CHARACTERISTICS AND COMPARISON OF CELLULAR AND HUMORAL IMMUNE RESPONSES IN *Busseola fusca* (FULLER) (LEP., NOCTUIDAE), *Chilo partellus* (SWINHOE) (LEP., PYRALIDAE) AND *Maruca testulalis* (GEYER) (LEP., PYRALIDAE) TO *Escherichia coli* AND *Enterobacter cloacae* INFECTIONS

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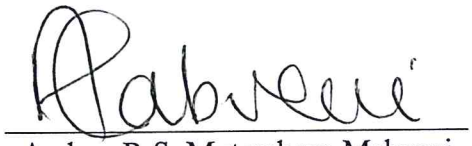
DECLARATION

I hereby declare that the material contained in this thesis is my own original work and has not been submitted for a degree in any other university.



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ABSTRACT

Characteristics of cellular and humoral immune factors were studied and compared in the fourth and fifth instar larvae of *Busseola fusca*, *Chilo partellus* and *Maruca testulalis*, using *Escherichia coli* and *Enterobacter cloacae* as the infection agents.

Haemocyte types were studied using light and electron microscopy techniques. Baseline levels of circulating total haemocyte numbers (THC) and differential haemocyte types numbers (DHC) were determined using the counting chamber method. Haemocyte population changes in bacteria-injected larvae were monitored for 72 hours. Phagocytosis and nodule formation were studied in *B. fusca* and *C. partellus* larvae injected with 2% azocarmine. The effect of bacteria injection and time post-injection on haemolymph lysozyme and antibacterial activities were determined using the bacteria growth inhibition zone assays. Haemagglutination and phenoloxidase activities in larval haemolymph were also studied.

Five morphological types of haemocytes were identified: prohaemocytes, plasmatocytes, granular haemocytes, spherule cells and oenocytoids. All five haemocyte types were present in the larvae of the borer species. *B. fusca* larvae had the highest number of circulating haemocytes (THC). Differential haemocyte counts (DHC) showed that granular haemocytes were the most predominant haemocyte type in the haemolymph. Injection of bacteria into the haemocoel lowered the total number of circulating haemocytes (THC), but effects on the different haemocyte numbers (DHC) were not significantly evident. There was a marked decline in haemocyte numbers over time in bacteria-injected larvae. Phagocytosis occured in both plasmatocytes and granular haemocytes. Pinocytosis and endocytosis were the processes involved. Nodule formation was a result of cellular aggregation and degranulation around the foreign particles.

There were baseline levels of haemolymph lysozyme and antibacterial activities in uninjected larvae. *M. testulalis* larvae had the highest baseline haemolymph lysozyme and antibacterial activities, while *B. fusca* larvae had the lowest. Injection of bacteria into the haemocoel increased these activities above the baseline levels. Larvae injected with bacteria showed antibacterial and lysozyme activities above the baseline levels by 20 hours post-injection.

Larval haemolymph agglutinated a range of vertebrate erthrocytes. *En. cloacae* cells were also agglutinated. In *C. partellus* and *M. testulalis*, the plasma fraction had higher agglutinating capabilities. In *B. fusca*, the whole haemolymph had higher agglutination capabilities. Studies with a range of inhibitory carbohydrates showed that the haemolymph agglutinins had specificity for sugars containing D-galactose moieties. Activation of prophenoloxidase (PPO) activity was demonstarted in the larval haemolymph. The whole haemolymph had pronounced activity in *C. partellus* and *M. testulalis* larvae. In *B. fusca* the plasma fraction had more activity.

Busseola fusca larvae had strong haemocytic responses and humoral defense activities against bacteria injected into the haemocoel. This would make *B. fusca* a difficult species to manage using bacteria biocontrol agents. The intermediate cellular and humoral immune responses in *C. partellus* and *M. testulalis* larvae would make these crop borers easy targets for microbial control in nature, if effective methods of introducing bacteria into the haemocoel are devised.

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CHAPTER 1: INTRODUCTION

The cereal stem borers *Chilo partellus* (Swinhoe), *Busseola fusca* (Fuller) and the legume pod-borer *Maruca testulalis* (Geyer) are important crop pests especially in the tropical and subtropical regions of developing countries, where cereals and legumes are staple food crops.

Due to the complexity in borer infestations and the difficulty in controlling them effectively with insecticides, it has been advocated that integrated pest management (IPM) schemes should be practised (Saxena *et al.*, 1986; ICIPE, 1989). The pertinent alternative strategies for IPM include the development of resistant or tolerant crop cultivars, cultural practices and the release of natural enemies. The use of micro-organisms which cause various diseases in insects pests, such as viruses, bacteria, fungi, nematodes and protozoa as well as predators and parasitoids, has offered promising alternatives or complementary treatments to chemical pesticides. They do not attack non-target organisms, are non-deleterious to the environment and can be genetically engineered to broaden host range, increase toxicity and pathogenicity or improve predatory efficiency and other desirable attributes.

However, it has been noted that insects have an inherent capability to combat and eliminate pathogens from their systems, and this is one of the factors that has contributed to their enormous evolutionary success (Postlethwait *et al.*, 1988). Active immune responses entail mechanisms for the recognition of introduced material as non-self and regulated alteration of physiology that results in the inactivation, suppression or removal of the foreign material. Protection can depend on the presence of constitutive, preformed factors and/or inducible humoral and cellular responses.

Although cellular and humoral responses play a significant role in insect defence mechanisms against pathogens and foreign bodies that invade the haemocoel, little work has been done on the immunity of lepidopteran crop pests, especially in the tropics and subtropics, regardless of their economic importance and despite the current emphasis on biological control as the most feasible and appropriate control method (Lackie, 1988a).

Existence of differential susceptibility between races or strains of the same species of insects has been well documented and has been thought to be attributable to the differences in physiological and biochemical defence mechanisms (Briese, 1981).

Lackie (1988a) provided a list of insects most commonly used as model systems for studying immunity. Among the Lepidoptera, *Manduca sexta* (tobacco hornworm) and *Helicoverpa* sp. are important crop pests. As noted by Ourth (1988), on the studies of the tobacco budworm, *H. virescens*, no work or studies of antibacterial immunity of lepidopteran pests have been done or reported.

The large number of insect species creates one of the key problems in entomology. Boman (1981) posed the question "to what extent can results obtained with one or a few species be generalised to a larger group of insects?" Thus, from the list provided by Lackie (1988a), generalisations on immunity mechanisms are not possible. There is need to study the immune mechanisms of crop borers, particularly from the tropical and subtropical developing countries.

Kaaya (1989a) gave reasons why the study of immunity to pathogens in insect vectors is important. It is from these that the following justification for the study of immunity to pathogens in important crop pests, is based.

(i). The study of immunity to pathogens in the larvae of crop pests is important for shedding more light on how the larvae interact with pathogens, especially those used in biological control.

(ii). Once the structure, biochemical characteristics and modes of action of the immune factors are fully understood, it may be possible to find practical applications for the information. For example, immune depressing substances could be applied or incorporated into bio-insecticides used for pest control, so that the pest immune mechanisms are impaired thereby allowing for better activity of the disease causing pathogen.

(iii). Some micro-organisms, e.g. the bacteria *Bacillus thuringiensis* and *Serratia marcescens*, the nematode, *Neoaplectana carpocapse* (Boman and Hultmark, 1987), and the fungus *Metarhizium anisopliae* produce immune inhibitory

substances (Kaaya,1989b). The biochemical effects of some of these substances can be well understood if the immune mechanisms of the target pests are elucidated. As a result of this understanding the genes encoding these anti-immune factors can be combined in a single micro-organism in order to enhance its biological control potency. Genetically engineered bacteria producing highly potent toxins as well as immune inhibitors would be very effective for biological control.

(iv). Elucidation of insect immune mechanisms can lead to the genetic manipulation of the immunity genes in some of the pests to produce insect strains that may be susceptible, which can be released in the field so that they can mate with the wild populations and spread those genes.

Although *C. partellus*, *B. fusca* and *M. testulalis* are extremely important crop pests, no previous work has documented or characterised or compared their immune responses to bacterial challenge.

The aim of this work was to study and compare the characteristics of the cellular and humoral immune factors of *B. fusca*, *C. partellus* and *M. testulalis* to some bacterial infections. This had an obvious applied significance, with the aim of shedding more light on the intricacies of host-parasite interactions, which provide a basis on which anti-immune factors produced by some micro-organisms can be exploited and enhanced for ecologically effective biological control of the pest.

This was achieved by the following objectives:

(1). Characterising and comparing the different morphological types of haemocytes found in the fourth and fifth instar larvae of *B. fusca*, *C. partellus* and *M. testulalis*.

(2). Comparing the total and differential haemocyte counts between uninfected larvae and larvae challenged with the bacteria *Escherichia coli* and *Enterobacter cloacae*.

(3). Comparing the cellular immune reactions in the larvae of the three insect/borer species.

(4). Determining the concentration of bacteria that induced antibacterial activity; the time taken for induction to occur and the types of antibacterial factors present in the haemolymph of infected borer larvae.

(5). Comparing the differences in antibacterial activity due to the larval instar stage and among the species.

CHAPTER 2: LITERATURE REVIEW

2.1. The borers: Distribution and economic importance

2.1.1. Cereal stalk-borers

Stalk-borers are considered major pests of many Gramineae all over the world (Hill, 1983). All important graminaceous crops such as maize, millet, rice, sorghum and sugarcane, often the staple food crops in developing countries, may be attacked. In the temperate regions, relatively few important stalk-borers are known, while in the subtropics and the tropics stalk-borers are much more abundant (Seshu-Reddy, 1985; Chundurwar, 1989; Sithole, 1989).

Although different species of stalk-borers have been recorded from different regions, the species infesting sorghum and maize are by far the most important (Ogwaro, 1983). *Chilo partellus* (Swinhoe) (Lepidoptera: Pyralidae) and *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) are important insect pests on maize and sorghum (Hill, 1983; Teetes *et al.*, 1983; Alejandro, 1987), especially in Africa where sorghum and maize are the major cereals produced for consumption.
C. partellus is found in Eastern and Southern Africa, the Indian subcontinent and South East Asia, while *B. fusca* is a widespread pest in the maize growing areas of tropical and subtropical Africa, from the south of the Sahara down to South Africa (Hill, 1983; Teetes *et al.*, 1983; Alejandro, 1987; Harris, 1989a, b).

Published figures for reduction in sorghum yield caused by borers range from only 5-10% in some areas and up to 83% in others (Harris, 1985; Chundurwar, 1989). Losses in maize due to *C. partellus* vary widely in different areas from about 18% in Kenya compared to 26-80% in India (ICIPE, 1989).

2.1.2. Legume pod-borer

The legume pod-borer *Maruca testulalis* (Geyer) (Lepidoptera: Pyralidae) occurs throughout the tropics and subtropics of central South America, Asia and Africa (Hill, 1983; Odhiambo, 1984).

(IFA). Diagnosis of RVFV is an important component in its transmission, prevention and control. A study carried out by Njenga et al. (2009) reported a rapid diagnostic tool to assist in the detection of RVFV in rural settings. Field diagnosis is the initial component towards identifying the disease in animals in order to minimise the risk in humans. Although this component is vital, it is primarily influenced by socioeconomic factors such as lack of medical facilities, accessibility of the area and sparse and temporary settlements of nomadic communities. Environmental factors such as heavy rains, flooding and temperature may also contribute towards increased RVF vector population resulting in increased chances of RVFV transmission leading to abortion in animals and disease in humans who are exposed to infected animals before field detection (Geering et al., 2002; Wilson et al., 1994).

1.5 Classification of Arboviruses

Arboviruses are classified into seven major families, which include Togaviridae, Bunyaviridae, Flaviviridae, Orthomyxoviridae, Rhabdoviridae, Reoviridae and Alfaviridae. The classification of these virus families is based on characteristics such as morphology, mode of viral replication and antigenic relationships (Murphy et al., 1995). The majority of arboviruses that affect humans and animals belong to the Togaviridae, Flaviviridae and Bunyaviridae. Togaviridae constitutes viruses that are mainly transmitted by mosquito vectors including Sindbis and Babanki viruses. These viruses are spherical, enveloped viruses (diameter 50-70 nm) containing genomes with a single molecule of positive-sense single-stranded RNA (Freiberg et al., 2008). The family is sub-divided into three genera: *Alphavirus*, formerly known as the Group A arboviruses; the genus *Rubivirus*, containing a single member, the rubella virus; and the genus *Pestivirus,* which includes viruses that cause disease in domestic animals (Wilson et al., 1984). Examples of arboviruses that fall under this family include; Semliki forest, Chikungunya, O'nyong'nyong and Ndumu.

Flaviviridae includes the genus *Flavivirus*, which contains 65 related species (Westaway et al., 1985). The flaviviruses were previously known as Group B arboviruses. They are small enveloped viruses (diameter 45 nm) that contain a single strand of positive-sense RNA (Monath, 1994). These viruses have been shown to cause major epidemics that affect both humans and livestock. Flaviviruses include West Nile fever, Omsk haemorrhagic fever, Kysanur forest disease, yellow fever and dengue.

Bunyaviridae is a taxonomically vast arbovirus family that consists of five genera: *Bunyavirus, Phlebovirus, Nairovirus, Uukuvirus and Hantavirus* (Porterfield et al., 1975). These genera contain 97 species and a large number of isolates, encompassing 19 viruses in seven groups of related viruses and dozens of ungrouped viruses. These viruses are spherical and enveloped (90-100 in diameter). The genome of Bunyaviridae is comprised of single-stranded, negative-sense RNA, divided in three segments (S, M and L) (Elliott et al., 1991). These viruses are capable of genetic reassortment, leading to major epidemics such as the Ngari hemorrhagic fever virus outbreak that occurred in East Africa in 1997-1998 (Gerrard et al., 2004). Other examples of viruses belonging to the Bunyaviridae family include RVF, Bunyamwera, Pongola, Batai, Potosi and Cholul.

1.6 Genetic Diversity of RVF

The RVFV genome is approximately 11,979 nucleotides in length. The virus is an enveloped RNA virus whose genome is of negative polarity and comprises three segments designated L, M and S (Figure 1.1). The L segment encodes an RNA dependent polymerase that synthesises both mRNA and genomic RNA (Albariño et al., 2007). The envelope of this virus

is composed of a lipid bilayer containing the Gn and Gc glycoproteins forming surface sub-units (Collao et al., 2009).

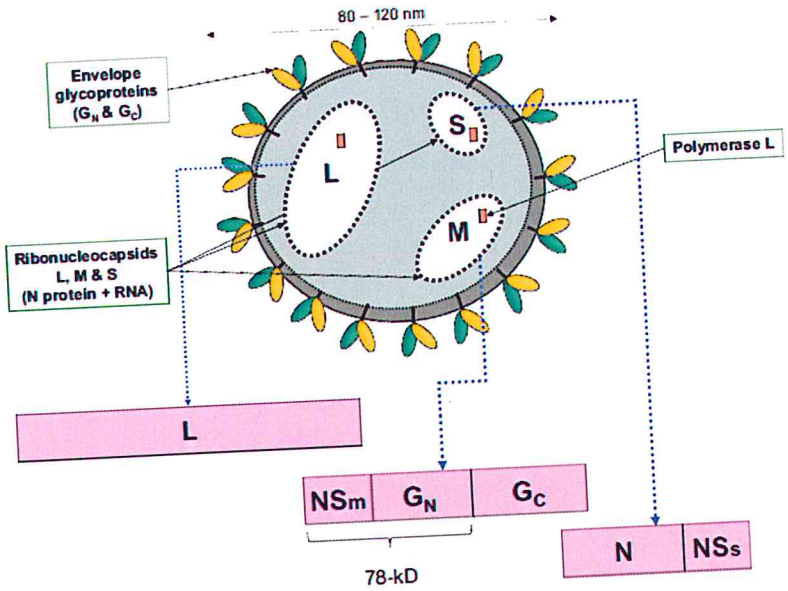


Figure 1.1: Structure of Rift Valley fever virus. Adapted from Linda Stannard (Tolou et al., 2009)

The viral RNP corresponds to each genomic segment and is associated with N and RNA dependent RNA polymerase L, which form the virion. The S segment encodes two proteins: N and NSs (Giorgi et al., 1991). The cRNA representing the copy of the S ambisense segment serves as a template for the synthesis of the NSs mRNA that plays a significant role in RVFV infection. The S segment has an ambisense coding strategy and contains two open reading frames (ORF); half of the N is encoded at the five prime of the viral antigenomic-sense molecule (cRNA) while the other half is encoded by the NSs of the five prime half of the genomic-sense RNA (vRNA) (Giorgi et al., 1991).

The two ORFs are separated by a C-rich intergenic region (IGR). The L and M segments encode the RNA-dependent RNA polymerase (L protein) and the glycoprotein precursor, which is co-translationally cleaved to generate mature envelope glycoproteins (G_N and G_C) and a nonstructural protein (Giorgi, 1996; Schmaljohn & Hooper, 2001). These segments are responsible for capsnatching or transcription termination.

RVFV virus is subdivided into 15 major genetic lineages based on the virus genotype and geographic location (Grobbelaar et al., 2011). However, virus strains with remote origins can be found within each of the lineages, which provides strong evidence of widespread distribution of RVFV genotypes throughout Africa. For example, RVFV strains can be found by the monophyletic linkage of isolates from regions as far as Egypt, Madagascar and Zimbabwe or Kenya, Mauritania, Burkina Faso, Zimbabwe and South Africa (Bird et al., 2008). Genomic reassortment of the RVFV RNA segments promotes genetic diversity and can allow for the emergence of novel RVFV strains. This reassortment has been shown to occur frequently in both *in vitro* and *in vivo* studies (Briese et al., 2006; Gerrard & Nichol, 2002) in spite of a lack of evidence of homologous recombination among RVFV (Bird *et al.*, 2007). Re-assortment influences RVFV replication, fitness and host virulence. In general, RVFV has relatively low genetic diversity: approximately 4% and 1% at the nucleotide and protein coding levels, respectively (Bird *et al.*, 2008; Bird *et al.*, 2007).

The low genetic diversity contrasts sharply with other *Bunyaviruses,* such as Crimean Congo haemorrhagic fever virus which has approximately 32% diversity at the nucleotide level (Carroll et al., 2010; Deyde et al., 2006). Furthermore, the low genomic diversity of RVFV suggests that the virus either has a very low tolerance for mutation within its genome or alternatively that the extant viruses collectively identified today as RVFV have a relatively

recent common ancestor. Previous studies on genetic diversity of RVFV were performed using partial gene sequences from a relatively small number of isolates; current studies are based on complete genomic sequences of a large number of viruses recovered from many geographic areas. As a result the number of identified viral lineages (distinct genetic groups sharing a common ancestor) of RVFV has increased from 3 in an early analysis (Sall et al., 1997; Sall et al., 1999) to 7 in a 2007 study (Bird *et al.,* 2007), and then 15 in the most recent report (mean pairwise distance ≤0.017 within lineages and bootstrap values ≥70%) (Grobbelaar et al., 2011).

1.7 Epidemiology of Rift Valley Fever virus

RVFV was first isolated in Naivasha in the Rift Valley province of Kenya in 1930 (Daubney et al., 1931). Since then, subsequent outbreaks have been reported in sub-Saharan countries including Tanzania, Somalia, South Africa, Senegal and Mauritania (El-Din Abdel-Wahab et al., 1978; Hoogstraal et al., 1979; Meegan, 1979; Siam et al., 1980) (Table 1.1 & Figure 1.2). Other cases of RVF were also documented in Madagascar in 1991 (Morvan (Morvan et al., 1992; Morvan et al., 1991) and in the Arabian Peninsula in 2000 (Ahmad, 2000). Evidence points to the spread and possible persistence of the East African strain of RVFV to Saudi Arabia and Yemen (Al-Afaleq et al., 2003).

Table 1.1: Incidences of RVF across different regions

Year	Study	Location	Significance
1918	Daubney *et al.*, 1931	Lake Naivasha region Kenya	Earliest epidemic of bovine hepatitis later considered as RVF
1930-31	Daubney *et al.*, 1931	Kenya	Major RVF epidemic, isolation of pathogen causing disease in human and sheep
1950	Joubert *et al.*, 1951	South Africa	First major epidemic, first description of ocular lesions
1974	Valden et al., 1977	South Africa	Return of RVF, recognition of VHF and encephalitis caused by RVFV
1977	Meegan *et al.*, 1979	Egypt	Massive epidemic and first epidemic out of sub Saharan Africa
1987	Jouan *et al.*, 1988	Mauritania, Senegal	First major west African epidemic, coincided with damming of Senegal River and high rainfall
1990	Morvan et al., 1992	Madagascar	Large epidemic related to ecological change
1993	Arthur *et al.*, 1987	Egypt	Disease recurred after absence of detectable circulation since 1980
1997	Bouloy *et al.*, 1998	East Africa	El Niño led to prolonged rainfall followed by largest recorded RVF epidemic
2000	Balkhy *et al.*, 2003	Arabian peninsula	Strain previously active in E. Africa spreads to Saudi Arabia and Yemen
2006-08	Sissoko *et al.*, 2009	Comoros Islands	RVFV epidemic in 2006. Activity in humans in 2007-2008
2006	Sang *et al.*, 2010	Kenya	Continued RVF activity
2008-10	Archer et al., 2010	South Africa	Low level activity

In West Africa, RVFV caused outbreaks in Mauritania and Senegal in 1987, which coincided with the damming of the Senegal River and high rainfall (Chevalier et al., 2005; Zeller et al., 1995). The outbreak led to 220 human deaths as the dam acted as a breeding ground for RVFV vectors (Jouan et al., 1989). Since then, active surveillance has led to the detection of several RVF animal cases in Mauritania and Senegal, which share the Senegal River basin, and other West African countries (HERVE, 1997; Thonnon et al., 1999; Zeller et al., 1995). Entomological and virological surveys conducted during the last decade have also confirmed

further RVFV cases in Senegal (HERVE, 1997; Thiongane et al., 1991; Wilson et al., 1994). For example, in 2002 and 2003, RVF was detected in the Ferlo region (Soti *et al.,* 2012; Empres 2003), and the potential role of *Aedes vexans* (Edwards) as a vector of RVF was established (Fontenille *et al.,* 1998). RVFV has also been isolated from *Culicoides* in Nigeria during inter-epizootic periods (Lee, 1979). However, the identification of vectors involved during epidemic/epizootic periods has not been achieved in West Africa, particularly in Mauritania where most of the epidemics/epizootics have been recorded (Mondet et al., 2005). In 1977, a massive epidemic of RVFV occurred in Egypt and this was the first epidemic recognised outside sub-Saharan Africa (El-Akkad, 1978). RVFV recurred in Egypt in 1993, sixteen years after the first outbreak. It is likely that this virus continued to circulate in Egypt at low levels until the first decade of 2000.

In South Africa, two large epizootics occurred on the interior plateau of the Free State, Eastern Cape, and Northern Cape provinces during 1950–1951 and during 1974–1976 (Pienaar & Thompson, 2013). However, smaller sporadic outbreaks have been regularly reported since the 1950s in 1969-71, 1974-75, 1981, 1996, 2007-08 and 2010 (Archer et al., 2011; Coetzer et al., 1994). Recent outbreaks of RVF were documented in South Africa between 2008-2011 (Archer et al., 2011). In 2008, RVF outbreaks in Mpumalanga, Limpopo, Gauteng and North West provinces resulted in 353 animal cases and 103 animal deaths. The animal species affected were cattle, goats, sheep and African buffalo (Grobbelaar et al., 2011). In 2009, more RVF outbreaks occurred in KwaZulu-Natal, Eastern Cape and Mpumalanga provinces with 210 animal cases and 66 animal deaths. The animal species affected were cattle and sheep (Grobbelaar et al., 2011). Major epidemics of RVF occurred in 2010. These outbreaks were first reported in the Free State province and spread across all provinces except KwaZulu-Natal. These outbreaks involved 14 342 animal cases and 8877

animal deaths. The epidemic affected mostly sheep, cattle, goats and wildlife (Directorate of Animal Health 2012). In 2011, RVF caused a minor epidemic, which occurred in the Eastern, Western and Northern Cape provinces and affected 4139 animals. Sheep, goats, cattle and wild animals were most affected (Directorate of Animal Health 2012).

The largest documented RVF outbreak was in 1997–1998 in the Horn of Africa (Woods et al., 2002). This epidemic was associated with 'El Niño' rainfall. This outbreak involved 5 countries, namely Sudan, Ethiopia, Somalia, Kenya and Tanzania, with a loss of approximately 100,000 domestic animals and 90,000 human infections (Woods *et al.,* 2002). This outbreak was of major significance due to the economic impact of a ban on livestock exports from the region. Subsequent epidemics have recently been reported in Somalia (2006–2007), Kenya (2006–2007), Tanzania (2007), Sudan (2007–2008), Mayotte (2007–2008), and Madagascar (2008) (Andriamandimby et al., 2010).

In East Africa, RVF outbreaks usually occur in the form of explosive epizootics with prolonged inter-epidemic periods in the order of 8 to 10 years. RVF outbreaks recurred during December 2006 in Northeastern, Rift Valley and Coast provinces of Kenya after an extended period of rainfall that caused floods and a subsequent increase in mosquito populations. These outbreaks were reported in 29 of the 69 districts in Kenya, with most human cases originating from Garissa and Ijara districts in Northeastern province, Kilifi district in Coast province, and Baringo district in Rift Valley province (Anyangu et al., 2010). The episodic nature of the disease and the rapid evolution of outbreaks create special challenges for its mitigation and control, which calls for a better understanding of the interaction between environmental conditions and vector species in the epidemiology of RVF.

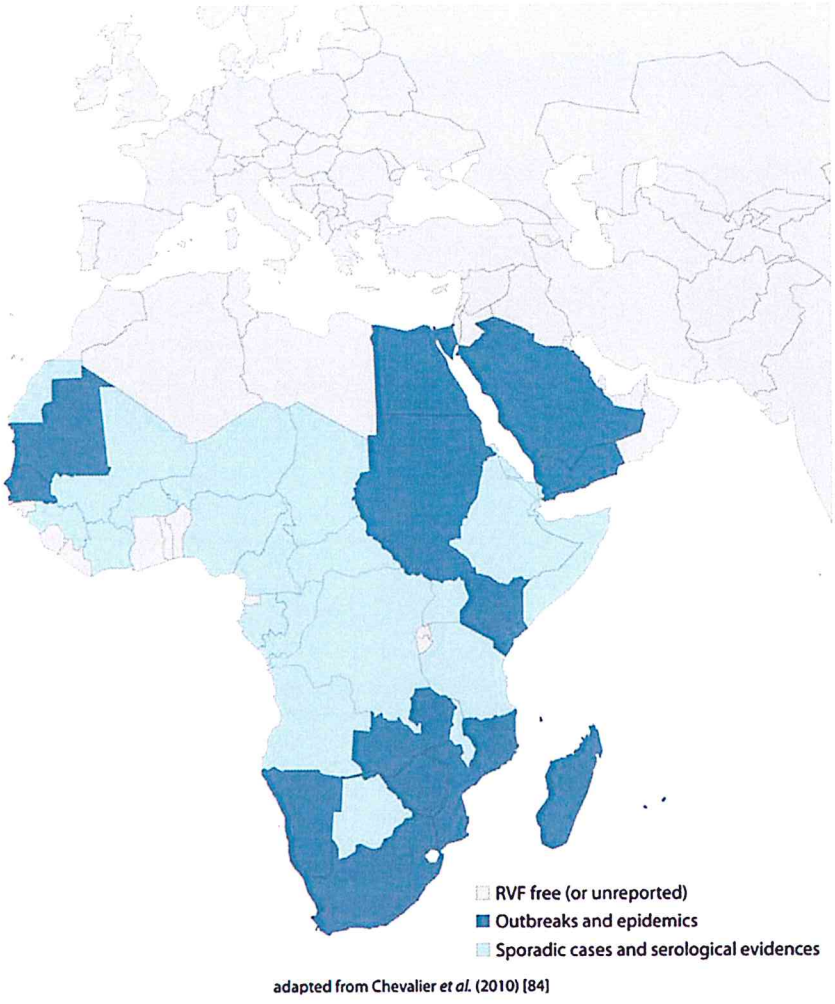


Figure 1.2: Geographical distribution of Rift Valley fever

1.8 Prevention and Control of RVF

1.8.1 Vector control

Control of RVF entails an integrated and multidimensional control strategy which is superior to a single line of approach (Shea & Chesson, 2002; Shea et al., 2002). Hence, vector control should include a strategy with a combination of different vector interventions (Coleman et al., 2006). Strategies such as; indoor residual sprays (IRS) and intradomicile application of insecticide-treated materials (ITM) have so far yielded positive output as combined interventions (Eisen et al., 2009; Kroeger et al., 2006; Nam et al., 2005). More emphasis

should be put on immature and adult mosquitoes for surveillance and control. This will contribute towards development of more informative and effective vector control methods, which will impact greatly on reducing morbidity and mortality.

1.8.2 Larval control

Larval control is a good practice in vector management, especially in areas with strong community participation and where conditions are particularly favorable. For example, in a study carried out in Vietnam by Kay and Nam (2005), larval mosquito biological control agents were available for treating a prominent and easily recognisable container class. Locations suitable for the development of the immature stages of the vector should be destroyed using both physical and chemical means as part of the integrated vector control programs (Coleman et al., 2006).

1.8.3 Adult mosquito control

Adult mosquito control involves protection of households using insecticides and in some cases insect repellants to prevent mosquito bites. This reduces mosquito longevity, which suppresses community level transmission (Killeen et al., 2002; Killeen et al., 2003). Modern technologies have also been embraced in an attempt to reduce malaria transmission by malaria vector species. This involves the use of genetically manipulated mosquitoes to generate populations with desirable genes that do not transmit malaria parasites, consequently reducing malaria cases (referred to as "strain/population replacement") (Alphey, 2002).

The use of insecticide treated nets (ITN's) for personal protection has been documented to significantly reduce morbidity and mortality due to vector-borne diseases such as RVF and malaria (Zaim et al., 2000). Personal protection measures include: screening of windows and

doors; electrically heated insecticide vaporizing mats; ITN's; pyrethroid-treated bed nets and long lasting insecticidal nets (LLITN's).

1.9 Ecology of Rift Valley fever vectors

1.9.1 Vectors of RVF

RVF is transmitted by vectors such as mosquitoes and midges (Hoogstraal et al., 1979; Moutailler et al., 2008). However, mosquitoes (Diptera: Culicidae) are considered the main vectors of RVFV from which most virus isolates have been obtained. Mosquitoes belonging to a wide range of genera, including *Aedes* (*Neomelaniconion* and *Stegomyia*), *Culex*, *Mansonia*, *Anopheles* and *Eretmapodites*, are capable of transmitting RVFV (Turell et al., 2010). The vectors of RVF can be categorised into two major groups namely ''reservoir" or "maintenance'' vectors, which include certain *Aedes* species associated with newly flooded temporary (Fontenille et al., 1998; Fontenille et al., 1994) or semi-permanent freshwater bodies (Gear et al., 1951) and "epidemic" or "amplifying'' vectors, associated with more permanent freshwater bodies (McIntosh & Jupp, 1981) Table 1.2. In Kenya, *Ae. ochraceus* and *Ae. mcintoshi* (Fig. 1.3) are the key primary vectors of RVF. These vectors belong to the sub genera *Neomelaniconion* and *Aedimorphus*, respectively (Sang et al., 2010). In West Africa, *Ae. ochraceus* alongside *Ae. vexans arabiensis* and *Ae. dalzieli* have been incriminated as important vectors of RVFV. *Aedes vexans arabiensis* is also known to be a vector of RVFV in Saudi Arabia (Fontenille et al., 1998). Amplification of RVFV during outbreaks involves secondary vectors belonging to the genera *Anopheles, Culex, Eretmapodites,* and *Mansonia*, which circulate RVFV after its introduction by primary vectors (Woods et al., 2002).

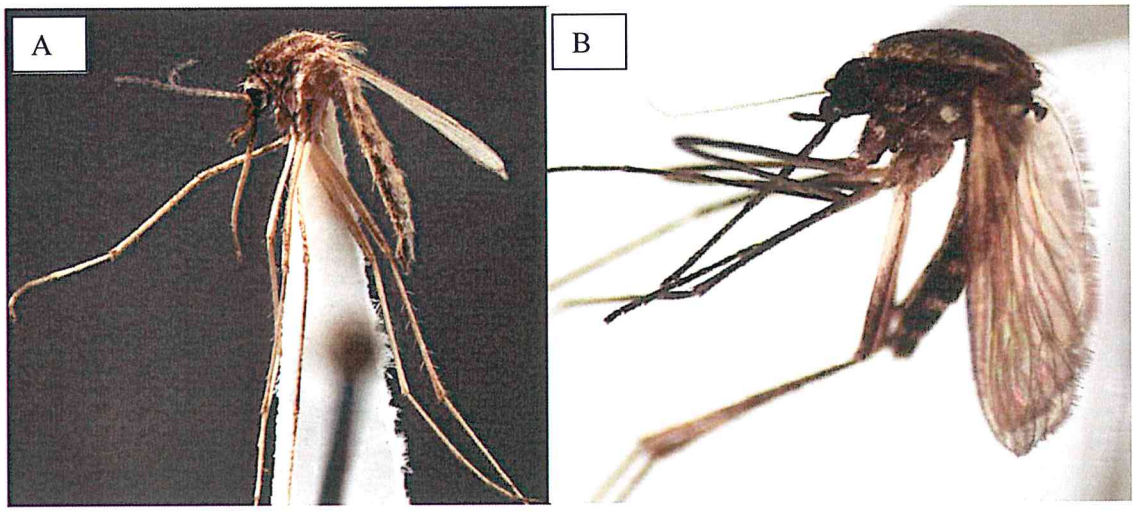


Figure 1.3: Photos of *Ae. ochraceus* (A) and *Ae. mcintoshi* (B) (Adapted from Walter Reed biosystematics unit and Biodiversity heritage library)

In the RVFV epidemiological model, it is thought that the virus is maintained in *Aedes* mosquitoes through eggs by vertical transmission during inter-epizootic periods, and that outbreaks depend on high vector densities following heavy rainfall or other environmental disturbances (Linthicum et al., 1999).

Table 1.2: Table showing primary and secondary vectors of RVF

Vector group	**Mosquito Species**	**Regional distribution**	**References**
Primary vectors	*Ae. mcintoshi*	East Africa	Linthicum, 1985
	Ae. ochraceus	East Africa	Sang et al., 2010
	Ae. vexans	West Africa	Fontenille, 1998
	Ae. dalzieli	West Africa	Fontenille, 1998
Secondary vectors	*Cx. pipiens*	East and West Africa	Sang et al., 2010, Fontenille, 1998
	Ma. africana	East Africa	Sang et al., 2010
	Ma. uniformis	East Africa	Sang et al., 2010
	Ae. sudanensis	East Africa	Sang et al., 2010
	Cx. poicilipes	East and West Africa	Sang et al., 2010, Fontenille, 1998
	Cx. univittatus	East and West Africa	Sang et al., 2010, Fontenille, 1998

1.9.2 Biology of RVF vectors

Knowledge of the biology of RVF vectors requires understanding of how both biotic and abiotic factors contribute to the success in their life cycles. It is therefore important to put into perspective the biology of the vectors in order to successfully control RVFV at different stages of its transmission cycle. The mosquito has four distinct stages in its life cycle: egg, larva, pupa, and adult. The adult is an active flying insect, while the larva and pupa are aquatic. Depending on the mosquito species, eggs are laid either on the surface of water or are deposited on moist soil or other ground that will often be flooded (e.g., floodwater *Aedes*) (Linthicum et al., 1985). The main breeding habitats for floodwater *Aedes* species associated with RVFV are "dambos" or shallow grassland depressions associated with river drainage systems and subject to seasonal flooding (Mäckel, 1974). In general, the life cycle of a mosquito can be completed in 5-14 days but this is dependent on species, ambient temperature and humidity (Blackmore & Lord, 2000). In tropical climates, development is rapid and therefore the egg-adult development time may be completed in 6 days (Gillies & De Meillon, 1968). However, it is important to note that the developmental parameters of *Ae. ochraceus* and *Ae. mcintoshi* under optimal conditions is not well documented and requires further investigation.

1.9.3 Egg biology

After mating and blood feeding, a gravid female mosquito lays about 50-500 eggs the second day after blood feeding (Clements, 2011). One common factor in all mosquito species is the association of their eggs with free water or moist surfaces. Eggs are white when first deposited, darkening to a black or dark brown within 12-24 hours. Single eggs are about 0.5 mm long, and those of most species appear similar when seen by the naked eye (the eggs of Anopheles species are an exception, which have floats attached to each side of the egg;

Coetzee, 2006). The hatching dynamics of floodwater *Aedes* (subgenera *Aedimorphus* and *Neomelaniconion*) mosquitoes depends on rainfall pattern. *Aedes* females lay eggs in pond mud or floodplains. Although these eggs become desiccated when ponds dry up, they remain viable for several years or even decades in a state of diapause (Bentley & Day, 1989; Service, 1977). This diapause is broken only when the fertile eggs are submerged in water of reduced oxygen content (Gjullin et al., 1950). However, not all eggs hatch uniformly in response to submersion in deoxygenated water, and some require repeated exposures to floods before hatching (Gjullin et al., 1950). This breeding pattern results in the emergence of enormous numbers of *Aedes* floodwater mosquitoes in floodplains and other habitats where they oviposit and consequently, the development of a large first generation vector population. In cases of vertical transmission of RVFV it is this mass emergence of the mosquitoes during flood seasons that results in amplification of the virus and subsequent dissemination by both primary and secondary vectors (Mondet et al., 2005; Vignolles et al., 2009).

1.9.4 Larval biology

The immature stages of *Aedes* mosquitoes have been neglected in many studies. They have never been used to help define the species of the group or to help place these species into a natural classification. This has created a gap in the understanding of the bioecology of most *Aedes* species. The larvae of all mosquitoes live in water and have four developmental periods or instars denoted 1^{st}, 2^{nd}, 3^{rd}, and 4^{th}, with each succeeding stage larger than the previous. At the end of each instar, the larva sheds its cuticle in a process called molting. The larva is an active feeding stage. The larvae feed on particulate organic material in the water (Gillies & Coetzee, 1987). The larvae of most species have a breathing tube and must occasionally come to the surface of the water to obtain oxygen. The total duration of time that larvae spend in the larval stage depends on the species and water temperature. Some can

develop in as little as 5 or 6 days (Gillies & De Meillon, 1968). Floodwater *Aedes* larvae develop in temporary woodland pools and dambos when sufficiently flooded (Vignolles et al., 2009).

1.9.5 Pupal biology

The mosquito pupa is very active, and, like the larva, it lives in water. It differs greatly from the larva in shape and appearance. The pupa has a comma-shaped body divisible into two distinct regions. The front region consists of the head and thorax (cephalothorax) and is greatly enlarged. It bears a pair of respiratory trumpets on the upper surface which must periodically come to the surface to permit gas exchange (Coetzee, 2004). The second region is the abdomen, which has freely movable segments with a pair of paddle-like appendages at the tip. Feeding does not take place during the pupal stage. The pupal stage lasts for a few days only and this is the stage when larval tissues are rearranged into those that are characteristic of the adult. The adult emerges directly from the pupal case on the surface of the water.

1.9.6 Adult biology

The adult mosquito is entirely terrestrial and is capable of flying long distances (Foster and Takken, 2004). Both females and males feed on nectar, which they use for energy (Dekker et al., 2001; Foster & Takken, 2004; Gary & Foster, 2004). Females and males mate only onceafter they have emerged. Only the females feed on blood, which they require in order to develop eggs (Clements, 2011; Ribeiro, 2000). Many mosquitoes feed on warm-blooded birds or mammals, although *Culex* species prefer birds and seldom feed on mammals and are known to transmit West Nile virus (WNV) (Gad et al., 1999). Many mosquito species feed on a wide range of warm-blooded mammals and humans. Once a female has completely engorged, it flies to a shaded environment until her eggs are completely developed (usually 2

to 5 days) (Takken & Knols, 1999). Once the eggs are developed the female is called a gravid female and she begins to search for a desirable place to lay her eggs. If a female survives her egg laying activities, she will very soon start searching for another blood meal after which she will lay another batch of eggs. Generally a female will only live long enough to lay 1 to 3 batches of eggs. During the daytime, the females normally rest in cool vegetated areas where the humidity is high so that they are protected from desiccation (Paaijmans & Thomas, 2011).

1.9.7 Mosquito breeding habitats

Mosquitoes breed in permanent or any temporary water bodies. The larvae live in shallow water, that is, 30 cm or less (Aditya et al., 2006). The presence of habitats conducive for breeding, and their quantitative (water depth) and qualitative characteristics (natural/artificial, permanent/temporary, shady/lighted, water movement, vegetation, and turbidity), determine the presence or absence of different mosquitoes species (Almirón & Brewer, 1996; Rueda et al., 2006). RVF vectors, for instance, have been associated with *dambos*, which are usually formed after heavy rainfall and extensive flooding (Davies, 1975; Sang et al., 2010). Relatively few mosquito species actually breed in permanent bodies of water such as marshes or swamps, and most of the mosquito species that make use of these habitats actually breed in temporary pools along their margins (Devi & Jauhari, 2005).

Mosquitoes select oviposition sites using visual and chemical cues. Chemical cues originate from water bodies due to breakdown of bacterial origin or from mosquitoes as oviposition pheromones (Bentley & Day, 1989). Plant volatiles from wetlands or mosquito breeding grounds have also been found to attract mosquitoes to oviposition sites (Rejmánková et al., 2005). These stimuli are responsible for the aggregation of eggs in sites suitable for egg development (McCall & Cameron, 1995). Oviposition activities and the time of oviposition

are determined by factors including ambient temperature, light conditions and timing of the blood meal (Clements, 1999). Studies have also shown that oviposition time is regulated by light-dark cycle and characteristics of the potential oviposition site (Sumba et al., 2004).

1.9.8 Survival patterns and longevity of mosquitoes

In tropical regions, the life span of adult mosquitoes range from a few days to several weeks and it is frequently longer in temperate regions. The life span of females for species that overwinter as adults may approach one year (Delatte et al., 2009; Joshi, 1996; Leisnham et al., 2008). The flood water *Aedes* of the sub genera *Aedimorphus* and *Neomelanconion* are known to survive the dry seasons through eggs that hatch when the dry habitats are flooded during heavy rainfall (Linthicum et al., 1985). The adults of these mosquitoes may only survive for a period not more than 30 days and occasionally attain two reproductive cycles in a flooding season (Delatte et al., 2009). Duration of lifespan is, however, influenced by environmental variables such as temperature and humidity (Leisnham et al., 2008).

1.9.9 Mosquito resting behaviour

Mosquitoes are known to have different resting preferences in different environments. Some of the mosquitoes are adapted to resting indoors while others may rest outdoors in natural vegetation or other structures (Burkett-Cadena et al., 2008). Other studies have reported that the resting behaviour of mosquitoes may influence their capacity in disease transmission (Takken & Knols, 1999). Consequently, this behaviour can be exploited in vector control in cases where resting sites are well known (Pates & Curtis, 2005). Factors such as availability of hosts and of resources necessary for mosquito life cycle largely influence the resting behaviour (Foster, 1995). The use of natural vegetation by mosquitoes as resting sites has not been well documented. However, some studies have reported that this interaction may be for sugar or nutritional benefits during the mosquito life cycle (Müller & Schlein, 2006). Other

plants are also known to produce chemical compounds that repel mosquitoes and may make them unsuitable resting sites (Tawatsin et al., 2001).

1.9.10 Feeding preference of the vector mosquitoes

Mosquitoes that are competent RVFV vectors obtain the virus by vertical transmission or feeding on viremic mammals (Linthicum et al., 1984). Therefore, it is important to understand mosquito host-feeding patterns and preference. Feeding patterns of RVFV vectors have important consequences for the amplification and transmission of diseases to vertebrate hosts (Sardelis et al., 2001). Knowledge of the feeding patterns of these vectors can help to define efficient disease-control policies, decrease the risks of outbreaks, and attain better knowledge of eco-epidemiology in both humans and other species of interest (Dye & Hasibeder, 1986). Vectors such as *Culex pipiens* L are potential amplifiers in the wild based on their diverse and non specific blood feeding preference (Gad et al., 1999; Kenawy et al., 1987). During the 1977 to 1978 epidemics, this vector was implicated as the primary vector of RVFV, based on a single virus isolation from an unengorged female (Hoogstraal et al., 1979). The incorporation of molecular techniques such as polymerase chain reaction (PCR) and sequencing into the study of mosquito ecology, as a complementary tool to the serological methods, has revealed a broad range of host species, and important local and seasonal differences in diet composition in the wild (Kilpatrick et al., 2008; Molaei et al., 2006; Muñoz et al., 2011). Mosquitoes use many strategies to obtain resources required to complete their life cycle. Factors that determine the host choice by mosquitoes largely depend on the surrounding environment (Lyimo & Ferguson, 2009). There is evidence that mosquitoes use both plant and vertebrate animals for their nutritional requirements. Studies have also shown that the feeding patterns vary across mosquito species with some reported to

show specialised feeding patterns (Takken & Verhulst, 2013). This specialisation may make them competent for transmission of diseases.

The role of mosquitoes as a vector of several emerging diseases necessitates assessment of host-feeding characteristics in native and newly invaded areas as a means of understanding the local risk of arbovirus outbreaks in humans and animals. This may provide insights to the contrasting feeding pattern among mosquito vectors found at different geographical regions and in the wildlife livestock interfaces along the nomadic routes and in disease endemic areas.

1.10 Circulation of viruses in the mosquito vectors

It is important to note that the level of virus activity may vary with geographical region. Ecological similarities or differences may contribute greatly to the level of circulation of different arboviruses. RVFV has been isolated from more than 40 species of mosquitoes from eight genera (Meegan & Bailey, 1988), and laboratory studies indicate that numerous species of mosquitoes and biting midges are susceptible to oral infection, some of which are able to transmit RVFV through bites (McIntosh et al., 1980). Evidence suggests that in certain *Aedes* species of the subgenera *Neomelaniconion* and *Aedimorphus,* the female mosquitoes may transmit RVFV vertically to their eggs (Linthicum et al., 1985). High viremias among infected animals may lead to the infection of secondary arthropod vector species including various *Culex*, *Mansonia* and *Anopheles* species, followed by the spread of this virus to other animals and humans (Davies, 1975). *Aedes albopictus* for example is native to east Asia and is a major vector of human arboviruses such as Chikungunya and dengue in tropical and non-tropical areas where it has caused several outbreaks in recent years (Aranda et al., 2006; Martin et al., 2010). The invasive *Cx. pipiens,* has also been noted as an important vector for

WNV transmission (Fonseca et al., 2004; Hamer et al., 2008; Turell et al., 2001a) but it is not competent for dengue or some other tropical viruses (Reiter, 2010; Vazeille et al., 2008). During outbreaks, other mosquito borne arboviruses have been observed to co-circulate with RVF, including Bunyamwera (BUN), Chikungunya (CHIK), Pongola (PO), Ndumu (NDU), Babanki (BBK), Semiliki forest (SF), Sindbis (SIN) and West Nile (WN) (LaBeaud et al., 2011a; LaBeaud et al., 2008; Sang et al., 2010). The co-circulating viruses were detected from the same mosquito species found transmitting RVFV during the East African outbreak that occurred in 2007-2008 (Crabtree et al., 2009; Sang et al., 2010). These viruses continue to actively circulate during RVF inter-epidemic periods with occasional isolations being reported from mosquitoes. Transmission of RVF and other arboviruses is largely dependent on the availability of critical populations of susceptible or naive vertebrate host populations at a given place in time, which serve as amplifiers of the virus when it coincides with high abundance of competent mosquito vectors. Competence of the vectors in circulation of arboviral pathogens may be influenced by a number of factors including susceptibility, abundance, survival, and feeding preference.

1.11 Overview

The majority of emerging human and animal infectious diseases are zoonoses, of which vector-borne diseases comprise a large percentage (Choffnes et al., 2007). Given the rapid growth of human and domesticated animal populations, and their increasing contact with each other and with wild animals, the zoonotic disease threat is expected to increase (Karesh et al., 2005). RVF occurrence is determined by ecological factors, which influence the periodicity of RVF epizootics (Anyangu et al., 2010). This among other vector-borne diseases have potential to cause enormous economic harm when they affect livestock, and even the threat of infection can severely limit trade of livestock and associated commodities.

It has been hypothesised by some studies that livestock become infected along the nomadic routes while in search of pasture and then bring the virus back to villages where vectors may amplify the viruses (Owange et al., 2014). Increasing southern ocean temperatures influence precipitation in Africa and elsewhere to a major extent, which results in prolonged rainfall that influences vector breeding and propagates circulation of vector-borne diseases (Anyamba et al., 2006). Although considerable attention and resources have been committed to important vector-borne diseases like malaria, relatively little is known about the ecology of the vectors associated with the transmission of RVF, which needs to be well understood to improve our options for control of the disease especially in areas which experience reoccurrence of the disease. Increasing geographical distribution and incidence of arboviruses at each epidemic across the world are pertinent reasons for conducting studies on the bioecology of potential vectors of RVF in the affected regions such as the northeastern region of Kenya. The ability to understand the dynamics of RVF maintenance, its transmission, and to develop effective control lies in an appreciation of the ecology of the important primary vectors that have been incriminated in the transmission of the virus. This thesis determined the ecology of vectors of RVF with a view to delineating their spatial distribution, abundance, survival, feeding preference and virus circulation among the vectors in sites along the livestock movement routes in north eastern Kenya.

1.12 Hypotheses

a) The distribution, abundance and diversity of the primary vectors of RVF, *Ae. mcintoshi* and *Ae. ochraceus* will vary between RVF hotspots in the northeastern region of Kenya as a consequence of spatial and temporal variation in environmental conditions.

b) Survival rates of *Ae. mcintoshi* and *Ae. ochraceus* in the selected sites along livestock movement routes (LMR) will vary due to differences in environmental conditions.

c) Host feeding preferences in *Ae. mcintoshi* and *Ae. ochraceus* will vary with their spatial distribution along livestock movement routes (LMR) and wildlife-livestock interfaces due to changes in the abundance of host species

d) Circulation of RVF and other arbovirus in *Ae. mcintoshi, Ae. ochraceus* and other mosquitoes along the LMR and livestock wildlife interface will vary based on environmental conditions, including host abundance.

1.13 Objectives

1.13.1 General Objectives

To understand the ecology of important primary vectors of Rift Valley Fever in the North Eastern region of Kenya.

1.13.2 Specific Objectives

a) To determine the spatial distribution and diversity of the vectors of RVF along livestock movement routes (LMR) in north eastern and coastal regions of Kenya.

b) To determine the survival rates and resting preference of *Ae. mcintoshi* and *Ae. ochraceus* in the selected sites and along LMR.

c) To determine host preference characteristics among RVF vectors in relation to their spatial distribution along LMR and livestock wildlife interfaces in north eastern Kenya.

d) To determine the level of circulation of RVF and other arbovirus in vector species along the LMR in north eastern Kenya.

Chapter 2

DISTRIBUTION AND DIVERSITY OF THE VECTORS OF RIFT VALLEY FEVER ALONG THE LIVESTOCK MOVEMENT ROUTES IN THE NORTHEASTERN AND COASTAL REGIONS OF KENYA

2.1 Introduction

Rift Valley fever (RVF) is a viral mosquito-borne disease that mainly affects livestock and humans, although many other mammalian species have also been shown to be susceptible (Kasari et al., 2008; Martin et al., 2008; Sissoko et al., 2009). It causes abortions and high mortality in young animals, and in humans it presents as a non-specific flu-like syndrome through to encephalitis, and ocular or hemorrhagic syndrome (Woods et al., 2002). RVF is caused by RVF virus (RVFV), one of the six hemorrhagic fever viruses that occur in Africa (Mandell & Flick, 2011; Métras et al., 2011). Although epidemics of the disease have been occurring in sub-Saharan Africa at irregular intervals, there is limited knowledge on how the virus is maintained during inter-epidemic periods, and the factors contributing to the re-emergence of the disease in hotspot areas are poorly understood. Importantly, gaps remain in our understanding of critical aspects of the ecology of potential vectors and how the vector-virus-host interaction influences the epidemiology of RVF (Anyamba et al., 2009; Chevalier et al., 2009; Métras et al., 2011; Sissoko et al., 2009).

RVF is a vector-borne disease usually transmitted to mammals by mosquitoes (Diptera: Culicidae), and mainly depends on the availability of competent vectors, susceptible hosts, and suitable ecological and environmental conditions that favour mosquito survival and reproduction (Diallo et al., 2000; Fontenille et al., 1998). RVF vectors can be classified into two major groups, namely primary and secondary vectors. In Kenya, the known primary vectors, *Aedes mcintoshi* Huang and *Aedes ochraceus* Theobald, are believed to serve as reservoirs for the virus (Fontenille et al., 1998; Fontenille et al., 1994; Traore-Lamizana et al.,

2001). Breeding of these vectors has mostly been associated with characteristic shallow depressions on land called "dambos" (Linthicum et al., 1985). The dambos are usually flooded after heavy rainfall, resulting in mass emergence of floodwater *Aedes* mosquitoes (Linthicum et al., 1984; Linthicum et al., 1985). The primary vectors maintain RVFV transovarially by transmitting the virus through to the eggs (Linthicum et al., 1985). The infected eggs can enter diapause in dry dambos for long periods and hatch into infectious mosquitoes during periods of extended rainfall. This may result in transmission of the virus to nearby animals and human beings when the vectors seek blood meals. Once primary transmission of the virus has taken place, secondary vectors belonging to the genera *Culex*, *Anopheles* and *Mansonia*, which take over flooded grounds for breeding, contribute to the amplification of the virus due to their ubiquitous biting patterns, consequently resulting in outbreaks (Anyangu et al., 2010; Linthicum et al., 1999; McIntosh & Jupp, 1981; Woods et al., 2002).

Northeastern Kenya is an important hotspot for RVF, being the region hardest hit by outbreaks in 1997/98 and 2006/07 (Anyangu et al., 2010; Nguku et al., 2010). These outbreaks affected over 18 districts, herdsmen lost their lives, and large economic losses were incurred due to animal abortions and deaths, as well as a ban on livestock trade and transportation (Nguku et al., 2010). In this region, pastoralism is the main source of livelihood and income. Pastoralism is a major production strategy in which people raise herds of animals, mostly in arid and semi-arid lands. Arid and semi-arid land covers about 80% of Kenya's landmass, and supports about a third of the country's human population and 70% of the national livestock kept in large herds. Due to limited and unpredictable rains, herders practice nomadic pastoralism, moving animals in large herds in search of pasture and water. This practice also favours convergence of domestic and wild animals from time to time,

which may create opportunities for cross transmission of diseases. Such an interface may serve as virus emergence points or reservoirs during the inter-epidemic period and also create variable risk points for infection of susceptible livestock.

Like most arboviruses, RVF is driven by a complex interaction of mosquito vector populations and vertebrate hosts in different habitat types under varying environmental conditions. (Fontenille et al., 1998; Linthicum et al., 1985; Lutomiah et al., 2013; Sang et al., 2010). During previous outbreaks, key primary vectors of RVF virus were identified (Sang et al., 2010) but the limited understanding of their ecology in diverse ecological zones and the interplay with the nomadic pastoral systems along the major livestock movement routes (LMR) was unknown. For these reasons, this study sought to determine the species composition and diversity of potential RVFV vectors along LMR in northeastern Kenya. The reported research represents part of an ongoing project to track RVF prevalence in nomadic herds along LMR to identify risk foci that can be targeted for RVF prevention measures. It is also envisaged that tracking of animal movement will permit identification of areas where introduction or amplification of the disease could potentially occur from wild disease reservoirs or hosts due to a high density of RVF vector populations, which could contribute to understanding of RVF epidemiology and present opportunities for strategic disease prevention.

2.2 Materials and methods

2.2.1 Study site

This study was conducted along nomadic livestock movement routes (LMR) established by the tracking of a sentinel herd, which moved in search of pasture and water in northeastern province and coastal parts of Kenya (Fig. 1) stretching between Garissa S00° 39' E40° 05' and Lamu S2° 16' E40° 54' Counties. Garissa County is traditionally occupied by the Somali ethnic group and over 80% of the land is earmarked for livestock production. The sparse population of approximately 7 people/km² of the district is found concentrated around the water sources and also around small market centers (Aklilu & Wekesa, 2002; Hogg, 1986). Mean annual rainfall varies between 200 and 500 mm with occasional torrential storms causing extensive flooding. Rainfall is bimodal; the long rains occurring from April and May and the short rains in October and November with occasional variation. The peak dry spell usually occurs between January and March. Generally, Garissa County is hot and dry with average daily temperatures ranging from 20°C to 38°C. Lamu County is a coastal cosmopolitan area with several communities practicing diverse cultures and economic activities including small scale farming, hunting and fishing. The expansive grasslands in the region form the major attraction for nomadic pastoralists from the neighboring Garissa County, who routinely migrate into the region during drier seasons with their livestock to access pasture. Seven sites along the LMR were sampled: Haney, Degurdei, Arbadobolo, Boni, Dondori Mlimani and Mangai (Fig. 2.1). Data collection in each sampling area targeted three seasons; the dry season and the long and short rainy seasons. The dry season (January-March) preceded the long rainy season. Over the course of the study, in the town of Garissa, the mean monthly precipitation in the dry season was 16 mm, with an average maximum temperature of 38.5°C, minimum average temperature of 28°C, and an average relative humidity of 51%. The long rainy season in the region usually commences in the month of

April and sometimes extends into late June. In Garissa, the mean monthly precipitation for the long rainy season was 63 mm, with an average maximum temperature of 34°C, an average minimum temperature of 26°C, and an average relative humidity of 64%. The short rainy season lasted for two months between November and late December before the recommencement of the dry season. The mean monthly precipitation for the short rainy season in Garissa was 58 mm, the average maximum temperature was 35°C, the minimum average temperature was 26°C, and the average relative humidity was 63%. The limited weather data available in this study was due to a lack of weather stations closer to the study sites, which were located in the remote areas of northeastern Kenya. Insecurity in the region also made it difficult to access, manage and download weather data from stations installed in previous studies. The weather in Garissa is largely representative of the region.

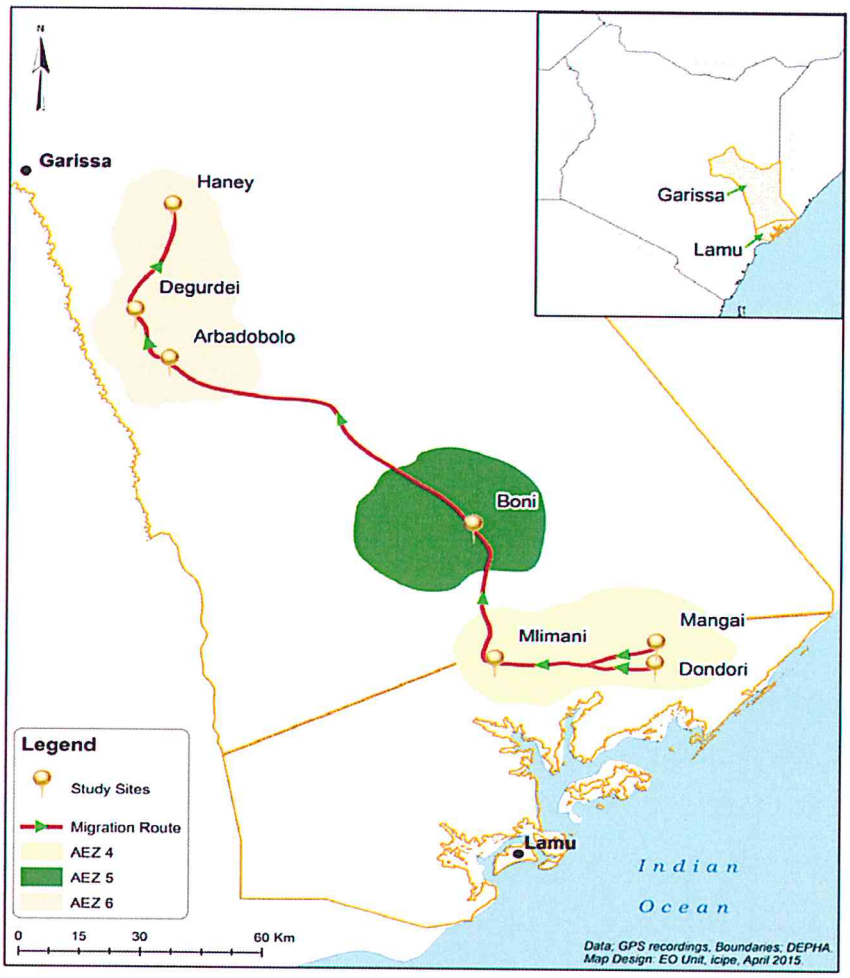


Figure 2.1: Map of Kenya showing sampling sites, livestock movement routes and ecological zones

The selected sites lie within three major ecological zones of Kenya. (Davenport & Nicholson, 1993; Lind et al., 1974). Haney, Degurdei and Arbadobolo are located in the semi arid zone (Zone VI), which is characterised by dry woodland vegetation and wooded or bushed grassland. Trees in the semi arid zone are typically *Acacia* species. The vegetation in this ecological zone is patchily distributed with most plant species being well adapted to dry conditions, with the exception of herbaceous plants found growing in areas that hold water for extended durations after it rains. Boni is located within the forest ecological zone (Zone

V), which is comprised of expansive indigenous woodland, with trees typically being the broad-leaved *Combretum*, alternating with patches of grassy fields. Mangai, Dondori and Mlimani areas are located within the humid to dry sub-humid zone (Zone IV), which is comprised of expansive grassland with patches of shrubby vegetation along the coastal region. The ecological zones represented in this study are important to pastoralists given that they provide pasture during different seasons and determine the livestock migration routes.

2.2.2 Sampling and identification of mosquito vectors

Mosquito surveys were conducted using CO_2-baited CDC light traps (John W. Hock Company-Model 512) during the long rains (April - June) and short rains (November – December) at each of the study sites between November 2012 and August 2014 (Figure 2.2). Dry ice was used as a source of CO_2/bait. During trapping one CO_2 dispenser was suspended alongside each trap to release CO_2. At each of the seven sites along livestock movement routes, sampling was conducted three times during the short rains in November and long rainy season between April and May, leading to three replicates for each site per season and a total of six replicates for both seasons over the period of study. During each trapping period, ten traps were randomly set at 1800 hrs and retrieved at 0600 hrs the following day for three consecutive sampling days at each site in both seasons. Trapped mosquitoes were anesthetised using Tri-ethyl amine for ten minutes, sorted, placed into 15 ml labeled vials, and transported to the laboratory in liquid nitrogen for identification. All mosquitoes were morphologically identified to species level using taxonomic keys (Edwards, 1941; Huang & Ward, 1981).

Figure 2.2: Photo showing CDC light trap set at night in the study area

2.2.3 Statistical analysis

Data on total mosquito catches among the different ecological zones and sites were compared using a negative binomial model (Hilbe, 2011). A negative binomial model was preferred over a Poisson model and other models that do not accommodate zero-inflated data based on Vuong test, $P<0.0001$ (Vuong, 1989). Driven by research interest/questions, mosquito captures were also compared separately across sampling sites for each of the three vector groups (primary vectors: *Ae. mcintoshi* and *Ae. ochraceus*; secondary vectors: species of the genera *Mansonia, Culex* and *Anopheles* with exception of malaria vectors; and other flood water *Aedes*: *Ae. sudanensis* Theobald and *Ae. tricholabis* (Edwards) while controlling for season (long rain =1, short rain=2). Mosquitoes were placed into these vector groups based on their importance/role in RVF maintenance and transmission (Sang et al., 2010). Risk ratios (RR) were computed for each site in comparison to Mangai, which had the highest number of mosquito catches. For ecological zones, zone IV was taken as the reference group by default. Overall factor effect in the NB model was assessed using Wald test (Agresti & Min, 2002), and different factor levels compared using appropriate contrast statements. To obtain

information on the rarity and commonness of vector species, we estimated the Shannon (H) and Simpson (D) diversity indices for each of the three replicate trappings during the short and long rainy seasons per site using the 'vegan' library (Oksanen et al., 2013) in R. The diversity indices were then compared across the sites and ecological zones, while controlling for season using analysis of variance (ANOVA). Species richness (that is, the number of individual species) across ecological zones, were also compared using ANOVA, which was deemed appropriate as the data were normally distributed (Shapiro test: $p = 0.2107$). This study did not focus on microhabitat differences around individual traps but larger-scale ecological effects in the entire LMR, hence the reason for pooling data for each trapping period. All analyses were performed in R version 3.1.1 (Kembel et al., 2010) for all three replicates combined at $\alpha = 0.05$ significance level.

2.3 Results

2.3.1 Abundance of vectors of RVF in diverse ecological zones and sites

A total of 31,727 mosquitoes (mean=755.5, variance=430045.8) comprising 21 species belonging to 6 genera were captured from the 7 sampling sites (Table 2.1). A goodness of fit (GOF) test indicated that the negative binomial model comparing total mosquito abundance across regions/sites fitted the data well (GOF Chi Sq. = 46.82, df = 39, P=0.1822). Overall mosquito abundance varied significantly by ecological zones (Wald test =14.8, df = 2, P = 0.0006). Compared to ecological zone IV, mosquitoes were significantly fewer in ecological zone VI (RR=0.34, 95% CI: 0.19-0.59), but not significantly different from zone V (RR=0.54, 95% CI: 0.26-1.26). The overall mosquito abundance also varied across sites (Wald test =171.9, df = 6, P< 0.0001). The highest number of mosquitoes was trapped in Mangai in ecological zone IV (n=10,740) while the lowest occurred in Haney in ecological zone VI (n=282). *Aedes* was the most diverse taxon, mostly represented by the floodwater

species *Ae. mcintoshi*, *Ae. ochraceus*, *Ae. tricholabis* and *Ae. sudanensis*, which were fairly well represented across ecological zones. Among the *Aedes* species, *Ae. ochraceus* and *Ae. mcintoshi* (the primary RVF vectors associated with previous outbreak) were the most abundant.

Table 2.1: Summary of mosquito catches across the sites and ecological zones in northeastern Kenya

		Mosquito species																					
Ecological Zone	Sites	*Ae. mcintoshi*	*Ae. ochraceus*	*Ae. tricholabis*	*Ae. sudanensis*	*Ma. africana*	*Ma. uniformis*	*An. squamosus*	*An. gambiae s.l*	*An. funestus s.l*	*Cx. pipiens s.l*	*Cx. poicilipes*	*Cx. univittatus*	*Cx. bitaeniorhynchus*	*Cx. tigripes*	*Cx. antenatus*	*Coquilletidia aurites*	*Ae. vitattus*	*Ae. hirsutus*	*Ae. africana*	*Ae. tarsalis*	*Ad. africana*	Total mosquitoes/site
Ecological zone IV	Mangai	190	1497	0	48	5838	593	953	785	227	408	93	56	2	21	13	9	1	0	0	5	1	**10740**
(Humid to dry sub-humid)	Dondori	73	467	0	21	4994	316	315	95	28	23	10	5	1	2	3	0	0	0	0	0	0	**6353**
	Mlimani	210	1690	43	481	409	0	193	16	5	593	99	25	0	9	2	1	0	0	0	0	0	**3776**
Ecological zone V	Boni forest	495	1234	977	122	16	0	35	12	0	305	500	75	2	2	0	0	0	0	0	0	0	**3775**
Ecological zone VI	Degurdei	2796	86	21	148	0	0	5	3	0	999	219	141	2	17	3	0	0	0	0	0	0	**4440**
(Semi-arid)	Arbadobolo	923	667	491	139	0	0	0	4	0	78	2	9	0	35	11	2	0	0	0	0	0	**2361**
	Haney	80	56	4	22	12	0	0	0	0	40	0	0	0	0	0	0	20	22	17	6	3	**282**

While the highest number of these primary vectors of RVF occurred in zone VI (n=4,608), there were zone and site specific differential abundances between the two species. .*Ae. ochraceus* dominated zones IV and V in Mangai, Dondori, Mlimani and Boni Forest, while *Ae. mcintoshi* was more abundant across zone VI in Haney, Degurdei and Arbadobolo (Table 2.2).

Table 2.2: Distribution and abundance of primary vectors of RVF a cross the ecological zones and sites

Ecological zone	Sites	Primary vectors		
		Ae. mcintoshi	*Ae. ochraceus*	Total
Zone IV	Mangai	190	1497	1687
	Dondori	73	467	540
	Mlimani	210	1690	1900
Zone V	Boni forest	495	1234	1727
Zone VI	Haney	80	56	136
	Degurdei	2796	86	2882
	Arbadobolo	923	667	1590

Other vectors also important in circulation of arboviruses comprised the genus *Culex* which was mostly represented by *Culex pipiens* L with the other species in this genus occurring in much reduced numbers especially in the ecological zone VI. *Mansonia africana* Neveu-Lemaire and *Ma. uniformis* Theobald represented the genus *Mansonia* with the former occurring in higher numbers relative to *Ma. uniformis*. Although *Mansonia* species dominated the overall captures from all sites (n=12181, 38.3%), these two vectors (*Ma. africana* and *Ma. uniformis)* were almost entirely found in ecological zone IV with only 0.13% (n=16) and 0.1% (n=12) abundance in ecological zones V and VI, respectively. Anopheline species trapped during this study comprised *An. squamosous* Theobald, *An.*

gambiae s.l. Giles and *An. funestus s.l.* Giles, which were mainly trapped in ecological zones IV and V

Negative binomial model results comparing the abundance within each vector group across sites are presented in Table 2.3. The table shows a significant difference in abundance of primary vectors across the sites (Wald test=250.4, df=6, P< 0.0001) and seasons with significantly higher captures recorded during the long rains compared to the short rains (RR=0.42, 95% CI: 0.33-0.53, P< 0.0001). After controlling for season, the numbers of primary vectors caught were significantly higher in Degurdei but lower in Haney and Dondori compared to Mangai (Table 2.3). For the secondary vectors, the catches in all the sites were significantly lower than Mangai, after controlling for season with Haney recording the lowest abundance. The other floodwater *Aedes* group of vectors also demonstrated significant differences in the abundance of mosquitoes across the study sites, with Arbadobolo, Boni forest, Dergurdei and Mlimani recording more catches than Mangai after adjusting for season. In terms of ecological zones, there were no differences in abundance of primary vectors. While the secondary vectors were fewer in ecological zones V and VI compared to ecological zone IV (Zone V: RR=0.16, 95% CI: 0.06-0.50; Zone VI: RR=0.09, 95%CI: 0.04-0.18), there was no difference in abundance of secondary vectors between ecological zones V and VI (Wald test=1.5, df=1, P=0.2200). The other floodwater *Aedes* were significantly less abundant in ecological zone IV (RR=0.18, 95% CI: 0.04-0.62) and zone VI (RR=0.25, 95% CI: 0.05-0.87), compared to zone V. GOF test indicated that the model fitted primary vectors data well (GOF Chi Sq=41.35, df=34, P=0.1804). Similar observations were made for other vector groups.

Table 2.3: Comparisons of catches of vectors by groups across the study sites in northeastern Kenya

Vectors group	Factors		RR (95%CI)	P value
Primary vectors				
	Site	Mangai	1	
		Arbadobolo	1.07 (0.71-1.61)	0.7451
		Boni forest	1.44 (0.96-2.17)	0.0773
		Degurdei	2.10 (1.40-3.15)	0.0003
		Dondori	0.44 (0.29-0.66)	0.0001
		Haney	0.09 (0.06-0.14)	<0.0001
		Mlimani	1.10 (0.73-1.66)	0.6344
	Season	Long rain	1	
		Short rain	0.42 (0.33-0.53)	<0.0001
Secondary vectors				
	Site	Mangai	1	
		Arbadobolo	0.01 (0.01-0.01)	<0.0001
		Boni forest	0.10 (0.08-0.12)	<0.0001
		Degurdei	0.15 (0.12-0.18)	<0.0001
		Dondori	0.65 (0.55-0.78)	<0.0001
		Haney	0.01 (0.00-0.01)	<0.0001
		Mlimani	0.15 (0.12-0.18)	<0.0001
	Season	Long rain	1	
		Short rain	0.43 (0.39-0.49)	<0.0001
Other floodwater *Aedes*				
	Site	Mangai	1	
		Arbadobolo	18.9 (9.99-36.09)	<0.0001
		Boni forest	32.52 (17.25-61.91)	<0.0001
		Degurdei	3.67 (1.93-7.02)	0.0001
		Dondori	0.46 (0.21-0.99)	0.0546
		Haney	0.53 (0.25-1.11)	0.1058
		Mlimani	11.8 (6.3-22.26)	<0.0001
	Season	Long rain	1	
		Short rain	0.24 (0.17-0.34)	<0.0001

2.3.2 Mosquito species diversity and richness

Shannon diversity index showed significant differences in mosquito species diversity across the ecological zones ($F=3.33$, $df=2,36$, $P=0.0465$) and sites ($F=10.82$, $df=6,36$, $P<0.0001$), after controlling for season. Multiple comparisons based on Tukey's test showed that the diversity varied significantly between zones VI and V ($P=0.033$) but neither between zones IV and VI ($P=0.800$) nor zones IV and V ($P=0.090$). Mosquito diversity indices across the sites are presented in Figure 2.2. Further, significantly higher mosquito diversity was observed during the long rains ($H=2.04$) relative to the short rains ($H=1.85$; $F=9.33$, $df=1,38$, $P=0.0041$). Similar conclusions were made using Simpson diversity index.

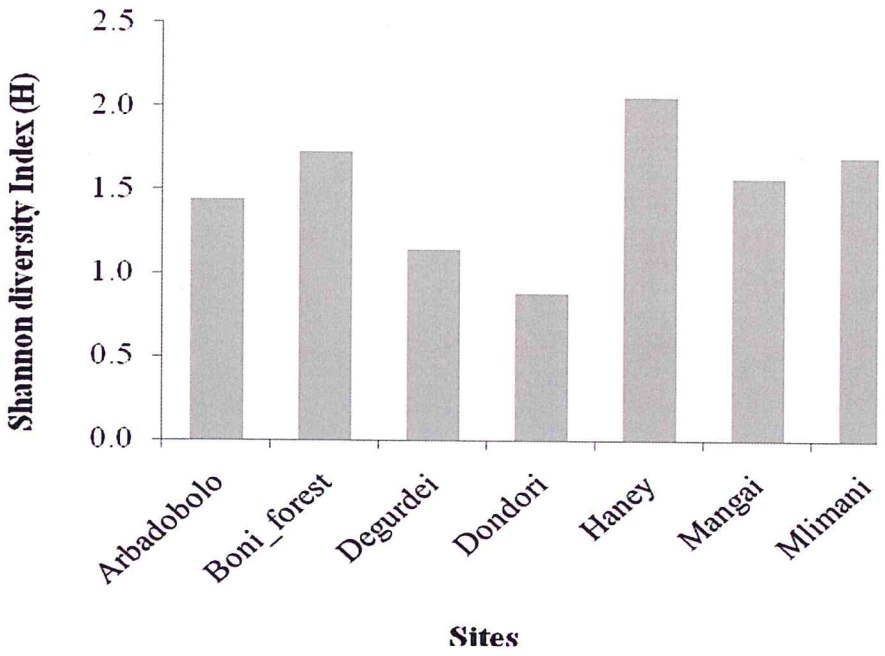


Figure 2.2: Mosquito diversity indices across the sites

Mosquito species richness varied significantly across the ecological zones ($F= 22.98$, $df=2,38$, $P<0.0001$). Ecological zone IV recorded a significantly higher number of species than ecological zone VI ($P<0.0001$) while there was no significant difference in species

richness between ecological zones IV and V. A significantly greater number of species were recorded during the long rains than short rainy season ($F= 26.68$, $df=1,38$, $P<0.0001$). For the sites, Mangai recorded the highest number of species (18), followed by Dondori (14) and Mlimani (14), Boni forest (12) and the rest sites with 11 species each. Residual plots indicated no problems with the fit of the two ANOVA models (i.e., the residual plots showed a random scatter around zero).

2.4 Discussion

An ecological assessment of RVF vectors is a fundamental aspect for the determination of high risk areas where emergence and circulation of RVF virus might occur. In this study, we have shown that the abundance and diversity of RVF vectors along the major nomadic livestock movement route (LMR) in northeastern Kenya vary across sites and ecological zones, which is likely to create variable points of risk for livestock exposure to the disease and subsequent human disease occurrence.

As demonstrated in this study, variation in RVF vector abundance across ecological zones indicates potential risk areas for RVF transmission and circulation. The semi arid ecological zone had a low abundance of vectors compared to other ecological zones but primary vectors of RVFV were associated with this ecological zone. The abundance of these primary vectors and other floodwater *Aedes*, especially in the semi arid zones, may be attributed to the nature of the terrain, soil types and vegetation cover, and rainfall which may influence availability of favourable vector breeding and resting grounds (Lutomiah et al., 2013; Sang et al., 2010). Our study also shows that more of the vectors were trapped during long rains than short rains across all the three ecological zones which may aid the amplification of RVF virus during epidemics. This finding is in agreement with other studies which also pointed out that during

periods of rainfall, mass emergence of mosquitoes may occur in their preferred breeding grounds and lead to epizootics of RVF (Daubney et al., 1931). Differential distribution patterns of RVF vectors may play an important role in understanding the epidemiology of RVFV. However, it is still unclear what causes the differential abundance among the vectors and how this may impact on RVF risk in sites along the LMR. The observed pattern suggests that large scale differences in environmental conditions possibly influence the choice of sites colonised by these vectors, and differences in the abundance of each species may drive RVF virus transmission separately at different sites, influencing levels of virus activity in different sites along the LMR. *Aedes ochraceus* has only recently been implicated as a primary vector of RVFV in northern Kenya, having been involved in circulation of the virus during the 2006/7 outbreak (Sang et al., 2010). The high abundance of *Ae. ochraceus* in ecological zone IV and V suggests the potential suitability of such environments for this species, meaning that *Ae. ochraceus* may drive the transmission of arbovirus in these ecological zones. Recent genetic analysis has also documented population expansion of this species in Kenya, with potential for greater epidemiological importance in future RVF outbreaks (Tchouassi et al., 2014). Similarly *Ae. mcintoshi* could also play an important role in the semi-arid ecological zone where it was the most abundant primary vector.

The data from this study showed an overall low occurrence of *Culex* mosquitoes, especially species known to play secondary roles in the transmission of RVFV such as *Cx. poicilipes* Theobald and *Cx. univittatus* Theobald. However, it was notable that there was variation in abundance of secondary vectors across the ecological zones. Vector populations involved in the circulation of RVFV are known to show a succession pattern with the emergence first of floodwater *Aedes* (primary vectors) whose populations are gradually replaced by those of secondary vectors comprising members of the genera *Culex*, *Mansonia,* and *Anopheles*

(Linthicum et al., 1985). The low occurrence of secondary vectors along the LMR in our study concurs with earlier studies conducted in parts of the Ijara region of northeastern Kenya (Lutomiah et al., 2013; Sang et al., 2010). However, the widespread distribution of species such as *Cx. pipiens* suggests its high level of adaptability to various ecological conditions in this region (Vinogradova, 2000). Due to this, *Cx. pipiens s.l* may be amongst the most important secondary vectors for amplification of the virus during epidemics in the northeastern region of Kenya, as was the case during epidemics in Egypt (Meegan et al., 1980).

The clear preference of *Mansonia* species, which are secondary vectors of RVF (Sang et al., 2010), to sites within the humid to dry sub-humid ecological zone may be related to their biology. These vectors were trapped at sites associated with marshy environments, which are characteristic of ecological zone IV in the coastal regions of Kenya. Even though their distribution was not widespread, *Ma. africana* and *Ma. uniformis* could also play important roles as amplifiers of RVFV in the coastal region of Kenya when the virus is introduced by livestock moving from potential virus circulation zones (Murithi et al., 2011).

This study also revealed that the mosquito assemblages along the LMR had high species diversity and richness. As expected, the species diversity and richness of the RVF vectors was higher during the long rains relative to the short rainy season across the ecological zones. This was likely due to an increased number of vector breeding habitats during long rains, which may have favoured the emergence of many vectors. This finding corroborates results of previous studies conducted during RVF outbreaks in the same region, which highlighted the potential role of prolonged rainfall and mass emergence of mosquitoes as one of the risk factors leading to the severe RVF epidemic in 2007 (Anyangu et al., 2010). Higher diversity

in ecological zone V compared to VI observed during this study could be attributed to the variation in climatic and environmental conditions between these ecological zones, which could potentially influence the adaptation of mosquito species populations in such areas (Minakawa et al., 2002; Reiter & LaPointe, 2007). Forest ecological zone V may, for instance, create humid conditions that improve survival of vector species in comparison with the extreme dry and hot conditions in the semi arid ecological zone VI. Other factors could also include differences in anthropogenic activities, including opening up water points for livestock in the forests, which could potentially influence mosquito breeding patterns between the ecological zones and promote mosquito diversity (Thongsripong et al., 2013). It is however, important to note that we found fewer mosquito species than in earlier studies conducted in Ijara areas of northeastern Kenya (Lutomiah et al., 2013). Factors that may contribute to this difference are the choice of sampling sites along LMR, frequency of sampling employed, method of sampling and duration of our study, which spanned a period of only two years. As such, a long-term, longitudinal study with more spatial replication will be required to unravel potential changes in the mosquito fauna across different seasons and sites in this region. Generally there was widespread occurrence of both primary and secondary vectors of RVFV in varying abundance and diversity across sites and ecological zones on the livestock movement routes used by nomadic pastoralists in northeastern Kenya. This may be important for understanding the epidemiology of RVF together with other mosquito-borne diseases in Northern Kenya. This pattern is likely to create variable risk areas of the disease with regards to infection of susceptible livestock. Mapping of these sites can be provided to the authorities for the purpose of implementing a focused RVF vector control and as a guide to formulating strategic animal vaccination plans for RVF prevention.

Chapter 3

SURVIVAL AND RESTING PREFERENCE AMONG KEY VECTORS OF RIFT VALLEY FEVER IN NORTHEASTERN KENYA

3.1 Introduction

Transmission and circulation of pathogens by mosquitoes is influenced by a range of biological factors including mosquito resting behaviour, survival and availability of suitable environmental conditions (Githeko et al., 2000). Prevailing environmental conditions, in particular, are key to the circulation of vector-borne diseases by insects because they dramatically influence the life history traits of vectors such as development rate, population growth rate and survival (Altizer et al., 2006). Resting preference and survival of mosquitoes may enhance their longevity and consequently increases their vectorial capacity in transmission of vector-borne diseases such as RVF.

Survival of the adult stage of mosquito vectors is highly variable, depending on species and environmental conditions (Delatte et al., 2009; Rueda et al., 1990). The average lifespan of an adult mosquito species can vary from 13 to 20 days. In the tropics, this duration may vary between 10-14 days in *Anopheles* species and may occasionally reach 21 days (Ndoen et al., 2012). Some mosquitoes are also known to live longer by entering dormancy to avoid unsuitable weather periods (Omer & Cloudsley-Thompson, 1970). Other mosquitoes hibernate during the winter as adults, so their lifespan as full-grown mosquitoes lasts for months (Sim & Denlinger, 2011). It is also known that temperature and its interaction with age and sex is an important variable that determines adult mosquito survival (Lyons et al., 2012). Survival in both excessively low and high temperatures is dependent on the ability of insects to thermoregulate through the selection of more moderate microenvironments (Harrison et al., 2012). Consequently, the resting behaviour of mosquitoes may contribute to

their survival in the field, which may then have a direct bearing on disease transmission and the epidemiology of vector-borne diseases.

It is often not practical to directly measure the lifespan of wild mosquitoes. However, some studies have conducted indirect estimates of daily survivorship for anopheline mosquitoes as a means of estimating their longevity (Charlwood et al., 1997). Age of mosquitoes can be determined by the age grading technique, which monitors changes to mosquito morphology (Detinova, 1962). Morphological methods can be performed using laboratory technology, such as fine dissection tools and a light microscope. However, the dissection techniques can be labour intensive when handling a lot of samples. The age grading techniques currently used include Detinova ovarian tracheation (parity), Polovodova ovariole dilatation, and daily growth line methods (Hugo et al., 2008). The most commonly applied morphological age classification technique for mosquitoes has been the ovary tracheation method of (Detinova, 1962). This technique proved successful in grading of mosquitoes as parous and nulliparous as a way of aging mosquitoes (Hugo et al., 2008). Survival is important for mosquitoes because it enables the vector to encounter with the hosts. This promotes the species' role as a vector as it may acquire an infection and transmit the pathogen to non-infected hosts during its gonotrophic cycles (Keesing et al., 2010).

Outdoor and indoor resting habits are common among mosquito vectors. However, it is unclear whether mosquitoes that rest outdoors prefer certain vegetation or resting sites over others. The knowledge of the resting sites of different mosquito species is important for targeted vector control, and may optimise sampling of fed mosquitoes for studies on their host preferences (Cupp et al., 2004; Hassan et al., 2003; Komar et al., 1995). Studies have documented variability in resting preference among mosquito species with some known

disease vectors showing variable resting tendencies including endophillic and exophillic resting behaviour (Coetzee et al., 2000; Paaijmans & Thomas, 2011). However, most studies conducted on survival and resting of mosquitoes have focused on vectors of malaria as opposed to vectors of other diseases such as RVF. Vectors of RVF such as *Aedes mcintoshi* (Huang) and *Aedes ochraceus* (Theobald) are widely distributed in diverse ecological zones in northern Kenya and have played an important role in transmission of the arbovirus in this region (Sang et al., 2010). When a blood meal is successfully obtained by these vectors, mosquitoes infected during the process often need to survive through the incubation period before they can successfully transmit the disease (De Moor & Steffens, 1970; Onori & Grab, 1980). However, survival of these mosquitoes at any given time may also be influenced by a number of factors such as climatic variables and parasite burden (Faran et al., 1987; Minakawa et al., 2002; Muturi et al., 2008; Reiter, 2007).

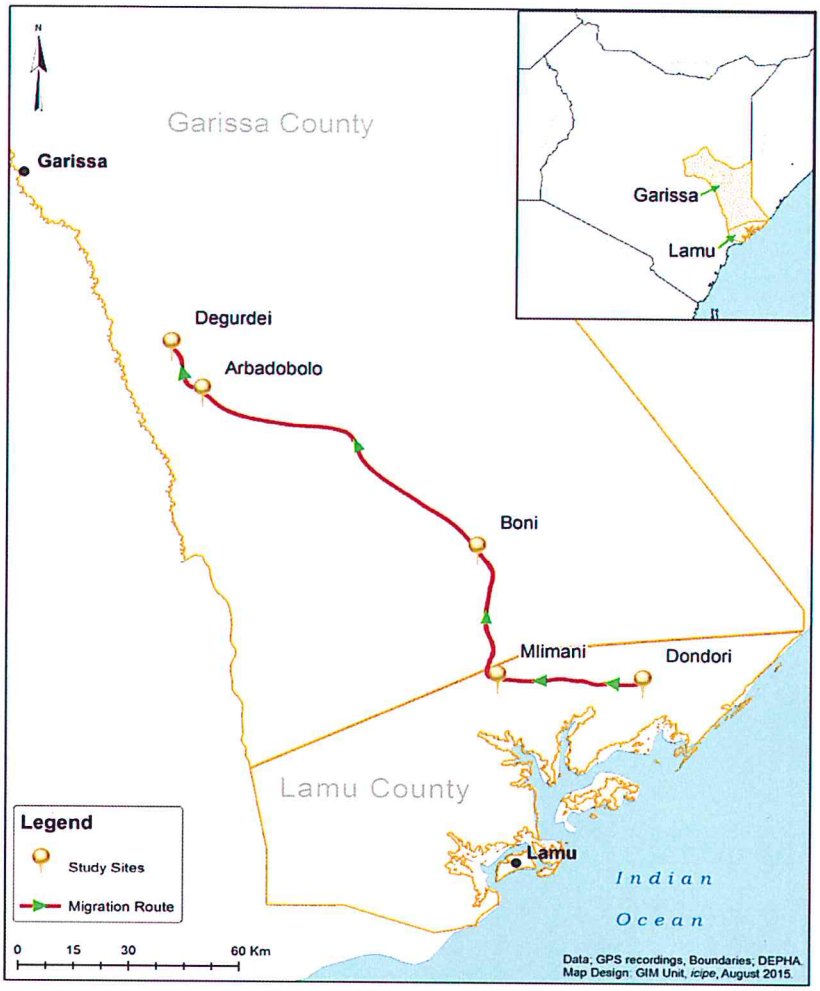
Northeastern Kenya is primarily occupied by nomadic pastoralists, and grazing areas have diverse plant species that could harbor disease vectors. Identifying preferred resting habitats could guide novel adult vector control efforts during outbreaks as a means of breaking transmission cycles. It may also help to identify plants which act as attractants or repellants to mosquitoes (Foster, 2008; Macel, 2011) . Information regarding resting preference of RVF vectors in natural environments remains unknown, which frustrates efforts to break transmission cycles during outbreaks using chemical control of adult populations. Even though sampling for outdoor resting mosquitoes can be complex (Mboera, 2006), studies on outdoor resting behaviour of RVF vectors remain an important component in managing RVF outbreaks, as it will assist in determining potential habitats used by mosquitoes for resting, which may then be targeted for vector control. This study examined survival rates among the key primary vectors of RVF, *Ae. mcintoshi* and *Ae. ochraceus*, and the resting preference of

RVF vectors among different plant species. The age of wild-caught *Ae. mcintoshi* and *Ae. ochraceus* was estimated by determining the parity of females. These data will provide insights into mosquito resting behaviour under field conditions that can be used for focused and site specific vector control to minimise the transmission of RVF and other diseases borne by these mosquito species.

3.2 Materials and Methods

3.2.1 Study area

The study was conducted in Garissa (S00° 39' E40° 05') and Lamu (S02° 16' E40°) Counties in the northeastern region of Kenya (Figure 1). Mosquitoes were trapped from five sampling sites: Degurdei, Arbadobolo, Boni, Mlimani, and Dondori. These sites were located within three distinct ecological zones: semi arid, dry humid forest and humid to dry sub-humid. The region is inhabited by the ethnic Somali community whose main source of livelihood is nomadic pastoralism. Annual livestock migration in the region occurs due to scarcity of rainfall. This region is known for the abundance of mosquitoes during the rainy seasons and risks of exposure to mosquito borne diseases. Climatic conditions range from extreme dry weather to extreme flooding conditions during the rainy seasons. The mean annual rainfall varies between 200 and 500 mm. Rainfall is bimodal; the long rains occurring from April to May, and the short rains in October and November with occasional variation. Generally, the region is hot and dry with average daily temperatures ranging from 20°C to 38°C. There is usually rapid growth of vegetation at most sites during the rainy season, which may form suitable resting habitats for mosquitoes. Climatic conditions during sampling periods are presented in 2.2.1.



Ficgure 3.1: Map of Kenya showing five sampling sites along livestock movement routes in Garissa and Lamu Couties

3.2.2 Sampling design

Mosquitoes were captured from vegetation to determine potentially preferred resting sites. There were no built structures at any of the sampling sites that could act as alternative mosquito resting sites. At each of the five sampling sites, a 1 x 1 km sampling area was identified and subdivided into four quadrants (A,B,C,D) each measuring 500 x 500 m. Each of the four quadrants were subdivided into four sub-quadrants (A1-4, B1-4, C1-4, D1-4) that

were 250 x 250 m each. Two of the sub-quadrants were randomly selected as sampling units for mosquito collection using CDC light traps (LTs) and direct aspiration from vegetation.

Mosquitoes were trapped during the rainy season (April-June) and dry season (August-October) in two consecutive years i.e., 2013 and 2014. Eight LTs, one in each of the two sub-quadrants, were used to trap host seeking mosquitoes. An aspirator, Prokopack (Vazquez-Prokopec et al., 2009), was used to trap resting mosquitoes from four randomly-selected plant species within the same sub-quadrants. The LTs were set at sites along LMR in random order at 1800 hours and retrieved at 0600 hours the next morning. Resting mosquitoes were aspirated between 1400 hours and 1600 hours. Aspiration from all selected plant species in each quadrant was conducted for 20 minutes and 5 minutes for each plant. Mosquitoes were identified morphologically and the plants from which mosquitoes were captured were collected and labelled for identification.

3.2.3 Examining the survival rates of the key vectors of RVF

Parity of the collected mosquitoes was used to determine survival rates among the primary vectors of RVF *Ae. mcintoshi* and *Ae. ochraceus.* The dissected mosquitoes were classified as parous to show evidence of previous blood feeding and egg production or nulliparous for mosquitoes without evidence of previous blood feeding and egg production. Given that the parous mosquitoes have one or more gonotrophic cycles, those with three gonotrophic cycles were considered to have survived the longest. The mosquitoes were individually dissected with the aid of a compound microscope (Leica DMRB). Anesthetised adult females were gently placed on a clean microscope slide and dissected into a drop of phosphate-buffered saline (Detinova, 1962). During dissection, the thorax was gently held by forceps and placed ventral side up with her abdomen in the phosphate buffered saline. A fine tip needle was used

to gently remove the 7th and 8th abdominal segments by grasping and gently pulling them away. Ovaries appeared as a pair of white oval objects attached to the removed segments, which were isolated, transferred to a new slide and allowed to air dry. The dry specimen was viewed under a compound microscope to determine the number of gonotrophic cycles (egg laying cycle) attained by the mosquitoes.

3.2.4 Data analysis

Data on proportions were compared using Chi square test. Given that there was no association between the collection method and species (χ^2=1.11, df=1, P=0.291), we analyzed data from the two collection methods (i.e., LT and aspiration) together. Although the data was collected from different zones, ecological zones were not taken into account in this objective given that our samples were pooled together and inference on survival was based on individual mosquito species. Data on the number of parous and nulliparous mosquitoes were compared using quasibinomial model with vector species (*Ae. mcintoshi* =0, *Ae. ochraceus*=1), season (rainy season=0, dry season=1) and collection method (aspiration=0, LT=1) as covariates in the model. Odds ratios (OR) were computed to quantify the effect of each variable. A quasibinomial model was used because (1) the outcome of interest was binary; and (2) to account for over dispersion in the data. To compare survival rate between the mosquitoes, daily survival rate was estimated based on the proportion of individuals that were parous using the formula p^n=M where (p) is the survival rate per day, (M) is the proportion of the population which is parous and (n) is the number of days between emergence of adult and first oviposition as described by Davidson (1954) (Davidson, 1954). This method was adapted based on the following assumptions; 1. That the population was static over the sampling period, i.e. that births equal deaths and immigration equals emigration, and 2. That the instantaneous mortality rate of the population was constant at all

ages. For both species, it was also assumed that $n = 3$, given that information on the species in question is not documented in literature and could not be determined experimentally in the laboratory. To investigate plant species resting preference by mosquitoes, we fit a negative binomial model for all mosquito species combined and separately for each mosquito species, with plant species and season as covariates in the model. Risk ratios (RR) were computed for each plant species in comparison to *Duosperma kilimandscharicum*, which was the most preferred plant species. In this case, a negative binomial model was adopted to account for excess variation in the data than could not be accounted for by a Poisson model, which is usually the model of choice for count data. All analyses were performed using R v3.2.0 (Kembel et al., 2010; Oksanen et al., 2015; Team, 2015).

3.3 Results

3.3.1 Mosquito diversity and abundance

Seven mosquito species belonging to belonging to two main genera (*Aedes* and *Culex*) were trapped during this study. 1422 primary vectors of RVF, *Ae. mcintoshi* 46% and *Ae. ochraceus* 54% were captured by LT (n=937) and aspiration (n=485) methods. Overall 1653 mosquitoes (mean= 8.61, SD=5.51) were aspirated from plants. As noted earlier, the primary vectors of RVF, were also caught in large numbers from plants. In addition, the secondary vectors *Ae. sudanensis* (Theobald) n=149, *Cx. pipiens* (L) n=149, *Cx. poicilipes* (Theobald) n=131 and *Cx. univittatus* (Theobald) n=252 and *Ae. tricholabis* n= 250 were also caught.

3.3.2 Survival rates among vectors of RVF

The number of gonotrophic cycles attained by mosquitoes in this study was used to determine how long these vectors may have survived to enable them to lay eggs either once or more and hence determining duration of survival. In total, 1422 mosquitoes were captured by LT

(n=937) and aspiration (n=485) methods. Of 1422 mosquitoes caught, 54% were *Ae. ochraceus* and 46% were *Ae. mcintoshi*. Our results indicated that 1124 (79%, 95%CI 76.8-81.1%) of the mosquitoes were parous. Proportion parous was higher among the *Ae. ochraceus* (80.2%) than in *Ae. mcintoshi* (77.7%). Quasibinomial model results indicated that this difference in the number of parous mosquitoes between *Ae. ochraceus* and *Ae. mcintoshi* was not significant (OR=1.04, 95%CI 0.71-1.51; P=0.847). A goodness of fit (GOF) test indicated that the model fitted the data well (GOF Chi Sq=336.54 df=313,P=0.1724). The number of parous mosquitoes was significantly greater among those caught during the rainy season than it was among those caught in the dry season (OR=0.08, 95%CI 0.04-0.14; P<0.001). There was no impact of collection method on the number of parous mosquitoes (OR=1.16, 95%CI: 0.72-1.82).

Dissected individuals of both *Ae. mcintoshi* and *Ae. ochraceus* represented the first, second and third gonotrophic cycles with relatively low variation between the two mosquito species. *Ae. ochraceus* showed higher survival rate than *Ae. mcintoshi* with a greater proportion (35.7%) with three gonotrophic cycles compared to *Ae. mcintoshi* (19.6%; (χ^2=35.02, df=1, P<0.001, Figure 3. 2). Figure 3. 2 show that in *Ae. mcintoshi*, 30% and 50% of parous mosquitoes were represented in the first and second gonotrophic cycles respectively.

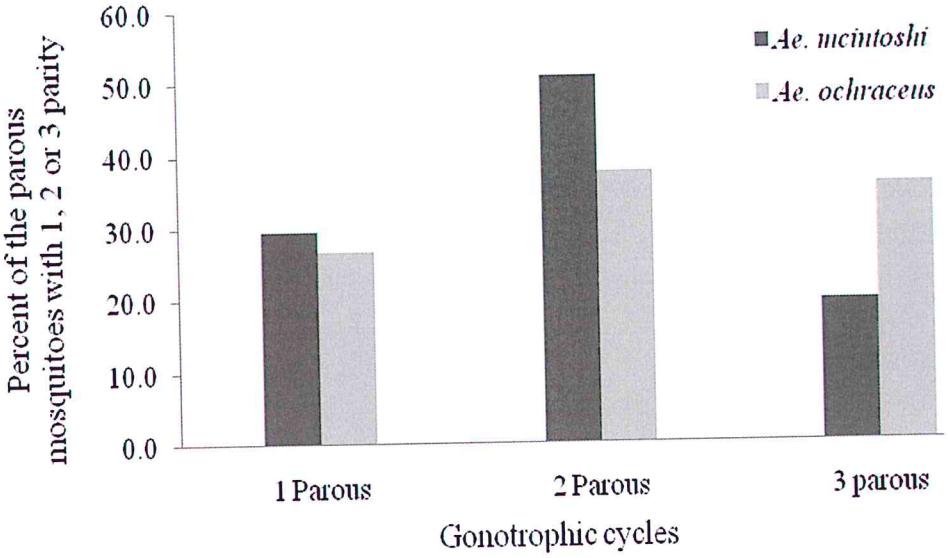


Figure 3.2: Proportion of parous mosquitoes at different gonotrophic cycles

It was evident that both primary vectors dissected during this study at least had representative individuals in each category of the three gonotrophic cycles with relatively low variation between the two species. The proportion of null parous mosquitoes were almost similar between the two species. In both *Ae. mcintoshi* and *Ae. ochraceus* higher numbers of the dissected vectors were parous while the null parous were present but in reduced numbers (Table 3.1).

Table 3.1: Survival of key primary vectors of RVF

	RVF primary vectors			
Parity	*Ae. mcintoshi*		*Ae. ochraceus*	
Null parous	144	(19.3%)	148	(20%)
Parous	529	(77.7%)	615	(80%)
Total counts	673	100%	763	100%

Based on the proportion of parous mosquitoes for each species, we estimated the daily survival rate for each mosquito separately. Daily survival rate *p* was estimated to be 0.93 for *Ae. ochraceus* while the daily survival rate for *Ae. mcintoshi*, was estimated to be 0.92. Our results also showed that difference in survival rate was not significant between the two mosquito species $F_{1,31}$=0.240, P=0.627.

3.3.3 Resting preference among vectors of RVF

A total of 1653 mosquitoes were aspirated from all 192 plants which comprised 12 plant species classified under 11 plant families. The mosquitoes aspirated from plants comprised both primary and secondary vectors of RVF. The plants from which the mosquitoes were aspirated were represented in varying proportions as presented below and they included *Duosperma kilimandscharicum* (Acanthaceae) Clarke 39.1%, *Commelina forskali* (Comelinacea) Vahl 13.0%, *Salsola kali* (Amaranthaceae) L. 10.9%, *Salvadora persica* (Salvadoraceae) Kharija 10.9%, *Cadaba ruspolii* (Capparaceae) 8.9%, *Mollugo nodicaulis* (Molluginaceae) 4.7%, *Cyperus giolii* (Cyperaceae) 4.7%, *Grewia tenax* (Malvaceae) 4.2%, *Gisekia africana* (Gisekiacea) 2.1%, *Polygola erioptera* (Polygalaceae) 0.5%, *Mollugo cerviana* (Molluginaceae) 0.5%, *Momordica rostrata* (Cucurbitacea) 0.5%. These percentages represented the number of each plant species that comprised the 192 sampled plants.

To avoid any bias caused by differences in plant abundance, we used the average mosquito catches per individual plant to determine resting preference. Mosquitoes were most often captured from *D. kilimandscharicum* (n=972), while the lowest number was captured from *G. africana* (n=27). Although the average mosquito captured per plant was higher in *D. kilimandscharicum* than the rest of the plants, the difference was minimal (Figure 3.3).

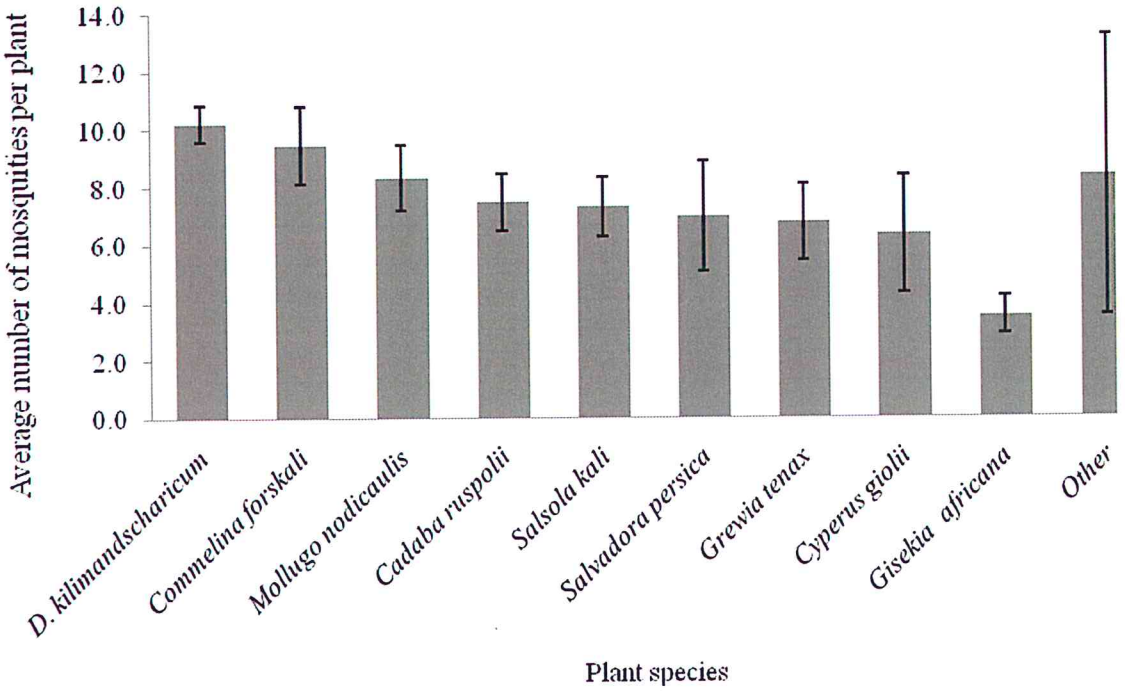


Figure 3.3: Average number of mosquitoes (all species combined) per plant species

A similar distribution pattern of the vectors among the vegetation was observed between the wet and dry seasons in which *Duosperma kilimachandricum* was the most preferred plant by mosquitoes with an average capture of 3.5 and 4.5 mosquitoes per plant for the rainy and dry season, respectively.

More vectors were trapped resting in *D. kilimachandricum, C. forskali, S. kali,* and *S. persica* during the dry season compared to the rainy season (Figure 3.4). This revealed the potential for high use of certain vegetation as refuges by the vectors of RVF during the dry season. Other plants such as *M. nodicaulis, G. tenax, C. giolii* and *G. africana* were mainly preferred for resting during the rainy season.

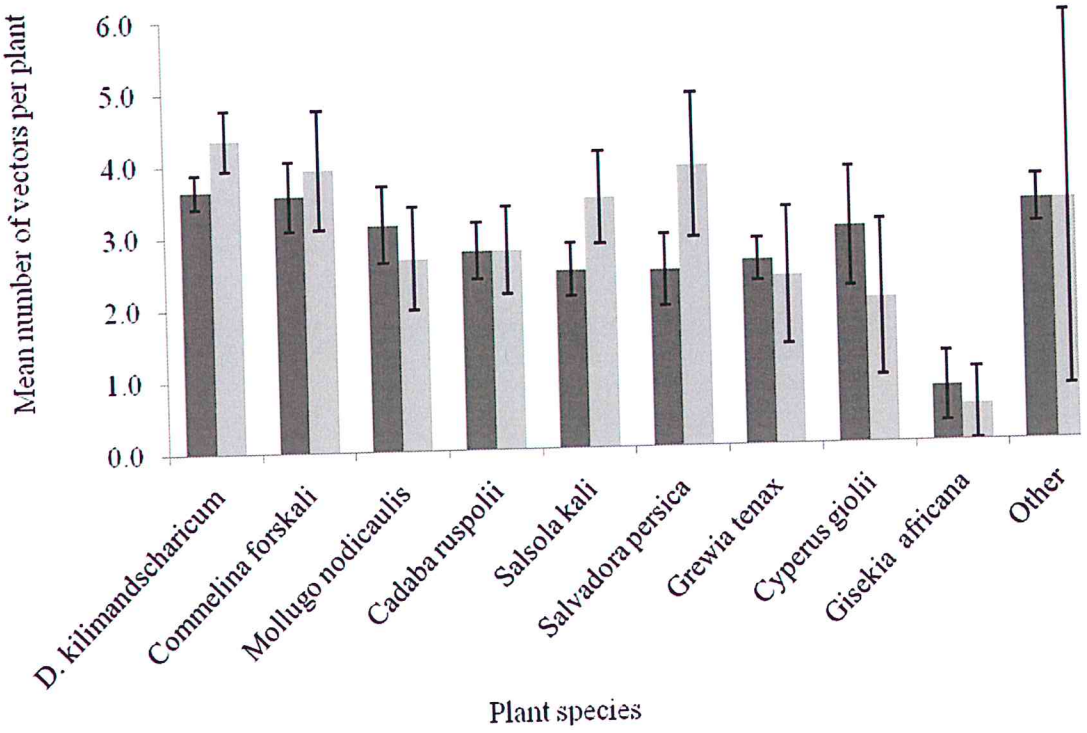


Figure 3.4: Average number of mosquitoes caught per plant during the rainy season (black) and dry season (light grey).

Primary and secondary vectors were captured in varying numbers from the plant species. The highest capture of the primary and secondary were from *Duosperma kilimachandricum* while the lowest capture of primary vectors were from *Gisekia africana* and the lowest capture of secondary vectors were from *Momordica rostrata* (Figure 3.5). Secondary vectors were most abundant than primary vectors in most plant species except in *Salvadora persica* which had slightly higher primary vectors than secondary vectors.

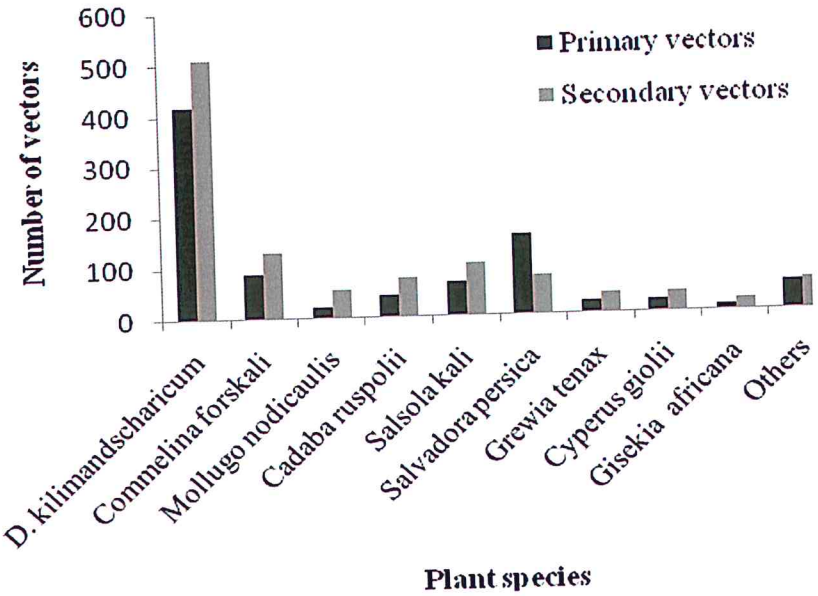


Figure 3.5: Abundance of primary and secondary vectors of RVF captured from different plant species

3.3.4 Abundance of specific vector species among different vegetation types

Different mosquito species were trapped from the twelve plant species which were sampled for resting adult mosquitoes. The vectors were comprised of mosquitoes mainly from genera *Aedes* and *Culex*. *Aedes mcintoshi* was the most abundant vector captured from different vegetation type followed by *Ae. ochraceus*. The two species were also most abundant in *Duosperma kilimachandricum* from which the highest number of vectors (n=972) were also captured (Figure 3.6).

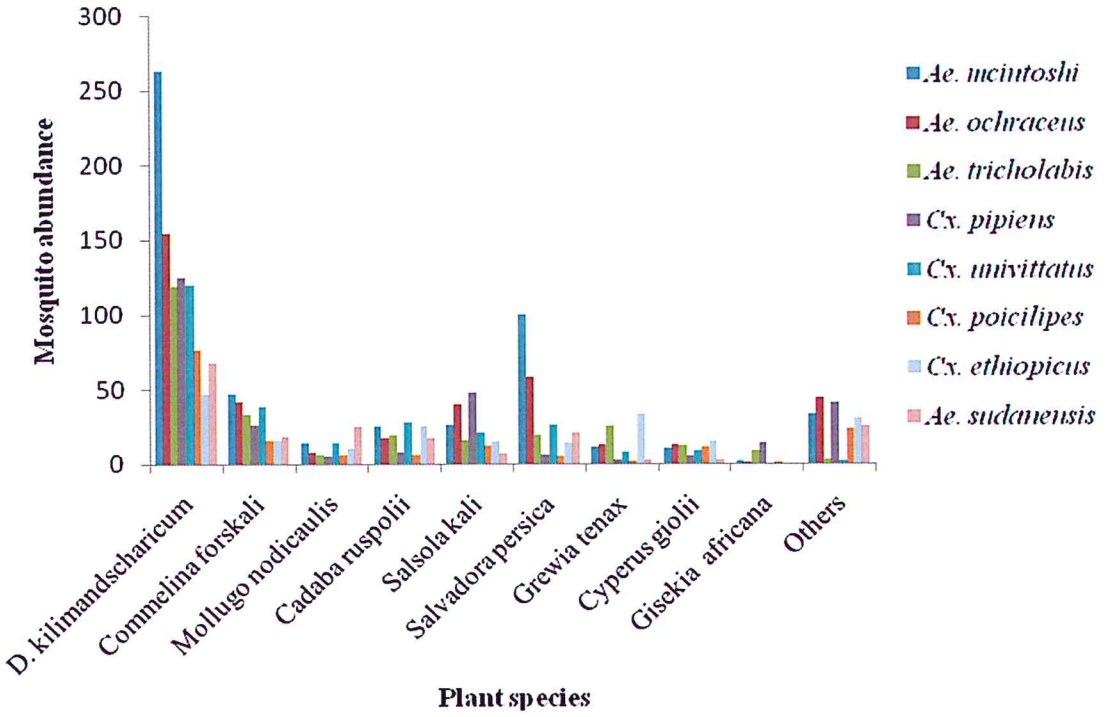


Figure 3.6: Abundance of different vector species found on vegetation.

Comparison of the overall captures of vectors from the vegetation between the season showed that there was significant difference in the captures obtained between the dry and rainy season (RR=1.36, 95% CI: 1.08-1.71, P=0.009). The difference in captures between the seasons was not significantly different among individual vector species captured during this study except in *Ae. sudanensis* (RR=2.42, 95% CI: 0.71-2.12, P=0.001). In this study, the preference of different vegetation types by mosquito species relative to *Duosperma kilimandscharicum* was assessed. In overall significant differences in captures were observed among different plant species such as *Salsola kali* (RR=0.68, 95% CI: 0.51-0.93, P=0.013). These differences were also observed in other plant species such as *Salvadora persica, Cadaba ruspolii, Cyperus giolii, Gisekia africana* as represented in Table 3.2.

Preference of vegetation types by different mosquito species also varied between mosquito species. However, only a few species showed significant difference in the captures of the vectors relative to *Duosperma kilimandscharicum.* The captures of *Ae. mcintoshi* for instance were significantly different (P=0.013) between *Salsola kali* and *Duosperma kilimandscharicum* (Table 3.2). The Goodness of fit (GOF) test indicated that these models fitted the data well (GOF Chi Sq P>0.06).

Table 3.2: Abundance of vectors of RVF among sampled vegetation types relative to *Duosperma kilimandscharicum*

Variables	Mosquito species									
	All species combined		*Ae. mcintoshi*		*Ae. ochraceus*		*Ae. tricholabis*		*Ae. sudanensis*	
	RR (95% CI)	P	RR (95% CI)	P	RR (95% CI)	P	RR (95% CI)	P	RR (95% CI)	P
Plant species										
Duosperma kilimandscharicum	1		1		1		1		1	
Commelina forskali	0.93 (0.71-1.22)	0.605	0.88 (0.59-1.29)	0.521	1.15 (0.76-1.72)	0.496	0.83 (0.44-1.6)	0.577	0.82 (0.44-1.60)	0.551
Salsola kali	**0.69 (0.51-0.93)**	**0.014**	**0.56 (0.34-0.88)**	**0.015**	1.29 (0.84-1.95)	0.238	**0.48 (0.22-1.02)**	**0.054**	**0.35 (0.22-1.02)**	**0.021**
Salvadora persica	**0.66 (0.48-0.90)**	**0.008**	0.71 (0.45-1.11)	0.140	0.80 (0.48-1.30)	0.379	0.60 (0.29-1.26)	0.173	0.95 (0.29-1.26)	0.886
Cadaba ruspolii	**0.71 (0.51-0.99)**	**0.040**	0.66 (0.40-1.06)	0.095	0.67 (0.37-1.15)	0.165	0.7 (0.33-1.53)	0.366	1.12 (0.33-1.53)	0.752
Mollugo nodicaulis	0.79 (0.53-1.19)	0.252	0.68 (0.36-1.24)	0.224	0.61 (0.27-1.21)	0.186	0.38 (0.12-1.14)	0.084	1.22 (0.12-1.14)	0.641
Cyperus giolii	**0.64 (0.41-1.00)**	**0.046**	0.52 (0.24-1.03)	0.077	1.00 (0.50-1.86)	0.990	0.85 (0.32-2.38)	0.742	0.37 (0.32-2.38)	0.150
Grewia tenax	0.69 (0.44-1.11)	0.119	0.67 (0.32-1.31)	0.262	1.13 (0.56-2.13)	0.719	1.11 (0.43-3.21)	0.832	0.53 (0.43-3.21)	0.333
Gisekia africana	**0.37 (0.18-0.78)**	**0.008**	0.25 (0.04-0.88)	0.065	0.18 (0.01-0.86)	0.092	1.44 (0.42-6.44)	0.590	0.00 (0.42-6.44)	1.000
Other	0.89 (0.44-1.86)	0.736	1.16 (0.43-2.88)	0.753	0.70 (0.16-2.17)	0.584	0.64 (0.11-4.26)	0.615	1.02 (0.11-4.26)	0.978
Captures from vegetation by season										
Rainy season	1		1		1		1		1	
Dry season	**1.27 (1.05-1.53)**	**0.012**	1.25 (0.95-1.63)	0.106	1.10 (0.82-1.47)	0.506	1.05 (0.67-1.64)	0.844	**2.17 (0.67-1.64)**	**0.000**

RR= Risk ratios

Table 3.2 (cont.): Abundance of vectors of RVF among sampled vegetation types relative to *Duosperma kilimandscharicum*

Variables	Mosquito species							
	Cx. pipiens		*Cx. univittatus*		*Cx. poicilipes*		*Cx. ethiopicus*	
	RR (95% CI)	P	RR (95% CI)	P	RR (95% CI)	P	RR (95% CI)	P
Plant species								
Duosperma kilimandscharicum	1		1		1		1	
Commelina forskali	1.02 (0.51-2.11)	0.947	1.09 (0.51-2.11)	0.738	0.65 (0.29-1.47)	0.292	1.03 (0.29-1.47)	0.935
Salsola kali	0.71 (0.31-1.60)	0.400	0.70 (0.31-1.60)	0.233	0.52 (0.21-1.28)	0.149	1.11 (0.21-1.28)	0.795
Salvadora persica	**0.18 (0.05-0.54)**	**0.004**	0.79 (0.05-0.54)	0.428	**0.18 (0.05-0.57)**	**0.006**	1.05 (0.05-0.57)	0.899
Cadaba ruspolii	0.43 (0.15-1.13)	0.088	1.13 (0.15-1.13)	0.680	**0.31 (0.10-0.93)**	**0.039**	0.62 (0.10-0.93)	0.331
Mollugo nodicaulis	0.63 (0.20-1.99)	0.423	1.18 (0.20-1.99)	0.645	0.63 (0.19-2.14)	0.448	1.59 (0.19-2.14)	0.339
Cyperus giolii	0.59 (0.16-2.06)	0.398	0.72 (0.16-2.06)	0.449	0.45 (0.11-1.75)	0.245	0.18 (0.11-1.75)	0.112
Grewia tenax	0.37 (0.07-1.53)	0.190	0.73 (0.07-1.53)	0.490	0.26 (0.04-1.33)	0.123	0.00 (0.04-1.33)	1.000
Gisekia africana	0.28 (0.01-2.40)	0.281	0.00 (0.01-2.40)	0.999	0.27 (0.01-2.61)	0.283	0.00 (0.01-2.61)	1.000
Other	1.11 (0.18-8.62)	0.910	0.51 (0.18-8.62)	0.418	1.08 (0.16-9.94)	0.935	1.16 (0.16-9.94)	0.874
Captures from vegetation by season								
Rainy season	1		1		1		1	
Dry season	1.46 (0.87-2.49)	0.152	1.24 (0.87-2.49)	0.229	1.37 (0.77-2.49)	0.268	1.30 (0.77-2.49)	0.310

3.4 Discussion

The ability of mosquitoes to survive environmental stresses and find suitable refuges are adaptations that may contribute to disease transmission efficiency among these vectors. Given that the transmission and circulation of mosquito borne diseases is primarily influenced by the ecology and physiology of the vectors, it is important to know the interactions existing between the vectors and their environment. Overall, most of the mosquitoes trapped were parous, which suggests that the majority of these mosquitoes survived for a relatively longer time to complete one or more gonotrophic cycles. High survival rate among mosquitoes may have a bearing on disease circulation. This is because mosquitoes that have already obtained one or more blood meals are more likely to transmit arboviruses when they seek a fresh blood meal after having acquired pathogens from their first feeding encounter. This is consistent with other studies, which reported that survival of mosquitoes under different ecological conditions influences transmission of mosquito borne diseases (Reiter, 2001). The differential survival of RVF vectors in north-eastern Kenya observed in this study may enhance their competence for transmission of arboviruses circulating in this region. This corroborates the findings of studies conducted elsewhere which documented that vector survival increases their disease transmission potential (Hardy et al., 1983). This may be further compounded by the fact that a high abundance of these floodwater *Aedes* has been reported in north-eastern Kenya (Arum et al., 2015; Lutomiah et al., 2013). Based on the mosquitoes we analysed, *Ae. ochraceus*, showed higher survival rate than *Ae. mcintoshi*. This vector was recently incriminated in the 2006-7 RVF outbreak in Kenya (Sang et al., 2010). The ability of this vector to survive more gonotrophic cycles than *Ae. mcintoshi* suggests that this vector may have the potential to efficiently acquire several blood meals and potentially initiate circulation of arboviruses including RVF virus. Furthermore, its increased geographic expansion despite recent introduction as reported in

recent studies (Tchouassi et al., 2014), may enhance the potential of this vector in circulating arboviruses into diverse ecological zones in East Africa.

This study found high survival rates among the two mosquito species *Ae. mcintoshi* and *Ae. ochraceus*. Although the daily survival rate did not significantly differ between the two species, the estimated survival rate could lead to increased opportunity for the incubation of pathogens with long extrinsic incubation periods (EIPs) relative to mosquito lifespan as reported in other studies (Chan & Johansson, 2012; Tjaden et al., 2013). Survival rate was higher during the rainy season than dry season. This may be due to the fact that gonotrophic cycle of mosquitoes depend on climatic conditions such as rainfall and temperature (Afrane et al., 2005; Minakawa et al., 2002; Muturi et al., 2007; Ndenga et al., 2006), as well as higher relative humidity (Afrane et al., 2006; Juliano et al., 2002), which largely influence mosquito biology. This is consistent with other studies conducted on the ecology of the primary vectors of RVF in this region (Arum et al., 2015; Lutomiah et al., 2013; Sang et al., 2010). Low survival rate was observed in the dry season, which may have been due to the extreme dry weather conditions. This may have led to mosquitoes diapausing and retaining eggs or entering dormancy as a way of avoiding dry conditions (Medlock et al., 2006). In view of our findings, it may be important to incorporate other means of assessing mosquito survival such as polovodova dissection technique in future studies other than parity in order to make conclusive comparisons of survival between dry and wet seasons. This may form a basis for informed and appropriate mosquito control approach across seasons.

We collected mosquitoes from different plant species, which supports the observation that mosquitoes usually seek shelter in different types of habitats including vegetation (Githeko et al., 1996; Silver, 2007). Our study focused on individual plant species with the view of

determining their specific roles in mosquito resting behaviour. It is on this basis that our analysis of resting preference focused on plant species as opposed to combining them into plant families, thus avoiding the pitfall of ecological fallacy; that is, misleading research findings due to formulating inferences at the individual level using group-level data (Diez-Roux, 1998). Generally, all the mosquitoes preferred *Duosperma kilimandscharicum, Commelina forskali,* and *Mollugo nodicaulis* to other plant species. From our findings, we may conclude that mosquitoes rest on vegetation around breeding sites or near animal hosts and may choose certain plant species as resting sites over others. The preference of these plants could be due to the nature of the plant and ability of some, such as *Duosperma kilimandscharicum* to grow in clusters, potentially creating a suitable microclimate beneath the plant that is preferred by vectors for resting. The mosquitoes could utilise this vegetation to seek protection from extreme temperatures, desiccation and predation in order to enhance their survival (Silver, 2007). These plants could also potentially produce attractants that mosquitoes use to choose resting sites, as has been demonstrated for malaria vectors (Nyasembe et al., 2012; Pitts et al., 2014). Some researchers have also found that mosquitoes often use plants as sources of sugar (Gu et al., 2011; Nyasembe et al., 2014; Yuval, 1992), which may also account for the observed variation in resting preference. It is not currently clear why mosquitoes were found in low numbers in some plants, such as *Gisekia africana.* However, some plants are known to produce chemicals which may repel insects (Dekker et al., 2011; Maia & Moore, 2011). The attractant and repellent properties of plants with regard to primary and secondary RVF vectors could provide information useful for a targeted environmental management approach such as the "push-pull" system that has been used successfully for the control of agricultural pests in Africa (Kfir et al., 2002). Further research to determine the basis for preference or non-preference is required before such a control tactic could become a reality.

The overall high capture of the mosquitoes from the vegetation during dry season may have been due to reduced vegetation cover in the dry season, consequently making the average capture per plant higher in the dry season than the rainy season. This pattern of distribution may also emerge as a result of the interaction between insects, plants and natural enemies (Benton et al., 2003; Söderström et al., 2001). This observation may also apply for *Ae. sudanensis*, which were caught in significantly higher numbers from plants during the dry season. Other than high plant density, alternative resting sites may have been present in the rainy season resulting in a more even distribution of adults and low average captures per plant in comparison to the dry season.

Differential survival rates among primary vectors of RVF observed in this study across the seasons shows how the two vectors may influence disease transmission in northern Kenya. High survival rate observed in *Ae. ochraceus* also reveals a greater potential role as a vector of RVF in the event of any future outbreak. Both primary and secondary vectors of RVF have resting preferences among certain plant species. Thus, areas dominated with such vegetation may constitute high risk zones where circulation and transmission of arboviruses to livestock and humans might occur during epidemics given that the vegetation may support the biology of the vectors to enhance their survival. The finding that RVF vectors utilise certain plant species for refuge, will in the future help to guide control operations targeting adult mosquitoes during outbreaks to interrupt transmission and minimise virus activity in the environment. This has been a very challenging step in past outbreaks.

Chapter 4

SPATIAL VARIATION IN HOST PREFERENCE AMONG RVF VECTORS ALONG LIVESTOCK MOVEMENT ROUTES IN NORTH EASTERN KENYA

4.1 Introduction

Most infectious diseases are vector-borne (Gratz, 1999) and their epidemiology is largely influenced by vector ecology. These diseases are circulated between diverse hosts by mosquitoes and other insect species during blood-feeding. Identification of mosquito hosts in habitats with diverse vertebrate species is an important ecological aspect that can help in understanding factors driving mosquito feeding behaviour in different ecological areas (Kilpatrick et al., 2006a; Muriu et al., 2008). These interactions may help disease ecologists to define disease transmission pathways and dynamics to control the spread of infectious pathogens of public health and economic concern (Kent, 2009). Mosquito host-seeking behaviour may be influenced by a number of factors including diversity of potential host species and the surrounding environmental conditions (Friend & Smith, 1977; Scott et al., 1993).

Mosquitoes usually acquire pathogens from hosts through feeding and transmit them to other animals within the immediate area in a multi-host environment (Kilpatrick et al., 2006a; Kilpatrick et al., 2006b; Pilosof et al., 2015). This feeding network can complicate efforts to identify disease drivers and control epidemics in a given community. This is because some mosquitoes can feed on more than one host and pathogens may also have the potential of infecting many hosts in multi-host and multi-parasite assemblages (Johnson et al., 2015). Some multi-host pathogens that have been isolated from mosquitoes include Rift Valley fever virus (RVFV), Semiliki Forest virus, Ndumu virus, and Chikungunya virus (Fontenille et al., 1998; Meegan & Bailey, 1988; Sang et al., 2010).

The epidemiology of these viruses is largely determined by the presence of competent mosquito vectors, animal hosts, disease-causing pathogens, and a suitable environment, which promote their circulation. This network presents a challenging situation in managing vector-borne diseases, especially when mosquito populations increase as a blood meal is crucial for female mosquito reproduction (Briegel, 1985). The origin of mosquito blood meals therefore provides an opportunity to understand mosquito feeding preference based on host availability and the surrounding ecological conditions. It also highlights the vectorial capacity of different species in areas with a diversity of host vertebrate species (Roiz et al., 2012; Townzen et al., 2008). Vector feeding ecology and the composition of the host community can play an important role in understanding patterns of disease transmission in a natural ecosystem (Kilpatrick et al., 2006a; Lyimo & Ferguson, 2009; Molaei et al., 2006).

The aim of this study was to establish the feeding preference of mosquitoes in diverse habitats. Our study focused on routine livestock movement routes (LMR) in northeastern Kenya. The LMR is a multi-host environment, which presents a diversity of wild animals, mosquito vectors and livestock that pass through these areas (Arum et al., 2015; de Leeuw et al., 2001). This creates an environment with complex interactions between disease vectors, pathogens and hosts that may determine the dynamics of disease-causing pathogens in the feeding network. Examining mosquito host preferences in such diverse ecological areas provides an opportunity to identify key vectors of arboviruses and understanding pathogen transmission networks.

4.2 Materials and methods

4.2.1 Study area

This study was conducted in northeastern Kenya along livestock movement routes (LMR). The region is occupied by nomadic pastoralists who migrate long distances with their livestock in search of pasture and water. The area is predominantly semi-arid. Pastoral and agricultural activities are only practiced along the River Tana along the border of Garissa County at a small scale. Occasional droughts often reduce or even deplete pasture, making it necessary for pastoralists to migrate long distances to neighbouring counties to seek water and pasture. During migration, the herders often graze and temporarily settle in some areas for a period of time before moving to other areas when pasture is depleted. Some areas where they settle while grazing are endowed with diverse species of wildlife, which creates an interface where livestock and wildlife closely interact during grazing. These sites also potentially provide mosquitoes with a wide array of hosts from which to feed.

Mosquitoes were captured from six sampling sites; Degurdei, Arbadobolo, Boni, Mlimani, Mangai and Dondori, which are all located along the major LMR. These sites are located in three major agro-ecological zones described in Chapter 2.2.1. Dedurdei and Arbadobolo are expansive herbaceous bushland and shrubland, which is usually suitable for grazing of livestock after rains. Large water pans in the area usually attract wild animals, and herders also settle in the area until the dry season sets in before migrating to other areas. Boni is a forest ecosystem usually preferred by pastoralists for grazing during the dry season. The forest is home to diverse species of wild animals protected by the Kenya wildlife service. Mlimani and Mangai are permanent settlements along the livestock movement route, located at the periphery of the forest, and occupied by hunters and fruit gatherers. Dondori is within Dondori National Reserve in the coastal region. This is a protected area with different wild

animal species. Pastoralists graze their animals in Dondori during prolonged droughts that completely deplete pasture in all other grazing areas. The weather conditions during the sampling period are presented in chapter 2.2.1

4.2.2 Distribution and diversity of wildlife in the area

The livestock movement route along which sampling sites were located traversed rangelands and national reserves in the northeastern region of Kenya. The main national reserves in the northeastern region include the Boni National Reserve (1,339 km^2) and Dodori National Reserve (877km^2) which lie on the north-east coast of Kenya near the Kenya-Somalia border. The two sites have been recognised for the conservation of biodiversity of global importance by being listed with the Eastern Arc Mountains and Coastal Forest of Biodiversity Hotspot (Burgess et al., 2007; de Leeuw et al., 2001) for containing endemic and globally threatened biodiversity. Over 13 ungulates are present in the region, including three Suidae Bushpig (*Potamochoerus larvatus),* Common warthog (*Phacochoerus africanus*), Desert warthog (*Phacochoerus aethiopicus*) nine Bovidae African buffalo (*Syncerus caffer*), Waterbuck (*Kobus ellipsiprymnus*), Bushbuck (*Tragelphus scriptus*), Lesser kudu (*Tragelaphus imberbis*), Kirk's dik-dik (*Madoqua kirkii*), Blue duiker (*Philantomba monticola*), Harvey's duiker (*Cephalophus harveyi*), Aders' duiker (*Cephalophus adersi*), and one hippopotamus (*Hippopotamus amphibius*) (Amin et al., 2015; Antipa, 2015; de Leeuw et al., 2001; Githiru, 2007). A total of 283 bird species have been recorded from more than 61 different families. This includes five threatened (Black bellied Sterling, Clark's Weaver, Yellow mantled weaver, Kretschmer's Longbill and the Grey Parrot) and six near-threatened bird species (Amani sunbirds, Caspian Tern, Ring-necked Francolin, Blue Quail, Moorland Francolin, Orange River Francolin), 27 Palaeactic migrants, 24 Afro-tropical

migrants, and 16 East African Coastal Biome species. Additionally, there are eleven forest specialists and 31 forest generalists (Burgess et al., 1998; De Jong & Butynski, 2009).

4.2.3 Trapping and identification of mosquitoes

Mosquitoes were trapped during the long rainy season (April-June) and short rainy season (November-December) in two consecutive years i.e., 2013 and 2014. To enhance the possibility of capturing both freshly fed and unfed mosquitoes for different laboratory assays, mosquitoes were captured using carbon dioxide-baited CDC light traps. Ten traps were set at 1800 h and ran overnight during each sampling period at sites along the LMR. These traps were randomly set at 100 m interval at each site and the same setup was replicated for three consecutive days at each site. The traps were retrieved early in the morning at 0600 hours and the trapped mosquitoes were preserved in a liquid nitrogen shipper for subsequent blood meal analysis.

In the laboratory, the mosquitoes were morphologically identified under cold chain to species level then pooled by species and site of collection. Fed mosquitoes were separated from unfed, then stored individually in 1.5 ml microcentrifuge tubes labeled with codes referencing species name, date and site of collection then preserved in a -80°C freezer. Samples of fed mosquitoes were stored for approximately one month until extraction of DNA and subsequent analysis of the blood meal source. Unfed mosquitoes were also preserved for other laboratory assays (Chapter 5).

4.2.4 Molecular identification of blood meal source

The abdomens of the mosquitoes were dissected for the extraction of DNA from each sample. One sterile dissection pin was used for each mosquito sample to avoid cross-contamination

between the samples. The abdomen of each mosquito was carefully dissected and removed from the rest of the body on a clean Petri dish. Each separated abdomen was transferred to a sterile 1.5 ml microcentrifuge tube and labeled for subsequent DNA extraction. DNA was extracted from the abdominal contents using the Qiagen protocol with the DNeasy blood and tissue kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. DNA extracts from the mosquito blood meal were used as the template in a standard polymerase chain reaction (PCR) assay using 12S mitochondrial rRNA gene ~500bp primers (12S3F-GGGATTAGATACCCCACTATGC and 12S5R- TGCTTACCATGTTACGACTT) (Roca et al., 2004).

The PCR cycling conditions incorporated: one cycle of 95°C for 5 min, 45 cycles of 95°C for 45 s, 56°C for 50 s, 72°C for 1 min, and 72°C for 7 min. The PCR amplicons were resolved in 2% agarose gel in Tris-borate EDTA buffer stained with ethidium bromide (Figure 4.1). The amplified PCR products were then cleaned up using ExoSAP-IT® PCR product clean up kit and sequenced by sanger method. The sequences were assigned to particular species by comparing them with the GenBank DNA sequence database (National Center for Biotechnology Information, 2008) and the BOLD Systems platform (http://www.boldsystems.org/ views/login.php). Positive identification and host species assignment was based on exact or nearly exact matches (>98%).

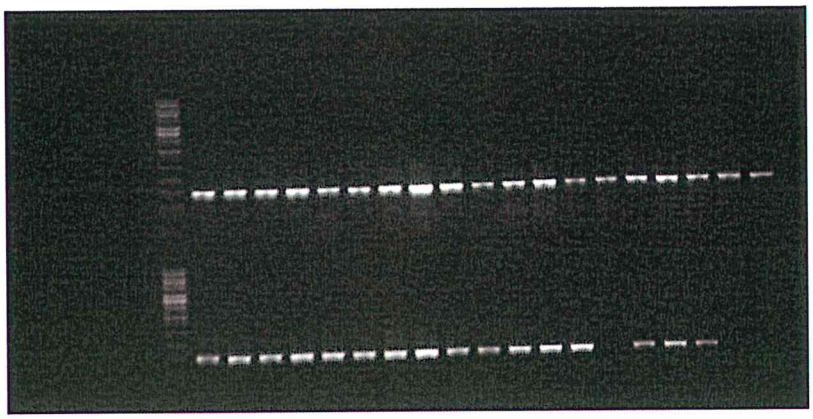


Figure 4.1: RT-PCR gel photo showing amplified amplicons from DNA extracts in this study

4.2.5 Data analysis

Chi-squared (χ^2) goodness of fit tests for proportions were used to establish whether feeding of all mosquitoes or primary vectors on wildlife, livestock or humans was associated with ecological zone.

4.3 Results

4.3.1 Composition of blood fed mosquito species

Over 300 fed mosquitoes were identified and their blood meals analysed to determine the hosts on which they fed. Identified and analysed fed mosquito species included, *Ae. mcintoshi* , *Ae. ochraceus*, *Ae. sudanensis*, *Ma. africana*, *Ae. tricholabis*, *Mn. uniformis* and *Cx. pipiens*. Vertebrate DNA was successfully amplified from 302 out of the 305 mosquito samples, which represented all seven mosquito species. Most of the fed mosquitoes were captured from Dondori national reserve, Mlimani and Boni forest, and the fewest captures of fed mosquitoes were trapped from Mangai, Degurdei and Arbadobolo. The fed mosquitoes were from different locations along the LMR and numbers that were fed varied among mosquito species (Table 4.1).

Table 4.1: Number of mosquitoes of each species from different sites tested for host blood meal

	Mosquito species							
Location	*Ae. ochraceus*	*Ae. mcintoshi*	*Ae. tricholabis*	*Ae. sudanensis*	*Cx. pipiens*	*Ma. africana*	*Ma. uniformis*	Total
Mangai	5	2	0	5	2	10	9	33
Dondori	20	1	5	13	5	45	10	99
Degurdei	5	8	2	5	4	0	2	26
Mlimani	10	0	5	20	9	16	2	62
Boni	16	4	10	5	12	14	2	63
Arbadoblo	11	1	0	6	4	0	0	22
Total	**67**	**16**	**22**	**54**	**36**	**85**	**25**	**305**

4.3.2 Hosts used by mosquitoes

The mosquitoes fed on 20 different animal species including; human, domestic cow (*Bos taurus*), African buffalo (*Syncerus caffer*), bushbuck (*Tragelaphus scriptus*), common duiker (*Sylvicapra grimmia*), ostrich (*Struthio camelus*), hyena (*Crocuta crocuta*), hippopotamus (*Hippopotamus amphibius*), waterbuck (*Kobus ellipsiprymnus*), Oribi antelope (*Ourebia ourebi*), warthog (*Phacochoerus africanus*), gerenuk (*Litocranius walleri*), dromedary camel (*Camelus dromedarius*), topi (*Damaliscus corrigum*), African bush rat (*Aethomys*), porcupine (*Erethizon dorsaum*), lesser kudu (*Tragelaphus imberbis*), spotted genet (Genetta genetta), porcupine (Hystrix cristata) and coucal bird (*Centropus*). Overall, our results show that the majority of fed mosquitoes analyzed in this study obtained a blood meal from hippopotami (n=85), followed by African buffalos (n=45), humans (n=38) and domestic cows (n=36), while only one mosquito had fed on each of hyena, porcupine, bush rat. Although hippopotamus appeared to be the animal species fed on by the highest number of mosquitoes,

our data indicate that it was mainly *Ma. africana* and *Ma. uniformis,* and a few other mosquito species, that fed on this host (Table 4.2).

Table 4.2: Animal hosts fed on by different mosquito species across ecological zones

Row Labels	Human	Cattle	African buffalo	Bushbuck	Waterbuck	Hippopotamus	Common duiker	Spotted genet	Gerenuk	Topi
Humid to dry sub-humid zone										
Ae. ochraceus	13	0	0	1	1	5	2	0	1	0
Ae. mcintoshi	3	1	0	0	0	0	0	0	0	0
Ae. sudanensis	19	5	3	3	7	0	0	0	0	0
Cx. pipiens	0	0	0	2	0	4	0	0	1	0
Ma. africana	0	0	0	9	3	60	11	1	0	0
Ma. uniformis	4	0	0	3	0	4	0	0	0	0
Forest zone										
Ae. ochraceus	5	18	13	1	0	0	0	0	0	1
Ae. mcintoshi	3	2	0	0	0	0	0	0	0	0
Ae. sudanensis	3	1	0	0	0	0	0	0	0	0
Ae. tricholabis	1	0	13	0	0	0	1	0	0	1
Cx. pipiens	4	1	1	6	0	0	0	0	0	0
Ma. africana	0	0	0	3	0	0	0	0	0	0
Semi arid zone										
Ae. ochraceus	1	7	0	0	0	0	0	0	1	0
Ae. mcintoshi	0	6	0	0	0	0	0	0	0	0
Cx. pipiens	2	0	3	2	0	1	0	0	1	4

Table 4.2 (cont): Animal hosts fed on by different mosquito species across ecological zones

Row Labels	Dromedari	Dik dik	Oribi antelope	Mouse	Lesser kudu	Porcupine	Ostrich	Warthog	Coucal bird	Spotted hyena
Humid to dry sub-humid zone										
Ae. ochraceus	2	0	2	0	0	0	0	0	0	2
Ae. mcintoshi	0	0	0	0	0	0	0	0	0	0
Ae. sudanensis	0	0	0	0	0	0	0	1	5	0
Cx. pipiens	0	0	0	0	1	0	0	0	2	0
Ma. africana	0	1	0	0	0	0	0	0	0	0
Ma. uniformis	0	0	0	0	0	0	0	0	0	0
Forest zone										
Ae. ochraceus	0	0	0	0	0	0	1	0	0	0
Ae. mcintoshi	0	0	0	0	0	0	0	0	0	0
Ae. sudanensis	0	0	0	1	0	1	0	0	0	0
Ae. tricholabis	0	0	2	0	1	0	0	0	0	0
Cx. pipiens	0	0	0	0	3	0	3	0	0	0
Ma. africana	0	0	0	0	0	0	0	0	0	0
Semi arid zone										
Ae. ochraceus	2	0	0	0	0	0	0	0	0	0
Ae. mcintoshi	2	0	0	0	0	0	0	0	0	0
Cx. pipiens	5	0	0	0	0	0	0	0	0	0

4.3.3 Host use across ecological zones by mosquitoes

The proportion of mosquitoes (all species combined) that had fed on wildlife varied significantly across the three ecological zones (χ^2=23.03, df=2, P<0.0001). The proportion of mosquitoes that had fed on wild animals was high in the humid to dry sub-humid zone and forest, but low in the semi-arid ecological zone. The proportion of mosquitoes that had fed on livestock varied significantly across the three ecological zones (χ^2=34.98, df=2, P<0.0001), with a high proportion having fed on livestock in the semi-arid ecological zone. However, there was no significant difference in the proportion of mosquitoes that had fed on humans across the three ecological zones (χ2=0.70, df =2, P=0.704).

Host use by primary vectors (*Ae. mcintoshi* and *Ae. ochraceus*) also varied across the ecological zones. Primary vectors were significantly more likely to have fed on humans in the humid to dry sub-humid ecological zone (χ2=10.11, df =2, P=0.006) compared with those from forest and semi arid zones. This pattern was also observed in mosquitoes that had fed on livestock (χ2=21.23, df =2, P<0.0001). Ecological zone had no effect on the proportion of primary vectors that had fed on wildlife (χ2=3.51, df =2, P=0.172). The secondary vectors did not show any significant variation among host preference across ecological zones (χ2=3.99, df =2, P=0.136).

4.3.4 Blood meals by abundant mosquito species across ecological zones

Overall, Chi-square goodness of fit showed that some mosquitoes fed on certain hosts than others. For instance, there was high feeding association of *Ae. ochraceus* with livestock (χ2=15.13, df =2, P=0.0005) and humans (χ2=6.01, df =2, P=0.04) across the three ecological zones sampled in this study. The two key primary vectors of RVF, *Ae. mcintoshi* and *Ae. ochraceus*, fed on different hosts across the ecological zones. *Aedes mcintoshi* consistently

fed on humans and cattle across the ecological zones. In the humid to dry sub-humid ecological zone, where there is human habitation, more *Ae. mcintoshi* blood meals were from humans than cattle, but sampled mosquitoes had mainly fed on cattle in areas without human settlements. *Ae. ochraceus* mainly fed on cattle, humans, African buffalo and camels across the three ecological zones. In the forest ecological zone 5, blood meals of *Ae. ochraceus* were mainly from Cattle humans and African buffalo.

The secondary vectors of RVF (*Cx. pipiens, Ae. sudanensis* and *Ma. africana*) obtained blood meals from diverse hosts across all ecological zones. *Culex pipiens* fed on more hosts in the rainy season compared to the dry season. *Ma. africana* mainly fed on bushbuck and hippopotamus in the rainy season, whereas humans were the main host for this mosquito in the dry season. *Aedes sudanensis* mainly fed on cattle and humans during the dry season. This mosquito fed on more than six hosts during the rainy season, although humans, cattle and waterbuck were the most common source of blood meals.

Other mosquitoes, including *Ae. tricholabis* and *Ma. uniformis*, fed on only a few host animal species across ecological zones. During the dry season, fed *Ma. uniformis* obtained their blood meal from humans in the humid to dry sub-humid zone where human settlements were present. However, in the same ecological zone, this mosquito species mainly fed on hippopotamus, waterbuck and bushbuck. *Aedes tricholabis* were mainly captured from Boni forest. African buffalo was the main source of blood meals for this mosquito, with only some having fed on lesser kudu, topi and common duiker.

4.4 Discussion

Zoonotic diseases can be transmitted through infectious bites of mosquitoes while these vectors seek blood meals from animal hosts. Pathogens causing such diseases can be multi-host and heterogeneity of the host animal species in a habitat may create a suitable environment for transmission and spread of infectious diseases by vectors (Keesing et al., 2006). Control of diseases in an environment with a high diversity of hosts may be challenging, especially when multihost parasites are involved in the network (Streicker et al., 2013). In some cases, mosquitoes that transmit pathogens may prefer to feed on specific hosts, which determine the vulnerability of animal species to infection. However, certain factors, such as ecological zone and even season, may interact to determine vector feeding patterns, and consequently define disease transmission pathways. This study, presents spatial variation in host preference by mosquito species. The results suggest that the feeding preference of different mosquito species varies across ecological zones. Interaction between animals along migration routes may potentially instigate differential mosquito feeding preference, which may facilitate the transmission of pathogens from infected animal species or reservoirs. These pathogens may spill over into other species and increase circulation of arboviral pathogens (Parrish et al., 2008; Vandegrift et al., 2010).

Mosquitoes fed on a diversity of animal species across the ecological zones, with differential preference characteristics among the mosquito species. *Aedes mcintoshi*, which is a key vector of RVF in northern Kenya, demonstrated a conserved feeding preference and mainly preferred humans and cattle across all ecological zones and between the seasons. Thus, the abundance and distribution of *Ae. mcintoshi* may play an important role in transmission and circulation of arboviruses that infect humans and consequently cause morbidity and death during epidemics. This mosquito has been documented as the primary vector of RVF in

northeastern Kenya (Linthicum et al., 1984). Its feeding preference may introduce the virus into human populations or cattle, which can subsequently be amplified by mosquitoes with diverse hosts such as *Ae. sudanensis* and *Cx. pipiens*. The feeding characteristics of *Ae. mcintoshi* may also make it a potential amplifier of arboviruses between livestock and humans. This is supported by other studies that reported the role of this mosquito in the 2007 RVF outbreak, which largely affected humans and livestock in Kenya (Nguku et al., 2010; Sang et al., 2010).

Circulation of disease-causing pathogens may be affected by the level of interaction by host, parasite and vector species in an environment (Holt & Roy, 2007; Ostfeld & Holt, 2004). Presence of diverse hosts may determine the feeding dynamics among mosquitoes and epidemiology of arboviruses. In this study, host switching across ecological zone was prominent among *Ae. ochraceus*. This mosquito mainly obtained blood meals from humans, African buffalo and cattle, but demonstrated diverse feeding preference across ecological zones. In the forest ecological zone, *Ae. ochraceus* mainly fed on African buffalo and cattle but a small number of sampled mosquitoes had fed on humans, topi and bushbuck. This flexibility to utilise various vertebrate hosts may increase its role in amplification of arboviruses, and explains its role in transmission of RVF that was reported by Sang et al. (2010). Recent studies have reported sero-conversion of RVF in buffalos in South Africa (LaBeaud et al., 2011b). The preference for buffalos and other ungulates by *Ae. ochraceus* suggests that this mosquito forms an important link in the transmission of arboviruses from wild ungulates to domestic animals and subsequently to humans during epidemics, thus making it a key vector of arboviruses in northern Kenya. This is also supported by the fact that Sindbis and Babanki viruses were also isolated from this mosquito in the current project (see Chapter 5). Mosquitoes such as *Cx. pipiens, Ae. sudanensis,* and *Ma. africana* are secondary vectors of RVF, which also play an important role in transmission of mosquito

borne pathogens. Different viruses have been isolated from these vectors in northern Kenya and elsewhere across the globe (Crabtree et al., 2009; Ochieng et al., 2013). The secondary vectors showed a generalised feeding pattern involving diverse host animal species across ecological zones and between the dry and rainy seasons.

Indiscriminate feeding patterns were identified in *Cx. pipiens* and its feeding preferences were not influenced by ecological zone or season. These vectors fed on hosts including humans, and wild and domestic animals, which corroborates the findings of Apperson et al. (2002). The generalised host use of *Cx. pipiens* implies that it can contribute to the amplification of mosquito borne diseases and actively transmit arboviruses to many host animal species (Thiemann et al., 2012). For example, *Cx pipiens* has been incriminated in the circulation of West Nile virus (Almeida et al., 2008; Molaei et al., 2006). This finding supports other studies, which reported that multiple host feeding enhances circulation of mosquito borne diseases (LoGiudice et al., 2003). *Aedes sudanensis* also fed on a number of host animal species. However, it was notable that *Ae. sudanensis* fed on more hosts in the rainy season than in the dry season. Consequently, this vector may contribute to circulation of arboviruses that emerge during the rainy season. It is not known whether the preference for only humans and cattle by *Ae. sudanensis* in the dry season may have been as a result of the absence of other preferred hosts. However, this observation may suggest the important role of this mosquito in the maintenance and circulation of arboviruses in the dry season.

Mansonia africana preferred to feed on hippopotami over other animal species across ecological zones. This feeding pattern may have been due to the ecology of the area with large marshy water pools inhabited by hippopotami and also suitable breeding grounds for this mosquito. *Mansonia* mosquitoes are known to be secondary vectors of RVF (Sang et *al.*,

2010). Their abundance in such areas and feeding patterns may impact on transmission of zoonotic diseases circulating in these wild animals as they are also known to be a vector of lymphatic filariasis (Ughasi et al., 2012). This finding is in agreement with other studies, which showed that vector abundance may play an important role in transmission and circulation of zoonotic diseases (Ostfeld & Keesing, 2000; Pepin et al., 2010).

Considering the abundance of mosquito vectors of arboviruses in this study area, their role in circulation of diseases such as RVF may have greater consequences during epidemics. This is because transmission of these diseases largely depends on the potential of the mosquitoes to acquire, maintain and subsequently transmit the disease-causing pathogen, and can successfully occur when these vectors are infected as they seek blood from hosts within their surroundings (Killeen et al., 2001; LoGiudice et al., 2003; Sellers, 1980). The findings reported here suggest that the feeding patterns of mosquitoes play a key role in the dynamics of mosquito-borne viruses circulating in north-eastern Kenya. Feeding preference of these vectors forms the basis upon which disease transmission pathways can be defined in the natural environment. This may be useful in the management of the spread and predicting epidemiology of these diseases. Host switching between different animal species by mosquitoes in the feeding network across different geographical locations creates an important link by which arboviruses can be transmitted from wild ungulates or disease reservoirs to livestock and subsequently to humans. Mosquito feeding dynamics reported in this study may therefore be used to map high-risk areas for potential transmission of arboviruses that can also be targeted during vector and disease control programs to stem the widespread transmission of arboviruses during epidemics.

Chapter 5

CIRCULATION OF ARBOVIRUSES AMONG VECTOR SPECIES ALONG NOMADIC LIVESTOCK MOVEMENT ROUTES IN NORTHEASTERN KENYA

5.1 Introduction

Arthropod-borne viral diseases are transmitted by different species of mosquitoes. Circulation of these viruses may involve a network of humans, arthropod vectors, and wild and domestic ruminants (Woods et al., 2002). These viruses have caused epidemics in many parts of the world and they continue to spread across wide geographical areas. Rift Valley Fever virus (RVFV), for example, was first discovered in Kenya and is currently causing epidemics in Africa and in the Arabian Peninsula (Balkhy & Memish, 2003). Epidemics of other arboviruses transmitted by mosquitoes, including dengue and yellow fever, are also of great public health concern (Gubler, 2002). Transmission and circulation of these arboviruses require a population of susceptible vertebrate hosts in a given area to enhance amplification of the virus. However, this phenomenon must be complimented with favourable environmental conditions and high abundance of competent mosquito vectors (Crabtree et al., 2009; Sang et al., 2010). These vectors form an important link within which viruses can be moved from one host to another as they seek blood meals and consequently spread the viruses.

In northeastern Kenya, nomadic pastoralism is a key source of income (Hogg, 1986). Pastoralists move with their large herds of livestock for long distances in search of pasture and water. As mentioned in earlier chapters (Chapter 2, 3 and 4), the natural environment of the region coupled with cultural nomadic pastoralism contribute risk factors that favour the occurrence of diseases such as RVF and other arboviruses. This region has experienced repeated epidemics of RVF, including one in 2006/7 (Nguku et al., 2010). The area is

endowed with a wide diversity of vectors including floodwater *Aedes* and other vectors of the genus *Culex* and *Anopheles* (Lutomiah et al., 2013). These vectors contribute to the transmission and circulation of a number of arthropod borne viruses (Chapter 2; also see Logan et al., 1991; Ochieng et al., 2013). Interaction, between livestock and wildlife in grazing areas and water points creates a unique environment in which polyphagous mosquitoes (Chapter 4) can transmit viruses from reservoir animals native to such habitats to migrating domestic animals and humans. Due to such exposures, viruses transmitted by these vectors can be amplified, resulting in spillover of the viruses to non-infected populations (O'Brien et al., 2011). This may cause a disease outbreak in livestock and febrile illness amongst the herdsmen who move along the livestock movement routes (LMR) alongside their livestock.

Vectors in the northeastern region of Kenya have shown great potential to circulate arboviruses. For example, during the 2006/7 outbreak of RVF in the region, RVF virus was isolated from a range of mosquito species (Sang et al., 2010). In addition, viruses such as West Nile, Pongola and Bunyamwera were found to co-circulate with RVF (Crabtree et al., 2009). While documentation is not sufficient to determine whether these viruses may be periodic, it is important to understand the level of circulation of these viruses in mosquito vectors captured from disease endemic regions during inter-epidemic periods. This may help in disease control and in mapping out potential point sources or geographic areas where the viruses could occur or originate and knowing the vector species involved in their transmission and maintenance in the event of outbreaks being reported in human or animal population. Currently, there is a significant increase in the geographic distribution of arboviruses with increase in frequency of hemorrhagic fever virus outbreaks like RVF and Dengue in Kenya and across the globe (Balkhy & Memish, 2003; Nguku et al., 2010). The

spread of these arbovirus infections have been attributed to cultural practices such as pastoralism, vector diversity and anthropological activities which may modify the environment to suit survival and reproduction of disease vectors (Anyangu et al., 2010). The co-circulating viruses were detected from mosquito species found transmitting RVFV during the East African outbreak that occurred in 2007-2008 (Crabtree *et al.*, 2009). These viruses continue to actively circulate during RVF inter-epidemic periods with occasional isolations being reported from mosquitoes and may be causing febrile illness of unknown origins in northern Kenya. Abundance of the competent vectors for these viruses in different locations is one of the factors that may instigate outbreak and circulation of arboviruses. Assessment of the level of circulation of mosquito borne viruses should consider analysis of vectors trapped from diverse habitats especially in disease endemic regions. This forms the basis of this study, which focused on the circulation of virus in mosquito species trapped from different geographical areas with diverse ecological conditions that may influence virus circulation along LMR. The molecular genetic approach used in this study provides an in-depth analysis of the isolates important for understanding the genetic diversity of the viruses.

5.2 Materials and methods

5.2.1 Study site

This study was conducted in the pastoral eco-zone of northeastern Kenya. Pastoralism is practiced in this region as a major source of income. Traditional livestock movement routes used by a sentinel herd in search for pasture and water within areas where RVF had been previously detected were used to select sampling areas for trapping mosquitoes used for virus assays in this study. The LMR covers approximately 600 kilometers from Garissa County (S00° 39' E40° 05') in the northeastern region to Lamu County (Lamu S02° 16' E40° 54') on the Kenyan coast. The seven sampling areas were Haney, Degurdei, Arbadobolo, Boni

forest, Mlimani, Mangai and Dondori (Figure 5.1). Livestock movement in the region is a common practice and groups of herders pitch temporary camps where they settle with their grazing livestock until the pasture in the area is exhausted and they relocate to other areas. This practice drives the pastoralists to different grazing areas including wildlife reserves where the interaction between livestock and wildlife at water points and grazing fields is high and may contribute to the emergence and transmission of viruses from potential reservoirs to naïve hosts. The weather condition for the region during the sampling period is presented in chapter 2.2.1.

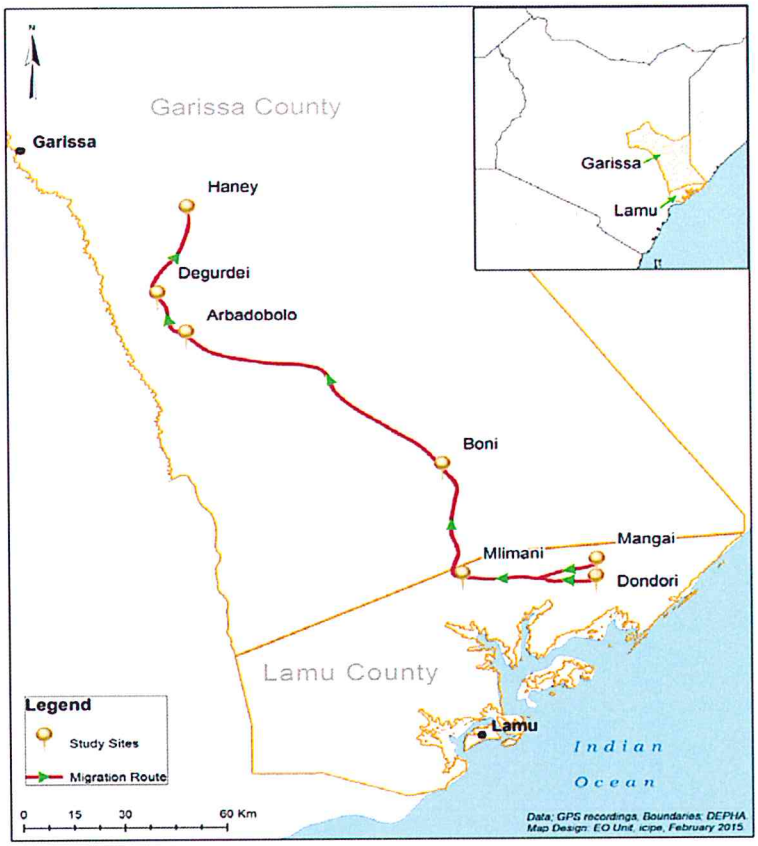


Figure 5.1: Map showing the sampling areas along livestock movement route in northeastern Kenya

5.2.2 Mosquito collection and processing

Mosquitoes were trapped using CDC light traps baited with CO_2 from November 2012 to August 2014 during the short and long rainy seasons in November and April, respectively (described in Chapter 2). Traps were set at 1800 hrs and retrieved at 0600 hrs. Captured mosquitoes in individual traps were sorted to remove other insects, preserved in a liquid nitrogen shipper and transported to the Emerging Infectious Disease (EID) laboratory at the International Centre for Insect Physiology and Ecology (*icipe*), Nairobi, Kenya, for further processing.

In the laboratory, mosquitoes were morphologically identified to species level using mosquito identification keys (Edwards, 1941). Those belonging to the same species were pooled to a maximum of 25 mosquitoes per pool and coded for screening in cell culture. Individual pools of mosquitoes were homogenised in a biosafety level 2 cabinet. In each microcentrifuge tube, a single 4.5-mm-diameter copper-clad steel bead (BB-caliber airgun shot) was placed together with 1 ml homogenisation medium consisting of Eagle's minimum essential media (MEM) (Sigma-Aldrich, St.Louis, MO) with Earle's salts and $NaHCO_3$, supplemented with 15% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), 2% L-glutamine (Sigma-Aldrich), and 2% antibiotic/antimycotic solution with 10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml (Sigma-Aldrich). The mosquitoes were then homogenised by shaking the tubes using a mini bead beater (Biospec) for ten seconds. The homogenate was clarified by centrifugation in a refrigerated bench-top centrifuge (Eppendorf AG Hamburg) at 12000 rpm for 10 minutes at 4°C and the supernatant transferred into 1.5 ml cryo vials for subsequent inoculations.

5.2.3 Screening of mosquitoes in cell culture

The supernatant obtained from mosquito pools were screened for viruses through cell culture inoculation in Vero cells (monkey kidney epithelial cells). This technique has been successfully used in the isolation of RVF virus in previous outbreaks (Fontenille et al., 1998; Nderitu et al., 2011; Sang et al., 2010). Fifty micro liters of each mosquito pool supernatant was inoculated into a single well of a 24-well culture plate containing a monolayer of Vero cells grown in MEM supplemented with 10% FBS, 2% L-glutamine, 2% antibiotic/antimycotic solution with 10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml. Inoculated cultures were incubated at 37°C in 5% CO_2 for one hour to allow virus adsorption to the monolayer, after which 1 ml of maintenance medium was added (MEM with 5% FBS, 2% L-Glutamine, 2% antibiotic/antimycotic solution). Cells were incubated at 37°C in 5% CO_2 and monitored daily for 14 days for any cytopathic effects (CPE). Cell culture supernatants were carefully harvested when CPE occupied more than 50% of the cell monolayer in the well (Figure 5.2 a and b). The harvested cultures were further sub-cultured to amplify the virus and confirm the reproducibility of the isolate.

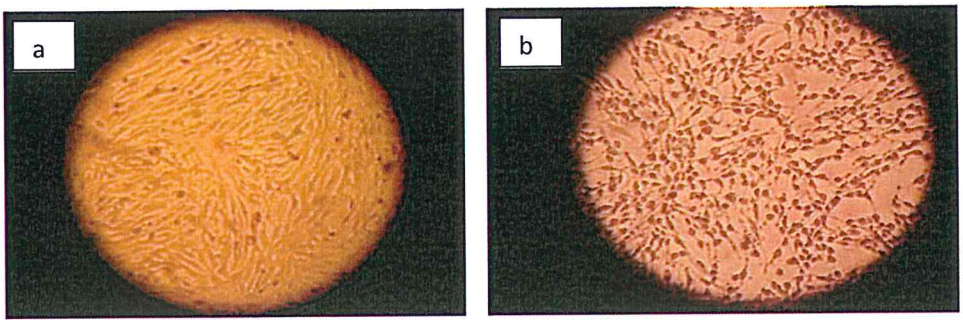


Figure 5.2: Photo of (a) Negative control and (b) Positive sample in this study showing cytopathic effects

5.2.4 Characterisation of virus isolates by RT-PCR

RNA was extracted using the Trizol®-LS-chloroform extraction protocol from 250 µL of each cell culture isolate. The resultant RNA pellet was dissolved in 11 µL of nuclease-free water at room temperature and either frozen or directly used to synthesise cDNA. Subsequently, 10 µL of RNA and 2 µL of random hexamer (100 nmol) were mixed in PCR tubes with sample labels. A PCR reaction was set up in a thermocycler programmed at 70°C for 10 minutes to denature the sample then cooled to 4°C for five minutes. To each sample, 4 µl of 5× first strand buffer (Invitrogen), 0.01 µM of dNTPs (Invitrogen), 0.02 µM of DTT (Invitrogen), 10 U of RNase out inhibitor (Invitrogen) and 100 U of Super Script III reverse transcriptase (Invitrogen) were added to make a volume of 20 µL and then incubated in the thermocycler set at the following cycling conditions: 25°C for 15 min, 42°C for 50 min, 70°C for 15 min and 4°C hold temperature. The volume of cDNA obtained was used to perform various amplifications to test for different genera and specific viruses. The reaction for specific viruses comprised 12.5 µl of Amplitaq Gold PCR master mix (Applied Biosystems), 0.5 µl of forward primer and a similar amount for reverse primer, 2 µl of the cDNA and 9.5 µl of water, making up a reaction volume of 25µl for each sample, as well as a positive and a

negative control. The specific reactions were conducted using cycling conditions for specific primers for family and specific virus tests (Table 5.1).

Table 5.1: DNA sequences of the primers used to characterise the isolates obtained from this study

Virus	Protein target	Primer sequence	Expected size	Position
Alphavirus	NSP4	VIR 2052 F; (5′-TGG CGC TAT GAT GAA ATC TGG AAT GTT-3’)	150bp	6971–6997
"		VIR 2052R; (5′-TAC GAT GTT GTC GTC GCC GAT GAA-3′)	150bp	7086–7109
Flavivirus	NS5	FU 1; (5′- TAC AAC ATG ATG GGA AAG AGA GAG AA-3′)	160bp	9007-9032
"		CFD2; (5′- GTG TCC CAG CCG GCG GTG TCA TCA GC-3′)	160bp	9308-9283
Bunyavirus	Nucleocaspid protein	BCS82C; (5′-ATG ACT GAG TTG GAG TTT CAT GAT GTC GC-3′)	251bp	86-114
"		BCS332V; (5′-TGT TCC TGT TGC CAG GAA AAT-3′)	251bp	309-329
RVF	Glycoprotein Mgene	RVF1; (5′-GAC TAC CAG TCA GCT CAT TAC C-3′)	363bp	777-798
"		RVF2; (5′-TGT GAA CAA TAG GCA TTG G-3′)	363bp	1309-1327
Sindbis	Non structural protein	SINV1; (5′-TTTAGCGGATCGGACAATTC-3′)	1084bp	5194-5213
"		SINV2; (5′-GCGGTGACGAACTCAGTAG-3′)	1084bp	6482-6500
Babanki	E1 envelop glycoprotein	Bab 3368 F; (5′- CAG CAG ATT GCG CGA CTG ACC-3′)	835bp	3368-3388
"		Bab 4203R; (5′- GCT CAC GAT ATG GTC AGC AGG-3′)	835bp	4184-4203

5.2.5 Gel electrophoresis

Electrophoresis was performed on a 1.5% agarose gel in tris-borate EDTA buffer stained with ethidium bromide for 1 hour to visualise the amplified samples. The amplicon bands were visualised by a UV transilluminator and prints of the gel obtained from the imaging system (Figure 5.3). The amplicons with clear positive bands were purified for sequencing by specific primers using a Wizard ® SV Gel and PCR Clean-Up System kit (Promega) according to the manufacturer's instructions, and the products were sequenced. Resultant sequences were compared with ones in the GenBank database using NCBI BLAST, to identify the viruses obtained from the samples.

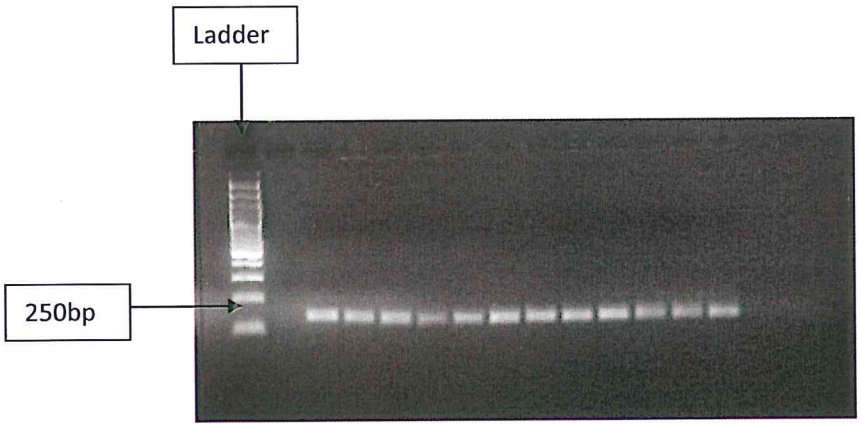


Figure 5.3 : Photograph of an RT-PCR gel showing samples positive for alpha viruses
The samples were run using a 100bp ladder with 0.1µg/µ molecular weight.

5.2.6 Data analyses

Virus sequences were analyzed in MEGA6 software and identified with reference to GenBank sequences. Mr Model test version 2.3 (Nylander, 2004) together with PAUP*4b10 (Swofford, 2002) using the Akaike information criterion (AIC) were used to predict the best parameters for reconstructing Bayesian trees. The Mr Model test predicted the JC and GTR evolutionary models as the best for the set of sequences for BBKV and SINV, respectively,

with only data for SINV subjected to a gamma distribution and a proportion of invariable sites. The Mr Bayes software package 3.1.2 (Ronquist & Huelsenbeck, 2003) was then used to run MCMC Bayesian inference (BI). The program was set to run for 10,000,000 generations with sampling every 1,000 generations. A 50% majority rule consensus tree was created from the trees remaining after a 10% burn-in removal. The included Bayesian sets of trees were sampled after likelihood scores reached convergence and the mean split difference values were almost 0.01. Trees were visualised using Fig tree v 1.4.0 with nodal support evaluated by posterior probabilities (PP) for the Bayesian inferences.

5.3 Results

5.3.1 Processing of vectors

In this study, over 31,000 mosquito vectors were collected from the seven sampling areas. These mosquitoes were mainly classified into four main genera; *Aedes, Culex, Anopheles* and *Mansonia*. The mosquitoes were pooled into a total 2240 pools. Each pool comprised the same species of identified mosquitoes and did not exceed 25 mosquitoes in number. These pools were screened for viruses and 30 reproducible virus isolates were obtained from pools of different mosquito species trapped from diverse locations in the study area.

5.3.2 Viruses isolated from mosquito species

Sindbis (SINV) and Babaki (BBKV) viruses were isolated from both primary and secondary vectors of RVF. However, no mosquito sample tested positive for RVF virus. SINV and BBKV were isolated from *Ae. mcintoshi,* and *Ae. ochraceus* (primary vectors of RVF), and other mosquitoes including *Ae. sudanensis, Ae. tricholabis, Mn. africana, Cx. pipiens* and *An. gambiae* (Table 5.2). The highest number of virus isolates (10) was obtained from *Ae. mcintoshi*. Among the secondary vectors of RVF, the highest isolation (6) was from *Ma.*

africana. All the viruses obtained in this study were isolated from mosquitoes trapped during the rainy seasons and none were detected in the dry season.

Table 5.2: Number of viruses isolated from mosquito species trapped from the sampling areas

	Viruses		
Moaquito species	Sindbis	Babanki	Pools tested
Ae. mcintoshi	5	5	303
Ae. ochraceus	3	2	342
Ae. sudanensis	0	2	175
Ae. tricholabis	2	0	112
Ma. africana	3	3	517
Cx. pipiens	0	4	218
An. gambiae	1	0	50
Total	14	16	1717

5.3.3 Circulation of Sindbis and Babanki viruses across the sampling areas

The isolated viruses were circulating in mosquitoes trapped from different sampling areas. These viruses were present in varying proportions in three of the seven sampling sites mainly from vectors trapped from Boni forest, Degurdei and Mangai, which are located in diverse ecological zones along the LMR namely forest, semi-arid and humid to dry sub-humid zone. These sites are common livestock and wildlife interfaces.

The majority of isolates (n=13, 43 %,) were from Mangai followed by Degurdei (n=11, 37%,) and Boni forest (n=6, 20%). In Degurdei area, SINV was circulating in *Ae. mcintoshi*, from which four isolates were obtained, while only one isolate of the same virus was obtained from *Ae. ochraceus*. Similarly five isolates of BBKV were obtained from *Ae. mcintoshi* while two isolates of the same virus were obtained from *Cx. pipiens* and *Ae. sudanensis* from the same area. In Boni forest, SINV was isolated from *Ae. tricholabis, Ae. ochraceus* and *Mn. africana.* It was notable that SINV virus isolates were mainly from *Ae. tricholabis* from

which 3 out of the five isolates were obtained. *Ae. ochraceus* and *Mn. africana* each had only one isolate of SINV from this site. In Mangai, BBKV and SINV were detected in diverse mosquito species. Eleven virus isolates were from four genera of mosquitoes; *Aedes, Anopheles, Culex* and *Mansonia. Mn. africana* and *Ae. ochraceus* circulated both SINV and Babanki viruses. *Cx. anulioris* only circulated Babanki whereas *An. gambiae* and *Ae. mcintoshi* mainly circulated SINV. Sindbis virus and BBKV were isolated from most mosquitoes in Mangai compared to other sites.

5.3.4 Phylogenetic analysis of virus isolates

Although 30 isolates were obtained, Sindbis (14) and Babanki (16), partial sequencing was performed for only twenty one isolates of BBKV and SINV and analyzed by comparing homologous sequences of other isolates from GenBank to determine any evolutionary relationship between our isolates and other strains (Figures 5.4 and 5.5). This was based on the fact that the remaining nine isolates were similar to the ones sequenced based on origin of isolates and to also reduce the costs associated with the sequencing of the samples. Babanki primers targeted the E1 envelope glycoprotein gene (835bp) while Sindbis primers targeted a non-structural protein gene nsP2 (1084bp). The reference sequences selected from GenBank originated from Cameroon, South Africa, Germany and the USA. The percentage identity of the Babanki and Sindbis isolates from this study ranged between 94-98% when compared with the similar sequence of isolates in GenBank. The isolates sequenced in this study are presented in Table 5.3. The results of the phylogenetic analysis reveal that there was less in group diversity in the study BBKV isolates since they clustered together in the phylogenetic tree irrespective of the mosquitoes they were isolated from or site of origin. These isolates also showed low phylogenetic relationship with GenBank sequences from different regions, which formed a separate clade. Sinbis virus demonstrated in group diversity of the sequences

obtained in this study. A close phylogenetic relationship was observed among isolates from diverse locations with minimal diversity between isolates from same sites. In comparison with sequences from GenBank, there was more differentiation observed among study isolates, which clustered in different clades. The study isolates appear to have more likely originated from the USA isolate and not those from GeneBank (Germany and South Africa)

Table 5.3: Isolates of Babanki and Sindbis viruses sequenced in this study

Virus	Isolate	Area	Site	Mosquito species	Season
Babanki	BBKV_270A	Garissa	Degurdei	*Cx. pipiens*	Long rains
	BBKV _239	Garissa	Degurdei	*Ae. mcintoshi*	Long rains
	BBKV _269	Garissa	Degurdei	*Ae. mcintoshi*	Long rains
	BBKV _270B	Garissa	Degurdei	*Cx. pipiens*	Long rains
	BBKV _271	Garissa	Degurdei	*Ae. sudanensis*	Long rains
	BBKV _287	Garissa	Degurdei	*Ae. mcintoshi*	Long rains
	BBKV _430	Garissa	Degurdei	*Ae. mcintoshi*	Long rains
	BBKV _584	Garissa	Degurdei	*Ae. tricholabis*	Short rains
	BBKV _818	Lamu	Mangai	*Ae. ochraceus*	Short rains
	BBKV _771	Lamu	Mangai	*Ma. africana*	Short rains
Sindbis	SINV _438	Garissa	Degurdei	*Ae. mcintoshi*	Long rains
	SINV _496	Garissa	Degurdei	*Ae. mcintoshi*	Long rains
	SINV _527	Lamu	Mangai	*Ma. africana*	Long rains
	SINV _228	Garissa	Degurdei	*Ae. mcintoshi*	Long rains
	SINV _549	Boni forest	Boni	*Ae. tricholabis*	Short rains
	SINV _562	Boni forest	Boni	*Ae. ochraceus*	Short rains
	SINV _566	Boni forest	Boni	*Ae. ochraceus*	Short rains
	SINV _868	Lamu	Mangai	*Ae. mcintoshi*	Short rains
	SINV _872	Lamu	Mangai	*Ma. africana*	Long rains
	SINV _894	Lamu	Mangai	*An. gambiae*	Long rains
	SINV _925	Lamu	Mangai	*Ma. africana*	Short rains

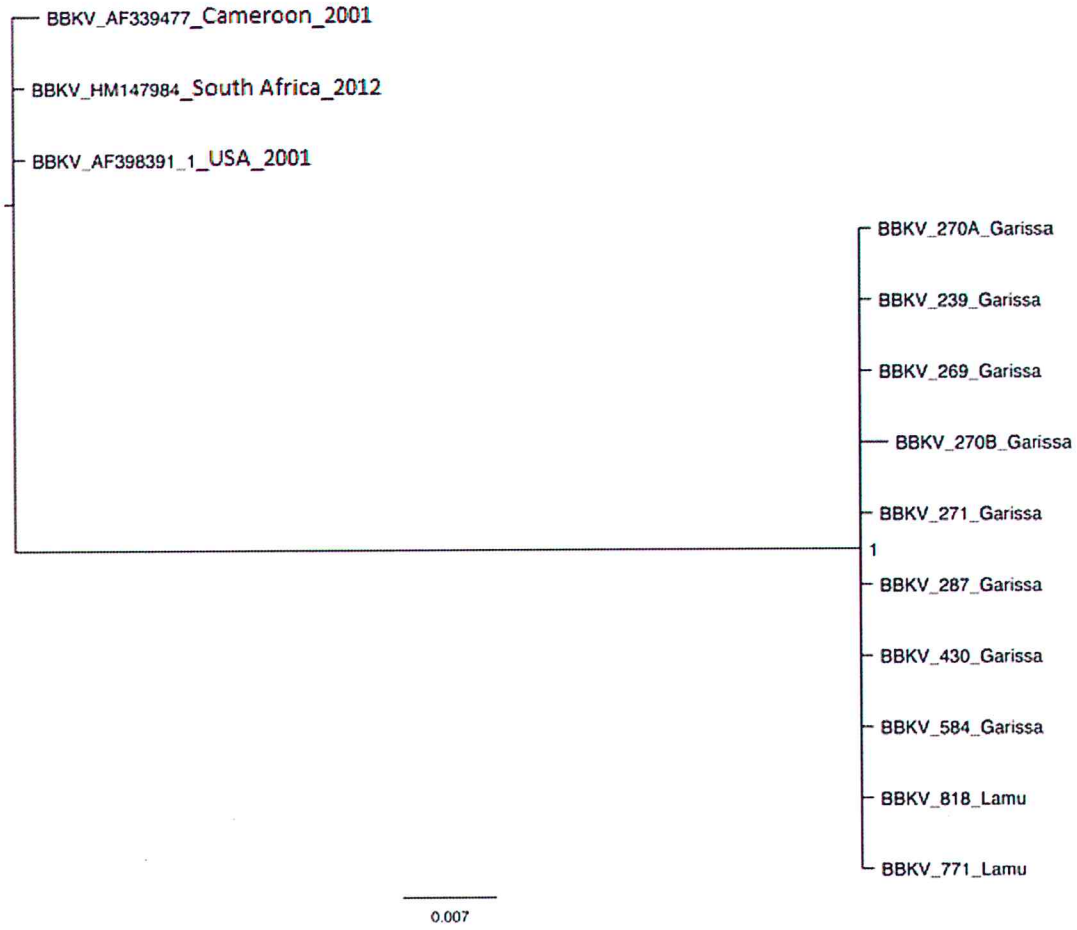


Figure 5.4: Phylogenetic tree of nucleotide sequences of Babanki virus isolates and reference sequences from GenBank; AF339477 (Cameroon), HM147984 (South Africa) and AF398391 (USA). The sequences from this study are BBKV_270A, BBKV_239, BBKV_269, BBKV_270B, BBKV_271, BBKV_287, BBKV_430, BBKV_584, BBKV_818, BBKV_871. The nucleotide sequences from GenBank and those of the selected Babanki virus isolates from this study were aligned using clustalW and the phylogenetic tree constructed using MEGA6. The included Bayesian sets of trees were sampled after likelihood scores reached convergence and the mean split difference values were almost 0.01.

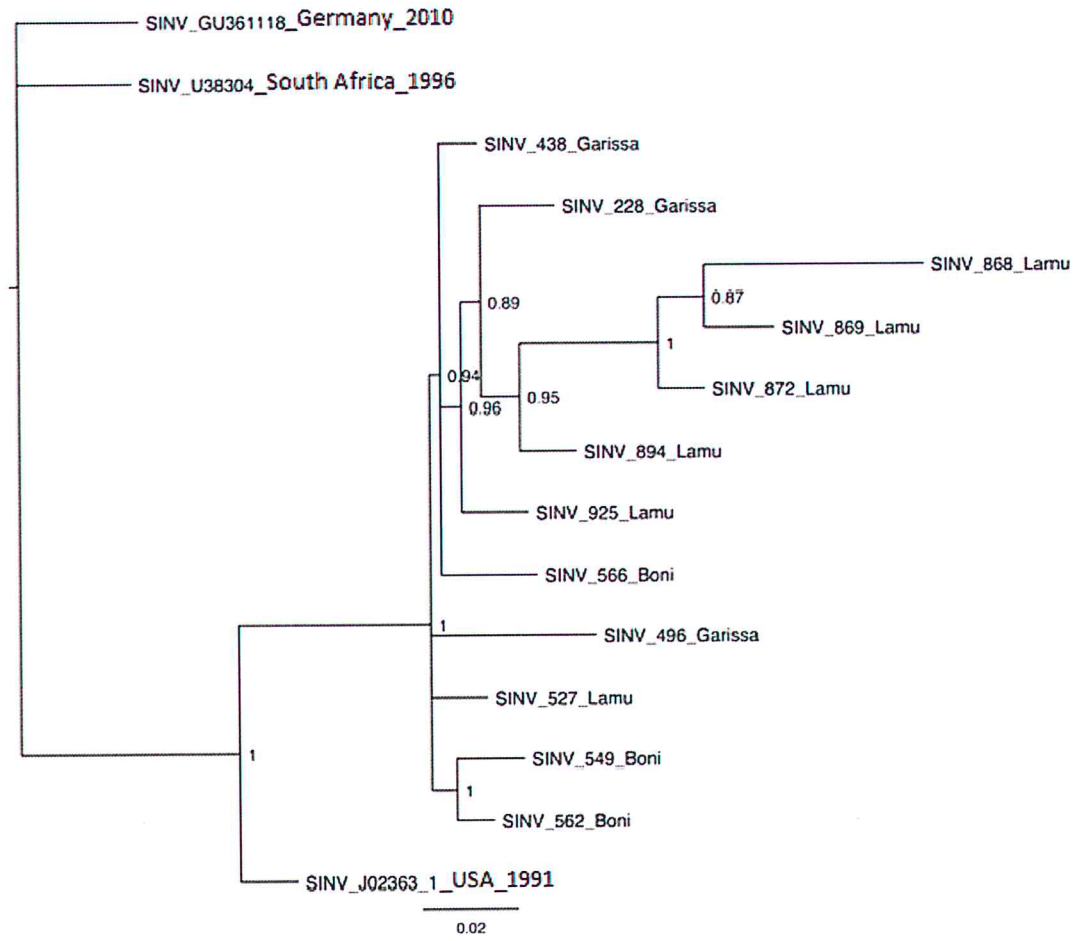


Figure 5.5: Phylogenetic tree of nucleotide sequences of Sindbis virus isolates and reference sequences from GenBank; GU361118 (Germany), U38304 (South Africa), J02363_1 (USA). Standard abbreviation (SINV) has been used to denote Sindbis virus followed by isolate number and area from which the isolate was obtained. The included Bayesian sets of trees were sampled after likelihood scores reached convergence and the mean split difference values were almost 0.01.

5.4 Discussion

Interaction between livestock, humans, wildlife and disease vectors may increase the risk of viral disease transmission to humans and domesticated animals from infected wildlife via the vectors (Gubler, 2002; Parrish et al., 2008; Vandegrift et al., 2010). In this study we isolated only alphaviruses, namely, SINV and BBKV virus in mosquito catches from three study sites; Degurdei, Boni forest and Mangai. Although part of this study has not reported high feeding preference for birds, the two viruses are usually associated with migratory birds (Kurkela et al., 2008). Such birds may nest along rivers, forests and lakes where they may be fed on by mosquitoes transmitting viruses. This creates the potential for transmission of these viruses from birds to humans by the some multi-host mosquitoes, which are known to feed on humans.

Sindbis virus has been isolated from mosquitoes in some African countries such as South Africa and has been reports in Australia, Northern Europe, and the Middle East. (Lundström, 1999; Shope & Meegan, 1997). In humans, it is known to cause febrile illness accompanied by fever, arthritis and rash. Similarly, BBKV has also been associated with human febrile illness. Babanki has been isolated from humans in Cameroon, Madagascar, and the Central African Republic (Gubler, 1996; Shope & Meegan, 1997). These two viruses are closely related. Babanki virus is known to be a Sindbis like agent circulating mainly in west and central Africa, its clinical significance in terms of infection has not been sufficiently documented (Shope and Meegan, 1997, Peters and Dalrymple, 1990). This study shows that the level of circulation of SINV and BBKV were almost similar. The level of circulation of these viruses could have been as a result of abundance of mosquito vectors and presence of potential vertebrate hosts in this region. Isolation of SINV and BBKV viruses in the current study shows that the human population in northern Kenya may acquire infection due to

persistent exposure to vectors along livestock movement routes (LMR) which may result into febrile illnesses of unknown origin.

Sindbis and Babanki viruses were isolated from diverse species of mosquitoes including *Ae. mcintoshi* and *Ae. ochraceus*, which have also been incriminated as primary and secondary vectors of RVFV and other arboviruses in northern Kenya (Crabtree et al., 2009; Ochieng et al., 2013; Sang et al., 2010). Circulation of viruses by mosquitoes depends on the capacity of the mosquito to acquire, maintain and transmit the pathogen (Sardelis et al., 2001; Turell et al., 2001b). Our results suggest that *Ae. mcintoshi* and *Ae. ochraceus* may play important roles in transmission of multiple arboviruses in the multi-host environment of north-eastern Kenya. Although these two mosquitoes are not known to feed on birds, they may have acquired viruses through a feeding network after potential introduction by avian feeding *Culex* mosquitoes like *Cx. pipiens and Cx univittatus* among others, which is known to feed on a diversity of animals (Molaei et al., 2006). This supports the findings of other studies, which documented the potential spill over of viruses from infected indigenous populations in a community (Kelly et al., 2009). This network may increase the level of circulation of these viruses, which pose health risks to the human population of the area.

Both viruses were mainly isolated in mosquitoes trapped in Mangai and Degurdei, rather than Boni forest, which are livestock grazing areas and also important wildlife interfaces. Degurdei is located in Garissa County, which is a key RVF hot spot and pastoral communities were most affected by RVF during previous outbreaks in this area (Anyangu et al., 2010; Nguku et al., 2010). Detection of Sindbis and Babanki viruses during this project suggest that these viruses may contribute to febrile illness between outbreaks of RVF and may also be part of the public health challenge during the outbreaks which go undetected as

people mainly focus on RVF. Thus, these viruses also need to be given adequate attention in both epidemics and inter epidemic periods. In this study, RVF virus was not detected in all the analysed mosquito samples. This could be attributed to variability in the distribution of the unknown reservoirs of the virus along the LMR during the interepidemic period. The other possibility is that the virus may be circulating below detectable levels during the inter-epidemic period. This is corroborated by the findings by Owange *et al.*, (2014) which detected elevated seroprevalence of RVF among livestock in the same areas during the rains as evidence of RVF virus circulation around this same period. More focus should therefore be directed towards identifying potential reservoirs of RVF including doing more extensive spatial scale sampling to cover a wider range of habitats to assess the variability of host preference in primary mosquito vectors as a means of identifying potential reservoirs.

Genetic diversity is an important factor in a population that allows different species of organisms to acquire genetic transformation in a given environment due to prevailing ecological pressures (Schneider & Roossinck, 2001). These pressures may cause the virus strains to undergo evolutionary changes resulting in diverse population structures. This study detected Sindbis and Babanki viruses isolated from diverse locations. These virus strains showed different population structure despite the fact that some were isolated from similar mosquito species. Phylogenetic analyses reveal that BBKV virus isolates clustered together and did not show a close phylogenetic relationship with reference sequences from GenBank. Clustering together of BBKV isolates shows less in-group diversity of this virus strain in northern Kenya. It is also notable that this relationship may not have been as a result the spread of similar virus strain by the same mosquitoes given that these sites are located far apart. This study shows that the SINV may have originated from Boni forest and subsequently moved to other locations of the region with over a period of time through

vertebrate hosts or vectors. Although, Sindbis did not cluster together with the GenBank sequences, there was a relatively close phylogenetic relationship with USA strain compared to that from Germany and South Africa. High in-group divergence was observed in SINV isolates with close association of isolates from the same geographical areas. These scenarios may be explained by the effect of diverse host animal species within which the virus strain replicated, subsequently generating strains with different levels of diversity (Ostfeld & Keesing, 2012). Alternatively, these viruses may have also been capable of generating levels of diversity in different hosts (Holmes, 2004; Plyusnin & Morzunov, 2001), but some selection pressure specific to a particular host limited the level of development resulting into the observed population structures between the two viruses. The presence and mobility of the primary vertebrate hosts of these viruses along livestock movement routes may thus be largely responsible for the diversity of these viruses in north eastern Kenya (Martella et al., 2010).

In conclusion this study confirms circulation of arboviruses in diverse mosquito species. Even though we did not isolate RVFV, SINV and BBKV were isolated from mosquitoes and these viruses could be causing febrile illness among pastoralists. Circulation of these viruses in mosquitoes merits further studies on the epidemiology and medical importance of these viruses in the northeastern region of Kenya.

Chapter 6

GENERAL CONCLUSION

Mosquitoes transmit approximately 50% of known arthropod-borne viruses (Karabatsos, 1985). Some of the arboviruses transmitted by mosquitoes include West Nile, Sindbis, Ndumu, Chikungunya and RVF, among many others (LaBeaud et al., 2011a). Critical in northeastern Kenya is RVF, which has caused recurrent outbreaks and significant economic impacts. This virus belongs to family *Bunyaviridae* and genus *Phlebovirus.* Since the emergence and identification of the virus in 1930's in Kenya (Daubney et al., 1931), the virus has spread across Africa, and for the first time in the year 2000, outside the borders of Africa into the Arabian Peninsula. Considering the changing climate and ecological conditions in different regions of the world, RVF has the potential to spread further into other regions where ecological conditions may favour mosquito vector species with the potential to transmit the virus, and where there is also close interaction with livestock through pastoralism and trade between countries in Africa and other continents. This may trigger the wide spread of the RVF virus during epidemics. RVF has caused serious epidemiological consequences in the recent outbreaks witnessed in African countries. However, there is limited knowledge on the maintenance of the virus during inter epidemics and subsequent re-emergence and circulation of the virus. The potential role of vectors in transmission of the virus in different ecological zones has not been given adequate attention in recent studies. In this study therefore, a comprehensive mosquito vector ecology study was conducted in the RVF hotspot of northeastern Kenya with the view of understanding the dynamics in vector diversity, survival and resting preference, feeding preference and circulation of RVF and other arboviruses in vectors of RVF as presented in the chapters 2, 3, 4 and 5 of this study. This region is inhabited by nomadic pastoralists who migrate for over 300 Km with livestock in search of pasture and water. Consequently, the pastoralists are exposed to risks of infection

by viruses circulating in diverse ecological zones of the region. The output of this study may support efforts by public health authorities in forecasting, managing and controlling RVF and other arboviruses through an informed approach.

In chapter 2 of this thesis, the diversity and distribution patterns of both primary and secondary vectors across diverse ecological zones along livestock movement routes was highlighted. As expected, *Ae. mcintoshi* and *Ae. ochraceus* were the key primary vectors present in the study area. Secondary vectors also included *Cx. pipiens, Cx. univittatus* and *Ae. sudanensis*. These findings are in agreement with the reports from entomological investigations conducted in the region in previous studies. However, this study presents a unique vector distribution pattern in which both primary and secondary vectors showed differential preference of ecological zones in the region: primary vectors were mostly present in the semi-arid ecological zone, whereas secondary vectors were dominant in the sub-humid ecological zone. In view of these findings, sites along LMR in the semi-arid areas form the major risk area areas where primary amplification of RVF virus by vectors in both livestock and human may occur. This supports other studies, which documented the adverse effects of previous RVF outbreak mostly in semi-arid areas of northern Kenya (Anyangu et al., 2010; Nguku et al., 2010). Other than the dynamics of the vector populations across ecological zones, the mosquito distribution pattern highlighted in this study therefore presents an opportunity to map risk areas for routine vector surveillance and control. More important for further investigation is the role other ecological factors in contributing to observed distribution patterns. This may help to unravel the factors underlying the re-emergence of arboviruses in this region.

In chapter 3, the survival rates and resting patterns of the vectors of RVF were presented. This chapter compared the survival rates between the primary vectors of RVF in Kenya, *Ae. mcintoshi* and *Ae. ochraceus*, and the resting preferences of primary and secondary vectors among different vegetation types in the study area. Differential survival patterns among these vectors may influence their role in transmission of RVF and other arboviruses. The results indicated that survival rate was much higher in *Ae. ochraceus* than *Ae. mcintoshi*, and during the rainy season compared with the dry season. Some studies have reported that mosquito borne viruses are mostly associated with rainfall, as it increases the capacity of the vectors to obtain suitable habitats for breeding. High parity among the primary vectors in the rainy season increases the risk of transmission and circulation of viruses by these vectors. *Ae. ochraceus* was recently incriminated in transmission of RVF. The high survival of this vector reported in this study shows the increasing capacity of this mosquito as a key vector of arboviruses in northern Kenya. In addition, the ability of this vector to potentially survive longer than *Ae. mcintoshi*, supports the recent involvement of this vector in RVF circulation. Part of this study investigated the feeding preference of mosquitoes; this may provide further information on the critical role of these mosquitoes in circulation of diseases in their natural habitat by determining their hosts which may be disease reservoirs. However, more studies should be conducted to enhance understanding of mosquito survival to support parity used in this study. This study also reports the preference of some plant species by mosquitoes as resting sites. The resting pattern of these vectors may provide insights into factors that contribute to the survival of these vectors in the surrounding environment. However, more studies will be needed to examine the interactive relationship between the vectors and plant species because it has been reported in other studies that some plants produce attractive or repellant volatiles. Whether the resting preference observed is on the basis of chemical

attraction is not explained by this study and determining whether it is for any physiological benefit will be a study worth undertaking.

In chapter 4, this study determined the host feeding preference of the vectors of RVF captured from different study locations. Female mosquitoes require a blood meal for egg development. It is imperative that these vectors seek hosts for a successful completion of their gonotrophic cycle. The findings show that there were differential feeding patterns among the vectors with varying preferences among humans, livestock and wildlife across the ecological zones. The results indicated that *Aedes* and *Culex* species fed on both wild and domestic animals. These variations in feeding patterns may provide crucial links that define disease transmission pathways along the LMR. Differential feeding patterns were evident when vectors were confronted with multiple hosts and especially in the wildlife-livestock interfaces. *Ae. sudanensis* and *Cx. pipiens* used a wide range of hosts in sites where there were both livestock and different species of wild animals, whereas *Ae. mcintoshi* obtained their blood meals from humans and domestic cows. On the contrary, *Ae. ochraceus* mostly fed on humans in areas close to human habitation, but mostly fed on African buffalo in sites where there were increased population of host animal species. This network can be used to determine risk areas where emergence of pathogens may occur in disease endemic areas. These findings may also help to identify vectors that transmit viruses from disease reservoirs to other populations. For instance, sero-conversion of RVFV antibodies during inter-epidemics has been reported among buffalo in South Africa (LaBeaud et al., 2011b). The study by (LaBeaud et al., 2011b) demonstrated low-level activity of the virus in African buffalo during an inter-epidemic period. This study highlights that *Ae. ochraceus* mostly preferred this host in some areas, suggesting that this vector could play an important role in transmission of RVF during outbreaks from buffalos to livestock and subsequently to

humans. Similar studies on viral activity in wildlife should also be conducted in Kenya to identify potential reservoirs of RVF given that the interaction existing between domestic and wild animals is unavoidable in LMR in northern Kenya.

Chapter 5 of this study investigated the level of circulation of RVF and other arboviruses in mosquitoes trapped from the study sites. We found that arboviruses were widely distributed in the study area and were circulated by vectors of RVF. This confirms the reports from other studies that demonstrated that primary vectors of RVF have the potential of transmitting other viruses other than RVF. The population structure of the two virus isolates in this study shows the potential of vertebrate host in determining the genetic diversity of the viruses and their distribution. Most of these viruses were isolated from mosquitoes trapped from Degurdei, Boni forest and Mangai. These sites form some of the risk areas where virus circulation might be instigated as they are important wildlife livestock interface in this region. Although we did not isolate RVF virus from mosquitoes, we isolated other arboviruses such as Sindbis and Babanki from both primary and secondary vectors of RVF. Isolation of these viruses have also been reported in other studies conducted in parts of the current study area (Crabtree et al., 2009). This shows that these viruses circulate in mosquitoes and can potentially cause febrile illness to populations in this region because Sindbis and Babanki viruses are known to cause febrile illness in human (Sane et al., 2012). The fact that these viruses were isolated from different mosquito species is a demonstration that these viruses have the potential to cause severe effects in the event that their amplification coincides with suitable ecological conditions for the vectors which can circulate them. Further research should be conducted with improved virus detection techniques to examine potential low level virus activity in mosquitoes during inter epidemics just as it has been reported in animals.

Overall, this work provides crucial vector ecological data showing that nomadic pastoralists are faced with serious health risks in their day-to-day life. There is evidence of potential exposure of residents of this region of northeastern Kenya to mosquito borne viruses including RVF due to pronounced presence and distribution of the vectors in the region. Since the diversity of vectors was high in rainy season, appropriate vector management strategies should be put in place especially in the rainy season to control mass emergence of vectors of arboviruses along livestock movement routes. This may minimise the emergence and rapid transmission of viruses to livestock and humans. Although RVFV was not isolated during this project, the findings show that some arboviruses circulate across the three ecological zones and the intensity of their circulation was high in the rainy season. Results also indicate high circulation of these viruses in semi-arid and forest ecological zones, identifying them as high risk areas for virus circulation during epidemics. Based on the findings of this project, measures such as treating livestock with repellants should be put in place, especially during seasons when the livestock graze in areas with diverse mosquitoes and other biting insects. Pastoralists should also be encouraged to use insecticide treated nets to protect themselves from mosquitoes. These measures may minimise the risk factors which may potentially trigger the onset of arboviral disease epidemics in northeastern Kenya.

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