternative hosts. Thus, pig-based zooprophylaxis should be encouraged in PNG as another vector-based disease control method.

APPENDIX

Appendix

Record of Deposition of Voucher Specimens

The specimens listed below have been deposited in the named museum as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the voucher number have been attached or included in the fluid preserved specimens.

Voucher Number: 2013-12

Thesis Title

THE HOST-FEEDING ECOLOGY OF MOSQUITO VECTORS IN THE *ANOPHELES PUNCTULATUS* (DIPTERA: CULICIDAE) SPECIES COMPLEX IN A MALARIA ENDEMIC PROVINCE OF PAPUA NEW GUINEA

Author

John Bosco Keven

Table 12. Record of mosquito species submitted as voucher specimens to the Albert J. Cook Arthropod Research Collection Museum at Michigan State University.

Family	**Genus-species**	**Life stage**	**Quantity**	**Preservation**
Culicidae	*Anopheles punctulatus* Donitz	adult	5	dried in vials
Culicidae	*Anopheles farauti* Laveran	adult	5	dried in vials
Culicidae	*Anopheles koliensis* Owen	adult	4	dried in vials
Culicidae	*Anopheles bancrofti* Giles	adult	4	dried in vials
Culicidae	*Anopheles longirostris* Brug	adult	5	dried in vials

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