UNIVERSITY OF NAIROBI

School of Biological Sciences

LABORATORY ASSESSMENT OF VECTOR COMPETENCE OF *PHLEBOTOMUS DUBOSCQI* TO A NOVEL SANDFLY-ASSOCIATED PHLEBOVIRUS

BY

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DECLARATION

I declare that this thesis is my original work and has not been submitted elsewhere for examination, the award of a degree or publication. Where other people's work or my own work has been used, this has been properly acknowledged and referenced in accordance with the University of Nairobi's requirements.

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List of Abbreviations

NPV	Ntepes virus
ZCL	Zoonotic Cutaneous Leishmaniasis
CBRD	Centre for Biotechnology Research and Development
KEMRI	Kenya Medical Research Institute
ICIPE	International Center for Insect Physiology and Ecology
EID	Emerging Infectious Diseases
WHO	World Health Organization
CO1	Cytochrome Oxidase subunit1
ICTV	International Committee for Taxonomy of Viruses
RNA	Ribonucleic Acid
SFTS	Severe fever with thrombocytopenia syndrome
EIP	Extrinsic Incubation Period
MEB	Midgut Escape Barrier
MIB	Midgut Infection Barrier
SGIB	Salivary Gland Infection Barrier
RVFV	*Rift Valley fever virus*
GFV	*Gabek Forest virus*
PRNT	Plaque Reduction Neutralization Test
MEM	Minimum Essential Media
FBS	Fetal Bovine Serum
CPE	Cytopathic Effects
GIBCO	Grand Island Biological Company
PFU	Plaque Forming Units
RH	Relative Humidity
HM	Homogenization medium
IR	Infection Rate
USA	United States of America
VHF	Viral hemorrhagic fevers
DPI	Days Post Infection
AFI	Acute febrile illness

ABSTRACT

Phleboviruses transmitted by sand flies are among emerging public health threats. A novel *Phlebovirus* named *Ntepes virus* (NPV) was recently described in Kenya and found to infect humans from a wider geographic area. However, the entomologic risk factors such as potential vectors and transmission efficiency remains poorly defined. This study assessed the ability of the sand fly *Phlebotomus duboscqi* to transmit NPV. Two hundred and five 5-day old laboratory colonized *P. duboscqi* were exposed to NPV by membrane feeding in a triplicate experiment with a viremic blood meal of a dose of about $10^{6.0}$pfu/ml. All the 205 NPV-exposed sandflies were randomly picked on the 6^{th}, 10^{th} and 15^{th} days post infection and individually dissected into abdomens, legs and salivary glands to test for mid-gut infection, disseminated infection and transmissible infection, respectively, by cell culture. Of the 205 NPV-exposed sandflies, 40 (19.51%) developed infections which were all limited to the mid gut and that did not disseminate to the legs nor the salivary glands. Mid gut infection rates decreased with increasing extrinsic incubation period (Spearman's correlation, $\rho = -0.7145$). These findings signify that *P. duboscqi* is an incompetent laboratory vector of NPV from ingestion of a viremic blood meal since the mid gut infections did not disseminate to the salivary glands to be transmitted by bites.

DEDICATION

I dedicate this work to my late father Ezekiel Okoth Yuko.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Study Background

Vector-borne diseases pose a significant burden to human health globally (Peters 2014). Serious arthropod-vector borne infections such as Rift Valley fever virus (RVFV), malaria, dengue and chikungunya are prevalent in the tropics while other new arthropod-vector borne pathogens continue to emerge (Takken and Koenraadt, 2013; Hill et al., 2005). Cases of vector borne viral diseases such as RVFV, chikungunya, and dengue fever continue to be reported in Kenya (Konongoi et al. 2018; Lutomiah et al. 2016; Sang et al. 2010). Of these vector borne diseases, the viruses transmitted by sandflies are inadequately researched on in the Sub-Saharan Africa (Alkan et al. 2013).

Sandfly fever is a group of diseases caused by sandfly borne phleboviruses which manifest as self-limiting febrile illnesses (Burrell, Howard, and Murphy 2017). Sandfly borne phleboviruses also cause severe and sometimes fatal encephalitis and meningitis (Sang and Dunster 2001; Killick-Kendrick 1999). Sandfly fevers often manifest as a "three-day fever" with influenza-like symptoms including fever, myalgia, retro-orbital pain and malaise with patients usually recovering fully within one week (Papa et al. 2015). Toscana virus; one of the sand fly borne phleboviruses, has however been shown to have a strong neurotropism and to sometimes cause meningo-encephalitis (Guler et al. 2012; Alkan et al. 2013).

Sandfly fever viruses occur in the Old World including Mediterranean basin, South and Central Asia, North Africa and middles East where they are transmitted by the genera *Phlebotomus* and *Sergentomyia* (Alkan et al. 2013). In the New World including South, North and Central America, they are tranmistted by the genus *Lutzomyia* (Killick-Kendrick 1999; Alkan et al. 2013). They have been isolated in both vertebrates and sandflies in Europe, Central Asia, America ,North and Central Africa (Guler et al. 2012). Their spread, locally and globally, is conditioned by different factors involving both the virus and the vectors. These factors include vector biology, the geographical distribution of both the virus and the vector as well as climate change. The spread of sandfly-borne viral diseases is restricted to the distribution of their potential vectors and their circulation can spread to a non-endemic areas with competent vectors if a viremic host is introduced (Depaquit et al. 2010).

Phlebotomine sandflies are distributed throughout the tropics and the subtropics including the Sub-Saharan Africa, Mediterranean regions of Europe and the Indian subcontinent of Asia (Alkan et al. 2013). Their distribution is limited to areas that experience temperatures of 15.6^0C for not less than three months in a year (Lawyer et al. 2017; European Centre for Disease Prevention and Control 2019).

Vaccines and therapeutic drugs have been developed for most of vector borne diseases in attempts to prevent and treat them, however, vector control as an intervention is lagging behind (Takken and Koenraadt 2013). This is despite the fact that vector control has been described as the most effective approach to interrupt disease transmission and can even lead to disease eradication (Takken and Koenraadt, 2013). Understanding the transmission ecology of a vector borne pathogen through assessments of vector competence of among endemic vector species is prerequisite in disease risk assessment and control (Hardy et al. 1983; Agha et al. 2017).

Tchouassi et al., (2019) described a novel *Phlebovirus* isolated from a pool of sandflies collected from Marigat, Baringo County, Kenya. The virus was isolated in an exploratory study and named *Ntepes Virus* (NPV) following the name of the village from where it was isolated. Cell lines of selected vertebrates including non-human primates, bats and rodents, livestock and humans, in in-vitro tropism studies, all showed susceptibility to the virus (Tchouassi et al. 2019). Swine and rodents are thought to be the amplificatory hosts of the virus since their cell lines produced higher copy numbers of the virus from the in vitro growth analyses.

Seroprevalence studies by Tchouassi *et al.*, (2019) on human serum samples showed evidence of infections to humans by NPV. Neutralizing antibodies specific to NPV were detected in 13.9% of the tested human serum samples collected from the area where NPV was isolated. Following the *CO1* gene sequence analysis of the pool of isolation, NPV is suggested to have been isolated from sandflies from the genus *Sergentomyia*. The isolation does not, however, guarantee vector status to *Sergentomyia* species.

Phlebotomus duboscqi (Order Diptera, Family Psychodidae) is one of the *Phlebotomus* species of sandfly in the same ecology from where NPV was isolated (Anjili et al. 2011) . It is also the principal vector of *Leishmania major*, the causative agent of Zoonotic cutaneous leishmaniasis (ZCL) (Muigai et al. 1987; Beach et al. 1984; Killick-Kendrick 1990) in the same ecology. Involvement of *P. duboscqi* in the transmission of NPV would be of an epidemiological significance owing to the fact that it has been shown to transmit phleboviruses under laboratory conditions (Hoch, Turell, and Bailey 1984; Turell and Perkins 1990; David et al. 2000). Could *P. duboscqi,* be responsible for this active circulation of NPV?

There is already evidence of a wider geographical spread of NPV in Kenya (Tchouassi et al. 2019; Marklewitz et al. 2020) but distribution can further be extended to non- endemic areas

with its potential competent vectors. According to Depaquit *et al.*, (2010), the distribution of sandfly-borne *Phlebovirus* diseases may not be confined to just the areas where the viruses have been recorded or isolated but as wide as the non-endemic areas where their potential vectors inhabit. Depaquit et al., (2010), therefore, emphasizes on a need for field work in terms of isolation of the viruses from sandflies as well as their possible vertebrate reservoirs, and lab work to establish the vector competence of colonized sandflies.

1.2 Statement of the Problem

The main promoter of the spread of a sandfly borne virus is the presence of a competent vector of that particular virus (Brett-Major and Claborn 2009). Despite being shown to be an efficient vector of several pathogens of human health importance, phlebotomine sandflies have been significantly neglected in studies to describe their role in the transmission of arboviruses. Most of the studies done on entomological risk assessment of viruses focus on mosquitoes and studies on sandfly as vectors are concentrated on leishmaniasis. This has limited the availability of knowledge necessary in understanding epidemiology and disease dynamics of sandfly-borne viruses.

Vector competence determination is always prerequisite in understanding the disease transmission ecology, epidemic potential and vector control (Hardy et al. 1983). The pioneer study by Tchouassi *et al.*, (2019) highlighted the circulation of a new *Phlebovirus* tentatively named as *Ntepes virus* (NPV) isolated from sandflies and with evidence of human infections widely distributed in Northeastern Kenya. The findings of that study suggest that sandflies may have been underestimated and neglected as potential vector for human pathogenic viruses in Kenya and East Africa. The transmission ecology of NPV, however, remains poorly understood.

There is therefore, a need to conduct vector competence investigations of suspected arthropod vectors in transmission of NPV.

Tesh, (1988) suggests an involvement of *Phlebotomus* species in *Phlebovirus* transmission in the Old World. *Phlebotomus duboscqi* is an efficient vector of leishmaniasis and is the incriminated vector of *Leishmania major* in Baringo, where *NPV* was isolated. Its implication in the transmission of NPV could have ramifications for possible epidemiological links between leishmaniasis and phleboviruses. This study purposes to determine the capacity of colonized *P. duboscqi* to transmit disseminated infection of NPV in the laboratory.

1.3 Justification

Out of the 10 ICTV recognized arthropod-borne *Phlebovirus* of human and animal health importance, seven are transmitted by sandflies (Tesh 1988). Like other arboviruses, the risk of spread of sandfly borne viruses are conditioned by the presence of a competent vector (Depaquit et al. 2010; Brett-Major and Claborn 2009). Although *Sergentomyia* are thought to be the genus from which NPV was isolated, this does not guarantee a vector status. Assessment of the vector status goes beyond merely the isolation of a pathogen from a field collected specimen (Azar and Weaver 2019).

The vector competence of a given arthropod to a particular disease agent is guided by an ecological relevance (Takken and Koenraadt, 2013; Young et al, 2013). According to Tesh (1988), each *Phlebovirus* tend to have a unique geographical distribution depending on the availability of its vector or the vertebrate host . *Phlebotomus duboscqi* co-occurs in the same ecology where NPV was isolated in Baringo County. This study focused on determining the vector competence of *P. duboscqi* to a newly identified *Phlebovirus;* NPV.

Understanding the vector competence of *P. duboscqi* to NPV will help understand the transmission ecology of the virus and this knowledge will be useful in managements of epidemics of the virus in terms of vector control.

1.4 Research Hypothesis

H1 *Phlebotomus duboscqi* is not susceptible to oral infection with NPV.

H2 *Phlebotomus duboscqi* does not efficiently transmit NPV after oral exposure.

1.5Objectives

1.5.1 Main Objective

To determine the vector competence of *P. duboscqi* to *Ntepes virus (*NPV*).*

1.5.2 Specific Objectives

i. To assess the oral susceptibility of *P. duboscqi* to NPV.

ii. To assess the transmission efficiency of NPV by *P. duboscqi.*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Sandfly borne Phleboviruses Classification

Phlebovirus (Order: Bunyavirales, Family: Phenuiviridae) is a genus characterized by its tri-segmented negative sense, single-stranded RNA genome (Liu et al. 2003). The three segments include the L, M and the S segments. The L (Large) segment codes for the RNA polymerase whereas the M (medium) segment codes for the envelope glycoproteins (Gn and Gc) for the virus. The S (small) segment usually codes for the nucleocapsid protein (N) and a non- structural protein (NSs) in an ambisense alignment (Alkan et al. 2015; Liu et al. 2003).

The International Committee for the Taxonomy of Viruses (ICTV) currently recognizes 10 arthropod borne *Phlebovirus* species (Adams et al. 2017; Maes et al. 2019) and several other described tentative species awaiting ICTV's classification. Phlebotomine sandflies transmit 7 out of the 10 classified phleboviruses including *Naples phlebovirus, Bujaru phlebovirus, Chilibre phlebovirus, Punta Toro phlebovirus, Salehabad phlebovirus, Candiru phlebovirus, and Frijoles phlebovirus*. *Rift Valley fever virus*, transmitted by different species of mosquitoes, is the best-known arthropod-borne *Phlebovirus* (Sang et al. 2010). *Severe fever with thrombocytopenia syndrome phlebovirus* (*SFTS virus*) and *Uukuniemi phlebovirus* are transmitted by ticks (Alkan et al. 2015; Marklewitz et al. 2019; Elliott and Brennan 2014; Jancarova et al. 2019; Adams et al. 2017).

2.2 Clinical Presentation of Sandfly Borne Phleboviruses Infections

Infections with sandfly borne phleboviruses usually present as acute febrile illness (AFI) that lasts about three days hence the name three-day fever (Woyessa et al. 2014; Marklewitz et al. 2019). Due to the unavailability of affordable clinical examination and diagnostic tests to determine the specific etiologies of AFI in developing countries, sandfly fevers have always been misdiagnosed as other infections that also present with AFI such as dengue, malaria, typhoid or influenza (Woyessa et al. 2014; Marklewitz et al. 2019). In addition, they also present with a wide range of unspecific symptoms including headache, photophobia, retro-orbital pain, anorexia, myalgia and low back pains (Brett-Major and Claborn 2009). Other cases, as in the case of toscana virus, however, present with mild to severe encephalitis and aseptic meningitis alongside the influenza-like symptoms (Burrell, Howard, and Murphy 2017; Papa et al. 2015; Marklewitz et al. 2019).

The intrinsic incubation period of sandfly fevers is 3-8 days (Brett-Major and Claborn 2009; Maroli et al. 2013) after which the patients recover in less than 7 days without recorded mortalities (Burrell, Howard, and Murphy 2017). The patients also develop an immunity from further attacks after the initial infection, an immunity specific to that particular strain of the virus (Young et al. 2013; Maroli et al. 2013b). Indigenous people in endemic areas benefit from this immunity following childhood exposures to the infections but travelers who are naïve to the infections are at risk (Burrell, Howard, and Murphy 2017).

Patients infected with phleboviruses are infectious to the arthropod vectors for about two days (Brett-Major and Claborn 2009). Due to the vector biology of phlebotomine sandflies, the infection cases of sandfly fevers are usually geographically restricted to near the typical vector habitats.

2.3 Maintenance Cycles of Sandfly Borne Phleboviruses

The maintenance of arboviruses in an ecology and vector competence are influenced by the intricate interactions among the pathogen, the arthropod vector, the vertebrate host and several external factors (Kramer and Ciota 2017; Tabachnick 1994). These include the intrinsic factors contributing to the immunity of both the vector and the vertebrate host and extrinsic factors that are usually environmental Elliott 2009).

Many phleboviruses are transmitted vertically by transovarial and venereal transmission among their insect vectors in nature (Horne and Vanlandingham 2014; Burrell, Howard, and Murphy 2017; Cusi, Gianni, and Giacomo 2010; Depaquit et al. 2010). This is very important for their inter-seasonal maintenance in the ecology during the dry seasons and/or during winters when the adult sandfly activity is low or when the vertebrate host is not available (Charrel 2014). Different laboratory studies have, however, shown that transovarial transmission cannot, solely, maintain the viruses in the ecosystem (Tesh 1988) since an absolutely perfect filial transmission cannot be achieved. It is therefore believed that vertebrates must be playing a role in the inter-seasonal and prolonged maintenance of the virus in the ecology (Tesh 1988; Endris, Tesh, Young 1983).

The availability of a susceptible vertebrate host allows for amplification of the virus within the vertebrate and subsequent infection of many sandflies (Tesh, 1988). These alternative cycles ensure the inter-seasonal survival of the viruses (Tesh, 1988). The short viremia of sandfly fever viruses in humans and lack of persistent infections in humans compromise the participation of humans in their maintenance in the ecology (Depaquit et al. 2010).

2.4 Vectorial Capacity

Vectorial capacity is comprised of different parameters including a set of intrinsic factors in both the vector and the host as well as the external factors, which are usually environmental. The host factors that affect vectorial capacity include the host susceptibility to the virus and its immunity (Ribeiro and Valenzuela 2011). Population density of the host also affects the frequency of the vector-host interaction which increases the probability of exposure of the host to the virus (Kramer and Ciota 2017). The factors that affect vectorial capacity in the vector include host preference, biting rates, biting habits, survival rates, population densities, vector longevity, the extrinsic incubation period (EIP) of the virus in the vector and vector competence (Brady et al. 2016; Catano-Lopez et al. 2019; Lefèvre et al. 2013).

Another important factor affecting the vectorial capacity is gonotrophy. Some sandfly species are gonotrophically discordant and therefore require more than one blood meal per gonotrophic cycle. This kind of gonotrophy increases the interaction between the arthropod vector and the vertebrate host contributing to the general vectorial capacity of the arthropod. A gonotrophically discordant vector is able to transmit to the next vertebrate host, a pathogen it acquired from a previous blood meal, during their subsequent blood meal(s) (Lewis 1971).

Vectorial capacity can be expressed by the equation below as described by Brady *et al.,* (2016)

$$Vectorial\ Capacity = \frac{ma^2p^Nb}{-\ln(p)}$$

Where, a = the biting rate,

m = the number of vectors available per host,

p = probability of the vector surviving 1 day,

b = vector competence and

N= extrinsic incubation period.

With this illustration, vector competence of an arthropod to a virus and the EIP of the virus in the arthropod are important variables contributing to the vectorial capacity of the arthropod.

2.5 Vector Competence

Vector competence, in the context of arboviruses, is the biological ability of an arthropod to serve in the life cycle of a pathogen to its infective stage (Ribeiro and Valenzuela 2011). It is an integral component of the general vectorial capacity which is described as the broad measure of the efficiency of the vector borne pathogen to be transmitted naturally by the vector (Brady et al. 2016). Vector competence is expressed as the proportion of the vector's population that can transmit the pathogen after an infectious blood-meal (Ribeiro and Valenzuela 2011). For an arthropod to transmit an arbovirus, a series of events must take place within the vector. Upon taking a viremic blood meal, the posterior mid gut epithelial cells must be infected with the virus. The virus then has to overcome the midgut infection barrier (MIB) composed of the action of the proteolytic enzymes, effects of the internal microbiota, RNA interferences, and the physical barriers presented by the mid gut epithelium itself (Azar and Weaver 2019).

Upon overcoming these factors, the virus initiates an infection in the mid gut, multiply actively on mid gut epithelial cells and escape the mid gut through the surrounding basal lamina into the hemocoel where the hemolymph transports it to secondary tissues including the fat body, muscles and nerves (Turell and Perkins 1990; Azar and Weaver 2019). As the virus dissemination progresses in the body of some vectors, another barrier, the salivary gland infection barrier (SGIB) must be overcome so that the virus may traverse the basal lamina surrounding the salivary glands into the salivary gland. The virus then infects the salivary gland acinar cells where it is shed to the apical cavity to be inoculated into the saliva for transmission during subsequent blood meals (Azar and Weaver 2019).

Some vectors may have a strong mid gut escape barrier which massively affect the particular vector's competence to a virus. In experimental infection of *P. duboscqi* with RVFV by Turell and Perkins, (1990), there were very low mid gut escapes by the virus but nearly all disseminated infections were transmissible. This insinuates that the escape of the virus from the mid gut into the hemocoel is the main determinant of RVFV transmission by *P. duboscqi* and predicts that the arthropod has a permissible salivary gland infection barrier. Different experimental infections of both mosquitoes and sandflies with viruses in the laboratory show higher transmission rates after intrathoracic inoculations than oral inoculations (David et al. 2000; Turell and Perkins 1990; Hoch, Turell, and Bailey 1984; Vogels et al. 2017). The findings of those indicate that sandflies tend to have a strong mid gut escape barrier (MEB) and that overcoming the MEB almost guarantees competence.

The dose ingested by vector may also influence the infection success (Chamberlain and Sudia, 1961). Studies by Turell and Perkins, (1990) and Dohm *et al.*, (2000) show the infection rates of RVFV, in *P. duboscqi* and other sandfly species increase as the infection dose of the virus was increased.

2.6 The Extrinsic Incubation Period (EIP) of Sandfly Fever Viruses

Most arboviruses develop in multiple segments of the arthropod's body following a viremic blood meal. This precedes multiplication of the virus in the mid-gut and a progressive dissemination through the gut into the hemolymph and eventually to the salivary glands (Titus and Ribeiro 1990). This period it takes the virus from the time the vector is exposed to the viremic blood-meal to when it becomes infectious is referred to as the extrinsic incubation period (EIP) (Chan and Johansson 2012; Hardy et al. 1983).

The EIP of arboviruses is affected directly by temperatures (Hardy et al. 1983) and other genetic factors within the vector (Ohm et al. 2018). For sandfly fever viruses, the EIP averages between 4-8 (Brett-Major and Claborn 2009) days after which the vector remains infectious for its entire life (Young et al. 2013). They are transmitted efficiently when the temperatures are above 25^0C which makes them common in the tropics and during the summers in The New World and the Mediterranean (Papa, Velo, and Bino 2011).

Extrinsic incubation period of an arbovirus and the lifespans of the corresponding arthropod vector are important for understanding the dynamics of the arboviral disease transmission and can be important for outbreak investigations and implementations of prevention and control programs (Chan and Johansson 2012). In this respect, if the EIP of the arbovirus and the longevity information of the vector are known, these variables can be used to determine the number of secondary cases that a single case of an arboviral disease can produce given the number of that particular vector available per vertebrate host (Ribeiro and Valenzuela 2011). Thus, EIP and vector competence become very important components of vectorial capacity.

2.7 Vector Profiles of Phleboviruses

At least 9 different species of sandflies can transmit phleboviruses across the world (Ayhan and Charrel 2017). The genus *Lutzomyia* transmits phleboviruses in America and the genera *Sergentomyia* and *Phlebotomus* transmit them in the Old World (Depaquit et al. 2010; Alkan et al. 2013; Ayhan and Charrel 2017). The sandfly-*Phlebovirus* association is highly influenced by the virus host range, the feeding preference of the sandflies and the geographical distribution of the virus, sandfly and the vertebrate hosts (Tesh 1988) .

Interestingly, the phleboviruses causing human infections are associated with the highly anthropophilic sandfly species such as *P. papatasi, P. perfiliwei, P. pernicious, Lutzomyia*

trapidoi, L. ylephiletor (Tesh 1988). Sandfly Fever Naples Virus has been isolated from *Phlebotomus papatasi, P. pernicious, P. perfiliwei* in the Mediterranean (Tesh 1988; Depaquit et al. 2010). Toscana virus has also been isolated from both female and male *Phlebotomus* species, confirming transovarial transmission of the virus in sandflies (Depaquit et al. 2010). *Punta toro phlebovirus*, endemic in Central America is transmitted by *L. trapidoi* and *L. ylephiletor* (Mellor 2000; Tesh 1988). *P. papatasi, P. duboscqi, P. sergenti, Sergentomyia schweitsi, L. longipalpis* have all been infected with RVFV in the laboratory although RVFV has not been isolated from any sandfly species in nature (Horne and Vanlandingham 2014).

Phlebotomus duboscqi is the incriminated vector of *Leishmania major*, the disease agent of Zoonotic cutaneous leishmaniasis (ZCL) in Baringo (Githure 1989). It has also been shown to be laboratory competent vector of RVFV (David et al. 2000). *Phlebotomus duboscqi* has been described to be gonotrophically discordant and are able to take up to three blood meals per gonotrophic cycle (Mukhopadhyay and Ghosh 1999). This trait gives *P. duboscqi* a high potential in epidemiology of diseases that it is competent in transmitting their pathogens.

Recent studies suggest that sandflies are not as specific to the viruses they transmit as thought earlier and a single species can possibly transmit more than one *Phlebovirus* species (Ayhan and Charrel 2017). Data presented from recent different seroprevalence studies on humans and animals also allude to a very active circulation of phleboviruses globally (Depaquit et al. 2010). Circulation of sandfly associated phleboviruses has also been recorded in East African countries (Woyessa et al. 2014; Tesh et al. 1976; Alwassouf et al. 2016) including Kenya (Tchouassi et al. 2019; Marklewitz et al. 2020). The studies also suggest that there may be a widespread unnoticed exposure of animals and humans to phleboviruses.

2.8 *Ntepes virus* and its Transmission Ecology

Ntepes virus (NPV) is a novel *Phlebovirus* that was initially isolated from a pool of sandflies collected from Marigat, Baringo County, Kenya (Tchouassi et al. 2019). The virus has had its entire genome sequenced displaying a characteristic tri-segmented genome architecture of a *Phlebovirus.* The virus possesses the conserved genome termini sequence of 5'-ACACAAAG and CUUUGUGU-3' typical to the genus phleboviruses (Liu et al. 2003). *Ntepes virus* also exhibit a close but distinct relationship in genomic composition with the *Gabek Forest virus* (GFV) with a 79% pairwise identity with GFV in the L-segment encoding the RNA dependent RNA polymerase (Tchouassi et al. 2019).

Ntepes virus is suggested to have been isolated from sandflies in the *Sergentomyia* genus following the *CO1* gene sequence analysis of the pool from which the virus was isolated (Tchouassi et al. 2019; Kuhn et al. 2020). Tropism tests in different animal cell lines predicted a wide vertebrate host range with cell lines from humans, bats, non-human primates, rodents, chicken, cattle, swine and goats all showing permissiveness to NPV, swine and rodents' cell lines showing the highest tropism. NPV, just like its closest relatives in the *Phlebovirus* genus including *Sandfly fever Naples virus, Sicilian virus,* and GFV that were isolated from sandflies, showed tropism only in sandflies cell lines but not mosquito and other arthropods' cell lines predicting a vector specificity to sandflies. *Ntepes virus* caused a rapid fatal illness in newborn mice in in vivo vertebrate pathogenicity tests. These tropism tests and the in vivo pathogenicity tests on vertebrates predict that rodents and sandflies may be the animals mainly involved in the maintenance of NPV (Tchouassi et al. 2019) in the environment.

Seroprevalence of humans with NPV infection of ~14% of the tested serums samples was observed in Marigat area, Baringo County following plaque reduction neutralization test (PRNT)

assays (Tchouassi et al.,2019). Even much higher exposure levels in humans was recorded in Northeastern Kenya, ~600km away from Ntepes, where the virus was first isolated, suggesting a wide distribution of NPV in Kenya. Since NPV was isolated in an exploratory study, its isolation suggest an active and unnoticed circulation of NPV and possibly, other sandfly associated viruses in the population. This has been supported by the isolation of four other novel phleboviruses (Marklewitz et al. 2020)

Although NPV has been characterized and its full genome sequenced, the knowledge on its transmission ecology remains poor including information regarding potential vector range and its transmission efficiency.

2.9 Distribution and Vector Biology of Phlebotomine Sandflies

Phlebotomine sandflies are the principal vectors of *Leishmania* species, the parasite which cause leishmaniasis in over 80 countries across the world (Boelaert and Sundar 2014). They also vector *Bartonella beciliformis* (Angelakis and Raoul 2013) which causes Corrions's disease, and viruses from the genera *Phlebovirus*, *Orbivirus* and *Vesiculovirus* (Depaquit et al. 2010). The distribution of phlebotomine sandflies is confined to regions experiencing temperatures of 15.6^0C for not less than 3 months in a year. They are found in both the New world and the Old world including Mediterranean Europe, Sub Saharan Africa, the Middle East and the India Subcontinent (Asimeng 1985).

Phlebotomine sandflies are strictly terrestrial and poor fliers, usually assumed not to disperse more than a kilometer away from their breeding sites and with an estimated flight speed of 1m/s (Killick-Kendrick 1999). This restricts their biting activities local to hatching points. They require warm and humid environments to thrive with just a few exceptions such as *Lutzomyia verucarum* which thrive in cooler and drier conditions (Lawyer et al. 2017). Adult sandflies rest

in cool humid places such as houses, cellars, caves, rodents' burrows, cracks on walls, tree holes and dense vegetation (Killick-Kendrick 1999).

Female and male sandflies alike, feed on plant sources of carbohydrates including floral nectars, plant saps and honeydew of aphids but only females feed on blood which they need for oogenesis (Schlein and Muller 1995). Female sandflies bite different vertebrates including mammals, reptiles, and birds for blood (Petriceva 1971) and feed in pools (telmophagous) (Berenger and Parola 2017).

Phlebotomine sandflies can only bite through uncovered skin due to the nature of their short mouthparts (Munstermann 2019). Their biting activity is usually nocturnal or crepuscular with few exceptional species which may bite in daylight (Berenger and Parola 2017). Females of many species bite outdoors (exophagic) or rest outdoors as their eggs mature (exophilic) making controlling them with house spraying unsuccessful (Killick-Kendrick 1999). Because they do not fly long distances, most of the diseases they transmit are localized in distribution (Urgunay 2014).

Depending on species, sandflies may require just one blood meal per gonotrophic cycle (gonotrophic concordance) or require multiple blood meals per gonotrophic cycle (gonotrophic discordance) (Mukhopadhyay and Ghosh 1999). Some species may produce the first batch off eggs autogenously but usually require blood meals for the subsequent gonotrophic cycles (Lawyer et al. 2017). Sandflies exhibit complete metamorphosis from eggs, larva with four instars, pupa to adults (Maroli et al. 2013b)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Samples

The stock *Ntepes virus* used in this study was isolated from a pool of sandflies sampled from Marigat, Baringo County, Kenya in 2014 (Tchouassi et al. 2019) and passaged in Vero cells. The sandflies used in the study were reared at the Kenya Medical Research Institute's (KEMRI) Center for Biotechnology Research and Development (CBRD) Vector Biology insectary from a pure pre-established colony of *P. duboscqi* that were collected from Baringo County, Kenya in 1984.

3.2 Sandfly rearing

The sandflies were reared as described previously (Lawyer et al. 2015; Lawyer et al. 2017) in 30×30×30cm polycarbonate cages at 26^0C and 80% relative humidity and maintained on fresh apple slices as energy source with a photoperiod of 12h:12h [L:D]. Adult females were blood-fed on adult mice anesthetized with pentobarbital (sagatal) and the fully engorged females were then aspirated into 500cm^3 oviposition pots for egg laying. They were maintained on 30% glucose-saturated cotton balls as energy source. The adult flies were removed from the pots 10 days post blood meal using vacuum aspirators, dead or alive to prevent excessive growth of mold, minimize mite infestation and deter the larvae from feeding on the adult carcasses, predisposing them to gregarines (Lawyer et al. 2015). The eggs were washed with 1% sodium hypochlorite, rinsed with clean water to remove the gregarine cysts that may have been adsorbed on the surface of eggs before returning them to the oviposition pots and checked daily for hatching. Once the eggs hatched into larvae, they were fed on freshly prepared larvae food prepared as previously described (Lawyer et al. 2017).

As the adults emerged, they were released into the polycarbonate holding cages where they were maintained on apple slices and eventually fed on mice blood for production of eggs. The cycle was repeated until the colony was populous enough for the study.

3.3 Virus Amplification

The stock NPV was passaged in Vero cells monolayer in T-25 cell culture flask to improve the titer and the virus volume. The cells were grown in (MEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) 2% L-glutamate, 2% antibiotic/antimycotic solution composed of 1000 parts of penicillin,25 micrograms of amphotericin B, and 10 mg streptomycin per ml and incubated at 37^0C in 5% CO_2 for 24 hours for the cells to attain 80%-90% confluence.

Two hundred microliters (200µL) of the stock NPV were inoculated onto the confluent monolayer of Vero cells then incubated for 1 hour with periodic rocking for the virus to adsorb onto the cells. The infected Vero cells was then maintained in MEM augmented with 2% heat-inactivated FBS, 2% antibiotic/antimycotic solution, and 2% L-glutamate and observed for cytopathic effects (CPE) daily. Once, 80% of the monolayer appeared to show CPE, the flask was frozen overnight at -80^{o}C, thawed in wet ice and centrifuged for clarification at 3000 rpm for 10 minutes. The resulting supernatant was harvested and aliquoted in 1.5ml cryovials and stored at -80^0C.

3.4 Virus quantification

The amplified virus was quantified by plaque assays by inoculating 100µl of serial 10-fold dilutions of the harvested virus onto each well of the monolayer of Vero cells in the 12-well culture plate and incubated for 1 hour with periodic agitation for virus adsorption. The Vero cells in the 12-well plate were seeded 24 hours prior to inoculation of virus that had been grown on

MEM supplemented with 10%heat inactivated fetal bovine serum (FBS), 2% L-glutamate and 2% antibiotic/antimycotic solution and incubated at 37^0C in 5% CO_2 overnight.

The infected cells were maintained on 2% methylcellulose overlay mixed with 2× MEM (GIBCO® Invitrogen corporation, Carlsbad, California) and incubated at 37°C with 5% CO_2 for 10 days then fixed for 1 hour with 10% formalin, stained for 2 hours with 0.5% crystal violet and the plaques counted and calculated to quantify the virus using the formula (Brady et al. 2016);

$$\text{PFU} = \frac{Number\ of\ plaques}{Dilution\ factor\ x\ Volume\ of\ diluted\ virus}$$

3.5 Vector Competence Assessment

3.5.1 Sandflies infection with NPV

For the first replicate, 213 Five-day old laboratory colonized female *P. duboscqi* were starved of the carbohydrate sources for 24 hours and allowed to feed on prepared viremic blood meal. The viremic blood meals were prepared by mixing 2ml of the harvested NPV stock with 2ml defibrinated sheep blood and then covered with freshly prepared mouse skin as the membrane feeder and maintained on Hemotek system (Discovery Workshops, Accrington, United Kingdom) at 35^0C as described by Denlinger et al., (2016) and the flies allowed to feed for one hour in darkness.

After 24 hours, all blood fed females were selected, aspirated into incubation cages and maintained on fresh apple slices for 15 days at 26^0C and 80% RH and photoperiod of 12h:12h [L:D].

The experiment was replicated twice with 200 sandflies exposed in the second replicate and 115 for the third replicate.

3.5.2 Infection and Dissemination Assays

After 6, 10 and 15 days of incubation, about 30% of the live virus-exposed sandflies were randomly selected and dissected in a cold chain to separate the legs and wings and the salivary glands from the body containing the mid-gut. Individual dissected body parts were placed separately in 1.5 ml microcentrifuge tubes containing 500microliters of homogenizing media (HM), consisting of MEM, supplemented with 15% FBS, 2% L-Glutamine, and 2% antibiotic/ antimycotic solution. Legs and wings of individual sandflies were put together in separate 1.5ml microcentrifuge tubes containing 500microliters of HM. Individual salivary glands were also placed in 1.5ml microcentrifuge tubes containing 200microliters of the homogenizing media. All the dissected samples were stored at -80^{o}C until they were.

3.5.2.1 Infection Success Assays

The individual bodies were homogenized using Minibeadbeater (BioSpec Products Inc, Bartlesville, OK 74005 USA) with an aid of plastic beads for 5 minutes and clarified by centrifugation at 12,000rpm for 10 minutes at 4^{o}C. The infection success assays were performed by inoculating 100µl of the supernatant of the clarified body samples onto 12 well plates of confluent Vero cells. The infected cells were incubated at 35^{o}C for 1 hour with frequent agitation for virus adsorption and maintained on 2% methylcellulose overlay mixed with 2× MEM (GIBCO® Invitrogen Corporation, Carlsbad, California) for 10 days at 35^{o}C and 5% $CO_{2.}$ On the 10th day, the plates were fixed with 10%formalyn for 2 hours and stained with 0.5% crystal violet solution for two hours and the plates checked for plaques.

3.5.2.2 Dissemination and Transmission Success Assays

The legs samples of the corresponding positive body samples were homogenized and clarified as described for the body samples and screened for virus recovery to show dissemination success by cell culture as described for body samples.

3.6 Data Analysis

Data analysis was performed using R statistical software *v* 4.1.0.

The infection rates were described by the test of proportions for mid gut infections, disseminated infections and transmissible infections with 95% confidence limits. The infection rates (IR) were expressed as the number of positive mid-gut infections over the total number of sandflies infected in the study. The dissemination rates were expressed as the proportion of the exposed sandflies that had the virus recovered from the legs. The transmission efficiency was described as the proportion of the exposed sandflies that tested positive for the virus in the salivary glands.

The data was tested for the significance of the extrinsic incubation periods to the infection success rates using Kruskal-Wallis Test with 95% confidence limits. Test of correlations between the infection rates and the extrinsic incubation periods was performed by Spearman's correlation analysis and linear regression analysis at 95% confidence limits.

CHAPTER FOUR

4.0 RESULTS

4.1 Virus Amplification and Quantification

The initial stock virus (passage zero) passaged twice in T25 culture flasks to the third passage showed cytopathic effects (CPE) on vero cells from day 5 post inoculation and by day 10, 80% of the monolayer of the vero cells were infected. The amplified NPV used to prepare the blood meals for infection experiments had a titer of 10^8 pfu/ml.

4.2 Sandflies Infection with NPV

The feeding success rates were moderate with just 48.3% (255 out of 528) exposed sandflies feeding on the viremic blood meal (Table 1). The titer of the virus in the blood meals reduced insignificantly within the 1 hour of blood feeding as shown in (Table 2).

Table 1. Feeding success rates of *P. duboscqi* on viremic blood meal by membrane feeding on hemotek system during the infection with NPV.

Replicate	Number Exposed	Number blood fed	Feeding success rate	Mortalities	Number dissected
1	213	97	45.54%	7	90
2	200	87	43.50%	23	64
3	115	71	61.74%	20	51
Total	528	255	48.3%	50	205

Table 2. Titers of NPV in the pre-feeding and post-feeding blood meals during the infections of *P. duboscqi* in Log10 pfu/ml.

Replicate	Pre-feeding (log10 pfu/ml)	Post-feeding (log10 pfu/ml)
1	5.72	5.15
2	5.60	5.30
3	6.60	6.30

Of the 255 exposed sandflies, 205 live sandflies were dissected (Table 3) following a 22.22% mortality rate (50/255) (Appendix 1). Significant mortalities were observed from day 5 of the EIP. The mortalities were also observed to be higher when the midgut infection rates were high following Spearman's correlation analysis of the data (ρ=0.609) (Figure 3). The mid-gut infection rates were 19.51% (40/205). None of the mid gut infections disseminated into the legs nor the salivary glands (Table 3).

Spearman's correlation analysis of the percentage mid gut infections against the extrinsic incubation period (EIP) shows a strong decline in the mid gut infection rates with increasing EIP (ρ=-0.71). The infection rates across the EIP are shown in a box and whiskers plot (Figure 1). This decline was, however, not statistically significant (Kruskal-Wallis Test, $p>0.05$).

Table 3. Infection, dissemination and transmission success of NPV in *P. duboscqi* across the extrinsic incubation period after oral feeding of the viremic blood meal.

	6DPI	10DPI	15DPI	TOTAL
No. dissected	90	71	44	205
Mid gut infections	22	11	7	40
Mid gut infection rates	10.73%	5.37%	3.41%	19.51%
Positive legs	0	0	0	0
Positive salivary glands	0	0	0	0

*DPI- Days Post Infection.

*Mid gut infections indicate oral susceptibility of P. duboscqi to NPV.

*Positive legs indicate dissemination success of NPV in P. duboscqi.

*Positive salivary glands indicate transmission success of NPV by P. duboscqi.

A linear regression analysis showing the association between the mid gut infection rates and the extrinsic incubation period (EIP) showed a strong negative association (y=15.7-0.845x) with the rates of infection shown to reduce with the increasing EIP (figure 2)

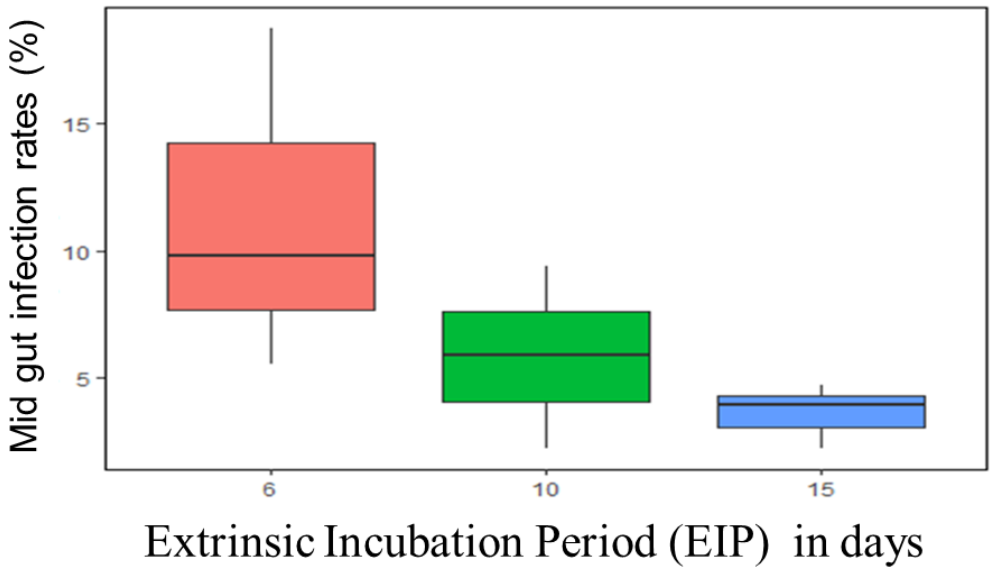

Figure 1 Box and whiskers plot showing percentage midgut infection rates of NPV in *Phlebotomus duboscqi* against the extrinsic incubation period in days.

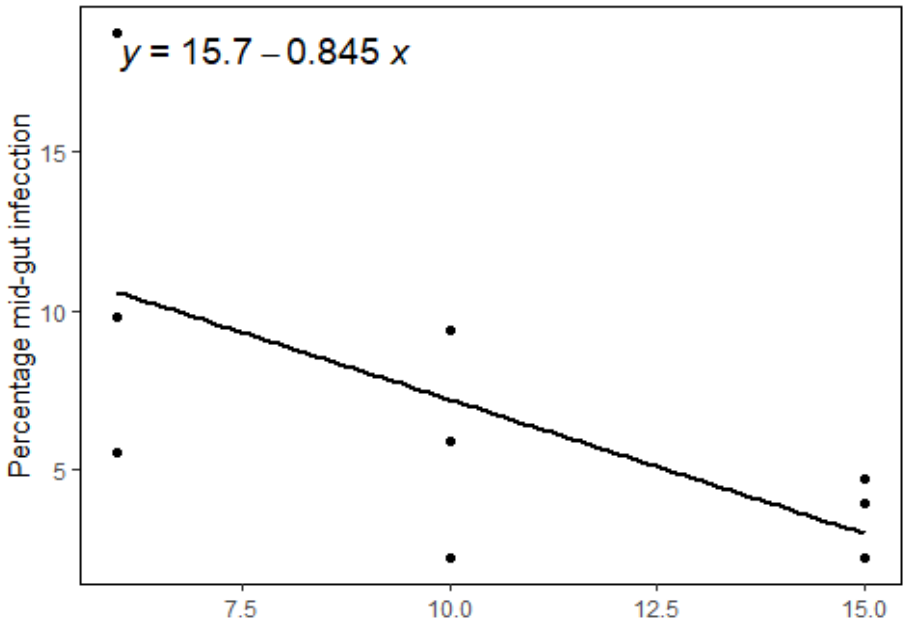

Figure 2. Regression line graph showing the correlation between midgut infection rates of NPV in *Phlebotomus duboscqi* against EIP in days.

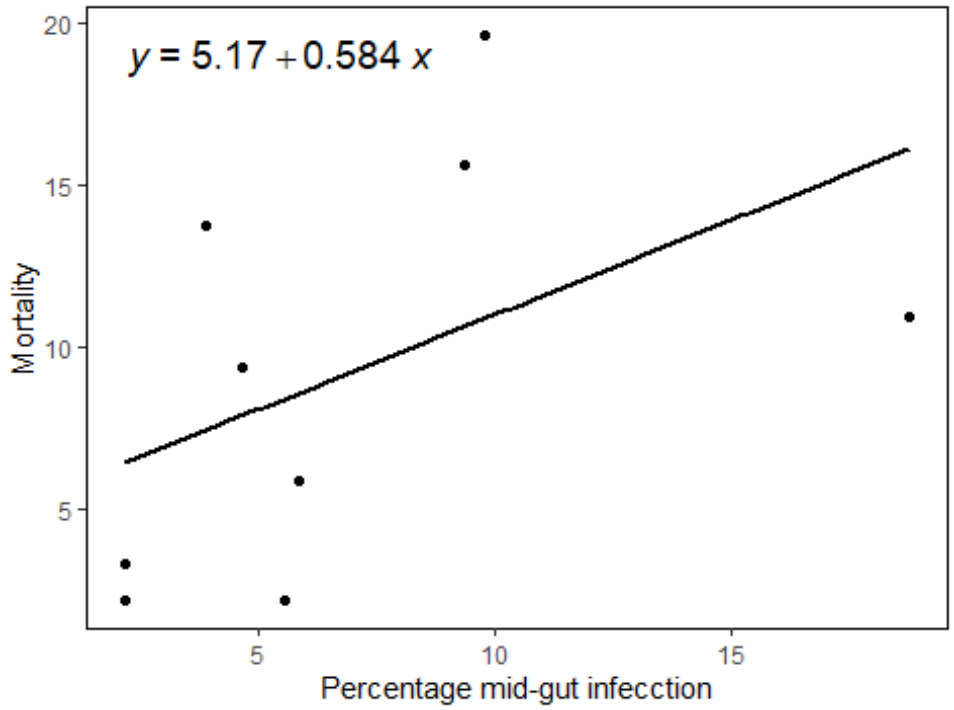

Figure 3. Regression line graph showing the correlation between mortalities of NPV-exposed sandflies against midgut infection.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Vector competence is one of the major factors that contribute to the vectorial capacity of arthropods to arboviruses (Brady et al. 2016).Vector competence assessment is therefore, fundamental for the entomological risk evaluation of a vector-borne infection (Hardy et al. 1983). Sandflies from the genus *Phlebotomus* have been linked to the spread and maintenance of different phleboviruses in the Old World (Depaquit *et al.*, 2010).

This study investigated the vector competence of *P. duboscqi* to NPV through artificial infection by membrane feeding system, to help gain insights into the possible involvement of *P. duboscqi* in the transmission of the virus. *Phlebotomus duboscqi* is abundant and is the principal vector of *Leishmania major* in the Baringo County, Kenya, where NPV actively circulates (Beach et al. 1984). Its involvement in the transmission of NPV could have ramifications for possible epidemiological relationship between NPV and leishmaniasis as has been reported for leishmaniasis and phleboviruses transmitted by common vectors (Thirion et al. 2011; Es-sette et al. 2014). Studies elsewhere also showed *P. duboscqi* to be laboratory competent of transmitting RVFV which is a *Phlebovirus* that has always just been associated with mosquitoes (David et al. 2000). *Phlebotomus duboscqi* being gonotrophically discordant and highly anthropophilic, could be of a significant epidemiological concern if it is shown to be a competent vector of NPV.

In this study, vector competence is estimated from the recovery of the virus from the salivary glands of dissected sandflies after an oral exposure to the virus. The observed blood feeding rates by membrane feeding in this study are extremely lower than those observed when they feed on live mice during rearing. This affects both the number of sandflies ingesting the virus as well as

the level of engorgement and therefore the amounts of virus ingested by the sandflies. This method is used due to the lack of a perfect animal model to test for the transmission success of most phleboviruses (Alkan et al. 2013; Tesh 1988).

This study is believed to be the first to investigate the vector competence of sandflies to a sandfly associated virus isolated in Kenya. The overall findings of this study demonstrate that *P. duboscqi* is not an efficient vector of NPV after oral infections with the virus in the laboratory. Only 19% (40 out of 205) of the orally exposed sandflies were infected with NPV and none of these infections disseminated for transmission. The low midgut infection rates suggest that *P. duboscqi* have a poorly permissible mid-gut infection barrier to NPV. The findings also predict that *P. duboscqi* has a very strong mid-gut escape barrier since none of these mid-gut infections were transmissible.

Non-transmissible virus infections of the mid-gut of sandflies after oral exposures have been reported in various studies. In a study by Hoch, Turell and Bailey, (1984) to demonstrate the replication of RVFV in *Lutzomyia longipalpis* , none of the sandflies that ingested RVFV transmitted the virus to hamsters and there were only transmissions after intrathoracic inoculations when the midgut escape barrier was surmounted. In that study, both the titers of the virus and the percentage of infected sandflies reduced with increasing EIP from the first day of the EIP. There were also very low transmission rates (6 out of 145) of RVFV by *P. duboscqi* in a study to establish the vector competence of *P. duboscqi* to RVFV by Dohm *et al.*, (2000). This was even after 35% of the orally exposed sandflies developed mid gut infections. Virus infections limited to the mid gut have also been observed in mosquitoes in *Culex pipiens* by Turell *et al*,.(1984). In the study, *Culex pipiens* did not transmit RVFV even though 75% of the orally exposed mosquitoes developed mid gut infections.

The findings of this study mirror those of the above-mentioned studies with the demonstration of non-disseminated infections of the mid gut of the sandflies. This could be due to low grade infections of the mid-gut that could not meet the threshold for or could be a result of residual virus from the initial ingested viremic blood meal as observed by a study by Hoch, Turell and Bailey, (1984). Low infection rates of sandflies by viruses from blood meals is a common observation in laboratory infection experiments (Brett-Major and Claborn 2009).

The EIP of sandfly borne phleboviruses in sandflies averages 7 days (Brett-Major and Claborn 2009). For a competent vector, the virus has to replicate to higher titers in mid gut and disseminate to the salivary glands of the vector within the EIP so that it may be transmitted to the vertebrate host through bites (Azar and Weaver 2019). The existence of strong mid gut escape barrier in *P. duboscqi* as has been described by (Turell and Perkins 1990) may have also limited the dissemination of NPV in this study. This physical barrier is believed to be genetic.

The escape of the virus from the mid gut may also be limited by the formation of the peritrophic membrane that forms after the blood meal (Ciurolini et al. 1989). In sandflies, the membrane forms in less than 48 hours, creating a physical barrier enveloping the blood meals and may block the dissemination of the virus into the secondary tissues. Physical barriers of the mid gut have also been shown to limit the dissemination of western equine encephalomyelitis virus in *Culex tarsalis* (Kramer *et al.,* 1981).

Although the salivary glands were tested for the virus to represent transmission success of NPV in this study, *P. duboscqi* has not been shown to possess a strong salivary gland infection barrier to viruses. Studies show that dissemination of the virus from the mid gut into the hemocoel is enough to determine viral vector competence of *P. duboscqi* (David et al. 2000). This is supported by the fact that nearly all disseminated infections from virus ingestion or infections

introduced by intrathoracic inoculation are always transmissible in laboratory studies (David et al. 2000; Turell and Perkins 1990). Intrathoracic inoculation deposits the virus directly into the hemocoel, surmounting the mid gut infection and escape barriers (Vogels et al. 2017).

Titers used in this study are similar to those used to demonstrate the transmission of RVFV by *P. duboscqi* (Turell and Perkins 1990). There is lack of evidence on the viremic levels of *Phlebovirus* that establish infections in sandflies in natural transmission setting. Possibly, the virus titers of the infectious blood meals used in this study were suboptimal to achieve transmissible infections.

More mortalities of *P. duboscqi* were observed when mid gut infection rates were higher in this study (Spearman's correlation analysis, $\rho = 0.609$). It is, however, not clear whether the mortalities were an effect of the virus. Studies report that laboratory colonies show high mortalities after the first oviposition (Killick-Kendrick 1999) which is often 4-8 days after the blood meal. This could explain the mortalities observed in this study.

5.2 Conclusions

1. *Phlebotomus duboscqi* has a poor oral susceptibility to NPV with mid gut infection rates of only 19.51%.
2. *Ntepes virus* forms a non-disseminating infection in *P. duboscqi* which cannot progress to the salivary gland of the arthropod to be transmitted by bites.
3. This study therefore concludes that *Phlebotomus duboscqi* is not a competent vector of *Ntepes virus*. This study has, however, provided a baseline for future vector competence studies in sandflies which will be important in establishing vector potentials of sandfly species for the entomological risk assessments of *Ntepes virus* transmission.

5.3 Recommendations

1. Since *P. duboscqi* has been shown to be unable to transmit NPV under laboratory conditions, there is need for experimental infections of other sandfly species, especially from the genus *Sergentomyia* with NPV to assess their vector competence to the virus.
2. Also, further studies should attempt the use of a suitable animal model to assess for vector competence of sandflies to NPV and other sandfly-associated viruses. The use of animal models can also improve the blood feeding success rates and engorgements to ensure maximum exposure of the sandflies to the virus.
3. Theirs is also need for further studies to decode the possible genetic barriers of dissemination of NPV in P. duboscqi.
4. There is also need for standardization and optimization of laboratory methodologies for the study of vector competence of sandflies to viruses.

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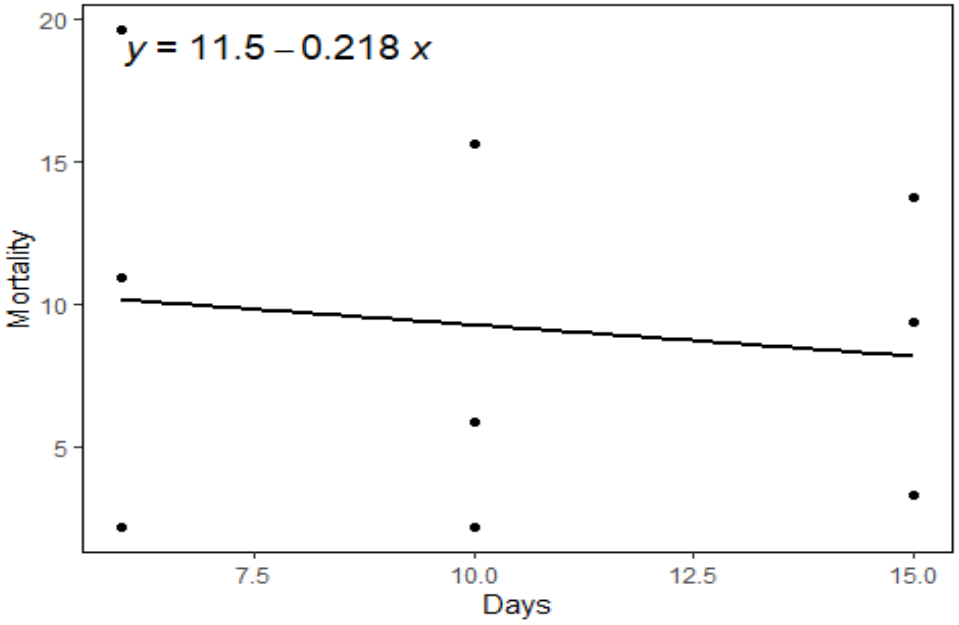
APPENDICES

Appendix 1. Mortalities of *Phlebotomus duboscqi* across the EIP after ingestion of NPV

Replicate	Number fed	6DPI	10DPI	15DPI	Total mortalities	Percentage
1	97	2	2	3	7	7.2%
2	87	7	10	6	23	26.4%
3	71	10	3	7	20	28.2%



Appendix 2. Regression of mortalities of *Phlebotomus duboscqi* against EIP in days