The role of *Rhipicephalus (Boophilus) decoloratus*, *Rhipicephalus appendiculatus* and *Amblyomma hebraeum* in the transmission of lumpy skin disease virus

By

Jimmy Clement Lubinga

Submitted in fulfilment of the requirements for the degree

Philosophie Doctor (PhD)

In the Department of Tropical Veterinary Diseases

Faculty of Veterinary Science, University of Pretoria

This work is dedicated to my wife, Enala and the boys: Diwa, Chabota, Javan, Timothy and Edward for their tolerance and support.

I also dedicate this work to my father (Timothy H. Lubinga) and mum (late Selina Makala Lubinga) for their great parental guidance and direction.

Delaration

I declare that this thesis, which I hereby submit for the degree **Philosophiae Doctor** at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at any tertiary institution.

Jimmy Clement Lubinga

May 2013.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation and gratitude to:

Prof. Estelle Venter- promoter: for all the guidance and support

Co-promotors Eeva Tuppurainen and Hein Stoltsz: for all the support and comments

Prof. Koos Coetzer: for critical editing and support

Sarah Clift and her team (Marie Smit, Naomi Timmerman and Rephima Phaswane at Pathology section): for their help with tick sections and immunohistochemistry

Post graduate colleagues: Mato, Iya, Mamostic, OK, B2, Babayani, Akin, Cavo, Betty, Devwald, Lerosky, Petunia, Benjamin and all the many other guys for the company, the jokes and the help.

Family: Many thanks to my brothers and sisters in the extended family for the big help to fill up the gaps where I was failing.

Joe and Mamoyo Kaunda: You were more than family.

Laboratory technologisists: Anna-Marie, Karren, Rebone, KG, Milana, Ilse. You guys made my study a success

All friends: Thanx to the many other friends who helped me through the encouragement and other forms of help.

The Infectious Diseases of Livestock for International Development (CIDLID) research programme, the Department of International Biotechnology and Biological Sciences Research Council (BBSRC), the UK government, the Department for International Development (DFID) and the Scottish Government (CIDLID project number BB/H009361/1) for funding the study.

The Director of the Department of Veterinary and Tsetse Control Services for allowing me to take time off to study.

SUMMARY

The role of *Rhipicephalus (Boophilus) decoloratus*, *Amblyomma hebraeum* and *Rhipicephalus appendiculatus* in the transmission of lumpy skin disease virus

By

Jimmy Clement Lubinga

Promoter — Prof E.H. Venter
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria
South Africa

Co-promoter — Dr. W.H. Stoltsz
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria
South Africa

Co-promoter — Dr. E.S.M. Tuppurainen
The Pirbright Institute
Capripoxvirus Reference Laboratory
Pirbright, Surrey
The United Kingdom

For the **PhD degree**

Lumpy skin disease (LSD) is an economically important and debilitating disease of cattle caused by the lumpy skin disease virus (LSDV), a poxvirus in the genus *Capripoxvirus*. The disease is of economic importance to farmers in endemic regions and is a major constraint to international trade in livestock and their products. It is characterised by fever, enlargement of superficial lymph nodes, loss of weight, inappetence, salivation, lachrymation and formation of eruptive circumscribed skin lesions. The quality of meat and milk are reduced; there is infertility due to reduced sperm quality, abortions and reduced calving rates. The hides are permanently scarred, thereby reducing their quality and trade may be affected following movement restrictions from affected areas.

Lumpy skin disease has the potential to become an emerging disease because of global climate change and changes in patterns of trade in animals and animal products. The disease has become endemic in Africa except in countries like Libya, Algeria, Tunisia and Morocco, where the disease has never been reported. It has also spread to the Middle East where outbreaks were first reported in Israel (1989), Kuwait (1991), Saudi Arabia (1990) , Lebanon (1993), The United Arab Emirates (2000) and Oman (2010).

In endemic areas, LSD outbreaks are common in summer. The persistence of LSDV between inter-epidemic periods has not been determined and there is no carrier state reported in either cattle or wild animals. Transmission of the disease has been associated with a high incidence of biting insects such as in wet conditions. The spread of LSD from Egypt to Israel e.g. was associated with movement of the stable fly, *Stomoxys calcitrans.* The virus has been recovered from *S. calcitrans* and *Biomya fasciata,* caught while feeding on infected animals and transmission by insects is suspected to be mechanical, which has been demonstrated in *Aedes aegypti* mosquitoes.

During the 1957 outbreak of LSD in Kenya, affected animals were observed to have high tick infestations, especially of *Amblyomma* species. In a pilot trial in 2008 at the University of Pretoria (UP), Department of Veterinary Tropical Diseases (DVTD), *Amblyomma hebraeum, Rhipicephalus appendiculatus* and *R. (B) decoloratus* ticks were implicated in the transmission of LSDV.

The overall objective of this study was to investigate the vector competence of three common sub-Saharan tick species (*R. (B) decoloratus, R. appendiculatus* and *A. hebraeum*) and their potential roles in the epidemiology of LSD. This was achieved by testing for persistence of LSDV in ticks and its subsequent transmission to recipient animals following interrupted feeding, transstadial and transovarial development of the ticks. The over-wintering of LSDV was also investigated during transstadial passage in *A. hebraeum* and transovarial passage in *R. (B) decoloratus.*

During the study, seven cattle were artificially infected with LSDV to serve as source (donors) of infection to ticks. To test for mechanical / intrastadial transmission and persistence in ticks, adult ticks (*A. hebraeum* and *R. appendiculatus*) were partially fed on donor animals and then transferred to recipient animals or collected for testing. To test for transstadial transmission/passage, nymphal stages of *A. hebraeum* and *R. appendiculatus*

were fed on donor animals until they engorged and dropped. Engorged nymphs were incubated to moult to adults. The emergent adults were placed on recipient animals and also tested for the virus. To test for transovarial transmission and passage *R. (B) decoloratus* (one-host tick) larvae were fed on donor animals until engorged adults. For *R. appendiculatus* and *A. hebraeum* (three-host ticks), adults were fed to repletion on the donor animals. Engorged females were collected and incubated to lay eggs and the eggs were allowed to hatch. The emergent larvae were placed to feed on recipient animals to test for transovarial transmission, while larvae were tested for the presence of the virus.

Over-wintering of LSDV in ticks was tested by transstadial passage in *A. hebraeum* and transovarial passage in *R. (B) decoloratus* under fluctuating reduced temperatures, simulating wintery climatic conditions. Engorged *A. hebraeum* nymphs and *R. (B) decoloratus* females were infected by intracoelomic injection.

The presence of the virus in LSDV- infected animals was tested by real-time PCR, virus isolation (VI), and the serum neutralisation test (SNT). Tick saliva was tested by real-time PCR and VI while ticks were tested by immunohistochemistry, transmission electron microscopy, VI and real-time PCR.

Mechanical/intrastadial and transstadial transmission is reported in *A. hebraeum* and *R. appendiculatus*. Transovarial transmission was reported in *A. hebraeum, R. appendiculatus* and *R. (B) decoloratus*. The virus was demonstrated in saliva and tick organs of *A. hebraeum* and *R. appendiculatus* adults following both mechanical/intrastadial and transstadial persistence. Transovarial passage of LSDV was demonstrated in *R. (B) decoloratus, R. appendiculatus* and *A. hebraeum* larvae. The virus also persisted through cold temperature exposure during transstadial passage in *A. hebraeum* and transovarial passage in *R. appendiculatus*.

This study confirms the vector competency of *A. hebraeum, R. appendiculatus* and *R. (B) decoloratus* ticks for LSDV. It also shows the potential for LSDV to over-winter in ticks and demonstrates that LSDV may persist in ticks during inter-epidemic periods.

Summary of articles published / manuscripts submitted arising from this study

Tuppurainen, ESM; Lubinga JC; Stoltsz, WH; Troskie, M; Carpenter, ST; Coetzer, JAW; Venter, EH and Oura, CAL	Evidence of vertical transmission of lumpy skin disease virus in *Rhipicephalus decoloratus* ticks	Published by Tick and tick borne diseases journal. Doi: 10.1016/j.ttbdis.2013.01.006	
Tuppurainen, ESM; Lubinga JC; Stoltsz, WH; Troskie, M; Carpenter, ST; Coetzer, JAW; Venter, EH and Oura, CAL	Mechanical transmission of lumpy skin disease virus by *Rhipicephalus appendiculatus* male ticks	Published by Epidemiology and infection journal. Doi: 10.1017/s0950268812000805	
Lubinga JC; Tuppurainen, ESM; Mahlare, R; Coetzer, JAW: Stoltsz, WH and Venter, EH	Evidence of transstadial and mechanical transmission of lumpy skin disease virus by *Amblyomma hebraeum* ticks	Published on line by Transboundary and Emerging diseases journal. Doi: 10.1111/tbed.12102	Chapter 3 of thesis
Lubinga JC; Tuppurainen, ESM; Stoltsz, WH; Ebersohn, K; Coetzer, JAW and Venter, EH	Detection of lumpy skin disease virus in saliva of ticks fed on lumpy skin disease virus- infected cattle.	Published on line by Experimental and applied acarology journal. Doi: 10.1007/s 10493-013-9679-5	Chapter 4 of thesis
Lubinga JC; Tuppurainen, ESM; Coetzer, JAW: Stoltsz, WH and Venter, EH	Transovarial passage and transmission of LSDV by *Amblyomma hebraeum*, *Rhipicephalus appendiculatus* and *Rhipicephalus decoloratus*	Submitted to Experimental and Applied Acarology Journal Manuscript number: APPA-D-13-00046	Chapter 5 of thesis
Lubinga JC; Clift, S; Tuppurainen, ESM; Stoltsz, WH; Babiuk, S; Coetzer, JAW and Venter, EH	Demonstration of lumpy skin disease virus infection in *Amblyomma hebraeum* and *Rhipicephalus appendiculatus* ticks using immunohistochemistry.	Submitted to Tick and tick-borne diseases Journal Manuscript number: TTBDIS-D-13-00090	Chapter 6 of thesis
Lubinga JC; Tuppurainen, ESM; Coetzer, JAW: Stoltsz, WH and Venter, EH	Evidence of lumpy skin disease virus over-wintering by transstadial persistence in *Amblyomma hebraeum* and transovarial persistence in *Rhipicephalus decoloratus* ticks	Submitted to Experimental and Applied Acarology journal Manuscript number: APPA-D-13-00065	Chapter 7 of thesis

Contents

List of tables

List of figures

Abbrevations

ARC	Agriculture Research Council
AUCC	Animal Use and Care Committee
BBSRC	Department of International Biotechnology and Biological Sciences Research Council
CCHFV	Crimean Congo haemorrhagic fever
CIDLID	Combating Infectious Disease of Livestock for Livestock Development
CPE	Cytopathic effect
DFID	Department for International Development
DNA	Deoxy-ribo nucleic acid
DVTD	Department of Veterinary Tropical Diseases
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
FVS	Faculty of Veterinary SciencesA
GPV	Goat poxvirus
IHC	Immuno histochemistry
LSD	Lumpy skin disease
LSDV	Lumpy skin disease virus
MCCI	Monte Carlo Confidence Interval
MEM	Minimum essential medium
OIE	Office of International Epizootics
OVI	Onderstepoort Veterinary Institute
ORF	Open reading frame
pa	Post attachment
PBS	Phosphate buffered saline
PBS^{+}	Phosphate buffered saline with calcium and magnesium
PCR	Polymerase chain reaction
pi	Post infection
RH	Relative humidity
SOP	Standard operating procedures
SPV	Sheep poxvirus
SNT	Serum neutralisation test
$TCID_{50}$	Median tissue culture infective dose
TEM	Transmission electron microscopy
UDG	Uracil DNA glycosylase
UP	University of Pretoria
UPBRC	University of Pretoria's Bioscience Research Centre
VI	Virus isolation

Chapter 1

Literature review

1.1. Introduction

Lumpy skin disease (LSD) is an acute, subacute or inapparent viral disease of cattle, characterised by high fever, formation of firm, sometimes eruptive, circumscribed skin nodules, necrotic plaques in the mucous membranes of the resipiratory and digestive systems as well generalised lymphadenopathy (Thomas & Mare 1945, Von Backström 1945, Weiss 1968). It is caused by lumpy skin disease virus (LSDV), a member of the genus *Capripoxvirus*, which it shares with sheep poxvirus (SPV) and goat poxvirus (GPV) (Alexander 1957, Capstick et al. 1959, Tulman et al. 2001). Most LSD outbreaks have been associated with high insect activity such as in summer as well as along water bodies (Weiss 1968).

Lumpy skin disease is a World Organisation for Animal Health (OIE) listed disease (World Organisation for Animal Health 2012) due to the high economic losses caused by decreased productivity of affected animals such as reduced quality of meat, milk and hides, lowered fertility, and trade restrictions (Body et al. 2012, Gari et al. 2011, Lalanne 1956, Weiss 1968).

Lumpy skin disease is endemic across the African continent and in the Middle East such as Israel, Yemen, Oman and recently Lebanon (Body et al. 2012, Brenner et al. 2006, Greth et al. 1992b, Kumar 2011, Tuppurainen & Oura 2012). More recently, molecular evidence of transmission of LSDV by ticks has been reported and more evidence is required to establish the roles that ticks may play in the transmission of LSDV (Tuppurainen et al. 2011).

1.1 History and geographical distribution

The disease was first diagnosed in Northern Rhodesia (Zambia) in 1929 (Thomas & Mare 1945, Von Backström 1945, Weiss 1968). At the time, the infectious nature of the disease was unknown and it was thought to have been caused by insect bites. The disease spread within Zambia for 13 years and by this time it was believed to be caused by plant poisoning. The disease also appeared in Ngamiland (Botswana), where it was called Ngamiland cattle

disease (Von Backström 1945). By 1944, the disease had spread to South Africa, where it affected over 8 million cattle (Bourdin 1971, Thomas & Mare 1945). In 1954, it was diagnosed in Madagascar (Lalanne 1956), in 1957, in Kenya (MacOwan 1959) and later reported in Sudan (1971), and Chad and Niger in 1973 (Ali & Obeid 1977). The disease first appeared in Nigeria in 1974 (Nawathe et al. 1982). The disease was reported in Ethiopia (1981-83), Somalia (1983) and Egypt 1988 (House et al. 1990, Mebratu et al. 1984, Mohamed et al. 1985). By 1986, LSD was endemic in sub-Saharan Africa and Madagascar (Yeruham et al. 1995). In 1989 it was reported outside of Africa, in Israel where it was suspected to have spread from Egypt (Yeruham et al. 1995). Other countries in the Middle East, that reported LSDV outbreaks include Kuwait (1991), Lebanon (1993), Yemen (1995), The United Arab Emirates (2000) and Oman (2009) (Body et al. 2012, Brenner et al. 2006, Greth et al. 1992b, Kumar 2011, Tuppurainen & Oura 2012).

1.2 Lumpy skin disease epidemiology

1.2.1 Disease occurrence pattern

Lumpy skin disease is most prevalent in the wet season or summer and along water courses (Ahmed, El-Desawy & Mohamed 2005, Ali & Obeid 1977, Nawathe et al. 1982) although outbreaks may also occur in the dry season (Haig 1957, Nawathe et al. 1982). In endemic areas, LSD has a tendency to disappear during the dry and cold months (Hunter & Wallace 2001, Woods 1988).

1.2.2 Host range, morbidity and mortality

Lumpy skin disease is primarily a disease of cattle though evidence of infection (antibodies) in some wild animals such as Arabian oryx (*Oryx leucoryx*) of Saudi Arabia (Greth et al. 1992a, Greth et al. 1992b), in black wildebeest (*Connochaetes gnou*), blue wildebeest (*Connochaetes taurinus*), eland (*Taurotragus oryx*), impala (*Aepyceros melampus*), the springbok (*Antiorcas marsupialis*), (Barnard 1997, World Organisation for Animal Health 2009) and Asian water buffalo (*Bubalis bubalis*) of Egypt were reported (Ali et al. 1990). Experimental infection was fatal to impalas (*Aepyceros melampus*) and girrafes (*Giraffa cameropardalis*) while it there was no clinical disease caused to the African buffalo (Syncerus caffer) and no antibodies were detected (Young, Basson & Weiss 1970). Experimental infection of LSDV in sheep and goats only caused a local erythematous swelling at the site of inoculation (Weiss 1968). In cattle, all age groups are susceptible,

although the young animals and milking cows are more susceptible. *Bos taurus* are more susceptible than *Bos indicus* breeds (Davies 1982, Salib & Osman 2011). The morbidity of the epidemics of 1989/90 and 2000/2001 in South Africa were estimated between 1-20% and in some occasions reached 50% (Coetzer 2004). During the 2006 outbreaks in Egypt morbidity were close to 100% (Salib & Osman 2011).

1.3 Economic importance

Lumpy skin disease outbreaks have serious impact on the economy of both the farmer and the country. The disease causes debility which leads to reduced meat quality. Pox lesions in the udder may lead to secondary mastitis (Weiss 1968). Permanent skin damage due to the development of scabs and secondary bacterial infection markedly lowers the value of the hides (Body et al. 2012, Gari et al. 2011, Green 1959, Green 1960). Other production losses are due to abortions and sterility in bulls (Irons, Tuppurainen & Venter 2005, Kumar 2011, Lalanne 1956, Weiss 1960). Movement restrictions imposed in affected areas contribute to loss of business and income and there is temporal loss in draft power due to the debility and lameness (Gari et al. 2011).

1.4 The virus

1.4.1 Taxonomy

Lumpy skin disease is caused by a double-stranded DNA virus, lumpy skin disease virus, a member of the genus *Capripoxvirus*. Other viruses within this genus include SPV and GPV. The genus *Capripoxvirus* is one of the 8 members of the subfamily, *Chordopoxvirinae* for vertebrates in the family *Poxviridae*. The other subfamily *Entomovirinae* is for insects (King et al. 2011, Tulman et al. 2001, Tulman et al. 2002). Poxviruses are the largest of all animal viruses (320x260 nm) (Fenner & White 1976, Fenner 1987, Weiss 1968). Lumpy skin disease virus has one prototype strain, the Neethling virus that has morphological and antigenic similarities with other capripoxviruses (SPV and GPV) (Davies 1976, Kitching 1986, Kitching, Hammond & Black 1986, Kitching 2003, Tulman et al. 2002).

1.4.2 Morphology

The virions of capripoxviruses are ovoid or brick shaped (Davies et al. 1971, Weiss 1968). They are characterised by the presence of three major structures: an outer coat (the envelope), two lateral bodies and the inner dumb-bell shaped core, containing the DNA (Catroxo et al.

2009). They are classified as either “M” (mulberry) or “C” (capsule) forms depending on the penetration of the stain during the negative staining preparation. The M-form, with poor stain penetration, is covered with thread-like surface ridges or interwoven particles while C-forms, with high stain penetration, lack the thread-like ridges but have an irregular capsule-like surface, with a dumb-bell core (Weiss 1968, Woodson 1968).

1.4.3 Lumpy skin disease virus genome

The LSDV has a linear double-stranded DNA with a genomic size of about 151 kbp (Tulman et al. 2001). The genome contains the genes required for the replication of the virus and has 30 genes for structural proteins (Tulman et al. 2001). The major structural proteins in a mature LSDV virion, as in other poxviruses, include proteins associated with the virion core, proteins associated with the intracellular mature virion and other membrane proteins and enzymes (Tulman et al. 2001). Lumpy skin disease viral genome encodes non-structural proteins responsible for virulence, inhibition of cell apoptosis, specific cell or tissue tropism and modulation or evasion of host immune responses (Banadyga et al. 2007, Banadyga et al. 2009, Johnston & McFadden 2003, Seet et al. 2003, Tulman et al. 2001).

1.5 Lumpy skin disease

1.5.1 Pathogenesis

Lumpy skin disease virus is epitheliotropic and multiplies at the site of entry. Poxviruses enter host cells by phagocytosis, into the cytoplasm where they undergo two stages of uncoating and multiplication (Purcell et al. 1972, Woodson 1968). After multiplication at the site of entry, the virus enters and multiplies in local draining lymph nodes followed by a primary viraemia. In experimental animals, the first viraemia has been detected as early as Day 2 to 7 post infection (Babiuk et al. 2008b, Chihota et al. 2001, Tuppurainen, Venter & Coetzer 2005). Infection also spreads to internal organs especially the lungs, liver, spleen, the rumen and abomasum (Babiuk et al. 2008b, Woods 1988). There is infiltration of epithelial and endothelial cells, pericytes and fibroblast causing lymphangitis and vasculitis with concomitant thrombosis and infarction, which leads to oedema and necrosis (Annandale et al. 2010, Prozesky & Barnard 1982). The virus has been demonstrated in saliva, semen, nasal secretion, skin lesions, kidneys, abomasum and rumen but not in urine and faeces (Babiuk et al. 2008b, Coetzer 2004, Prozesky & Barnard 1982, Weiss 1960, Weiss 1968).

1.5.2 Clinical signs

The incubation period under experimental conditions is 4-14 days (Tuppurainen, Venter & Coetzer 2005). Under natural conditions, it may take between 2-4 weeks (Weiss 1968). Early signs include a fever of 40-41.5°C, which will last between 4-14 days. During this time, the animal will manifest inappetence, salivation, lachrymation and mucoid to mucopurulent nasal discharge (Carn & Kitching 1995a, Carn & Kitching 1995b, Prozesky & Barnard 1982). The superficial lymph nodes especially the prescapular and precrural, become enlarged up to 10 times the original size (Body et al. 2012, Coetzer 2004, Kumar 2011, Prozesky&Barnard 1982, Salib & Osman 2011). Conjunctivitis develops and may be followed by corneal opacity and eventual blindness (Alexander 1957, Body et al. 2012, Haig 1957). Characteristic circumscribed intra-dermal, mostly eruptive, skin nodules develop. They are firm, raised, round about 10-50 mm wide and are readily seen in animals with short hairs unlike in long haired animals. Ulcerative lesions may develop in the conjunctiva, nostrils, muzzle and mucous membranes of the mouth, larynx, trachea, reproductive and alimentary tracts, especially the abomasum (Carn & Kitching 1995a, Prozesky & Barnard 1982, Tuppurainen, Venter & Coetzer 2005, Weiss 1968). In severe cases, nodules may erupt leaving a raw surface of the muscles. Nodules in the skin of the udder and teats may lead to mastitis. Subcutaneous swelling of the legs, leading to lameness may occur. Abortions or prolonged anoestrous as well as intrauterine infection may occur. Temporal sterility in infected bulls may be observed and secondary complications may develop. These include aspiration pneumonia due to inhalation of infected necrotic lesions from upper respiratory tract, stenosis of the trachea and secondary bacterial infections may complicate the necrotic nodules (Prozesky&Barnard 1982).

1.6 Transmission

The method of LSDV transmission is not fully understood (Kitching & Taylor 1985b). Direct contact between animals is said to be ineffective, although venereal transmission has been suspected through demonstration of the virus in semen of infected bulls (Annandale et al. 2010, Bagla et al. 2006, Irons, Tuppurainen & Venter 2005, Weiss 1960, Weiss 1968) and experimentally proven (Annandale C.H et al. 2013). Ingestion of infective saliva was also shown to transmit the disease (Haig 1957).

1.6.1 Arthropod transmission

Transmission of LSDV is considered to be mainly mechanical by biting insects. This follows observations that most outbreaks of LSD have been reported in the rainy seasons and along water-courses where there is increased insect activity (Carn & Kitching 1995a, Haig 1957, Weiss 1968). In 1957, the outbreak of LSD in Kenya was associated with high numbers of mosquitoes *Aedes natronius* and *Culex mirificens* (Burdin & Prydie 1959) while in September 1989, the LSD outbreak in Israel was suspected to have been transmitted by *Stomoxys calcitrans* wind-blown from Egypt (Yeruham et al. 1995). Since *S. calcitrans* was demonstrated to transmit other capripoxviruses i.e. SPV and GPV (Kitching & Mellor 1986, Mellor, Kitching & Wilkinson 1987), it is suggested that it has the capacity to transmit LSDV as well, although experimental transmission trials of LSDV with *S. calcitrans* failed (Chihota et al. 2003). The virus was however recovered from field caught *S. calcitrans* and *Biomya fasciata* feeding on infected animals (Weiss 1968). It was also experimentally demonstrated that *Aedes aegypti* mosquitoes can mechanically transmit LSDV (Chihota et al. 2001).

Insects have a short life span. For example, *A. aegypti* takes between 8-25 days to complete its life cycle (Teesdale 1955) while it takes 12 to 37 days for *S. calcitrans* to complete its life cycle (Shipley 1915). The short life spans for most blood-feeding insects combined with insignificant breeding during the cold and dry months (Teesdale 1955) and lack of evidence for biological transmission by these (flying) insects (Chihota et al. 2003), it is very unlikely that the flying insects play a role in the maintenance of LSDV during the inter-epidemic periods, which include the cold (winter) months.

1.6.2 Ixodid (hard) ticks as possible vectors for lumpy skin disease virus

The role of ticks in the transmission of LSDV has been suspected (Aiel 2009) especially with observations of the presence of ticks, mostly *Amblyomma* spp., on affected animals (Ali & Obeid 1977).

In 2008, a preliminary experiment on the transmission of LSDV by three common sub-Saharan tick species was carried out at the Faculty of Veterinary Science of the University of Pretoria. Different life cycle stages of *Rhipicephalus appendiculatus, Amblyomma hebraeum* and *Rhipicephalus (Boophilus) decoloratus* ticks were fed on LSDV-infected animals and were then transferred to feed on naïve recipient animals. The viral DNA was detected at the feeding sites of *R. appendiculatus* and *A. hebraeum* ticks. When the ticks were tested after feeding on recipient animals the viral DNA was detected in the mouthparts, salivary glands

and gut of *R. appendiculatus*, *A. hebreum* and *R. (B) decoloratus* ticks as well as in eggs of *R. (B) decoloratus* ticks (Tuppurainen et al. 2011). This evidence highly suggested the possibility of mechanical, transstadial and transovarial transmission of LSDV by *ixodid* (hard) ticks (Tuppurainen et al. 2011). Biological (transstadial / transovarial) transmission of protozoan, bacterial and other viral diseases by ixodid ticks such as theileriosis, heartwater, anaplasmosis, babesiosis, Nairobi sheep disease and tick-borne encephalitis have previously been demonstrated (Connell & Hall 1972, Labuda et al. 1993b, Meltzer, Norval & Donachie 1995, Norval 1991, Norval et al. 1994, Pegram & Banda 1990, Peter et al. 1999, Prozesky & Plessis 1987). In addition, camel pox virus was isolated from ticks feeding on infected camels in the United Arab Emirates (Wernery, Kaaden & Ali 1997, Wernery, Meyer & Pfeffer 1997).

1.7 Biology of ticks

1.7.1 Classification of ticks

Ticks belong to the phylum *Arthropoda* for invertebrates, in the class *Arachnida* together with spiders and mites, the order *Acari* (mites and ticks) suborder *Ixodida* for ticks (Cupp 1991, Sonenshine 1991, Walker 1994). The family *Ixodidae* (hard ticks) have a hard dorsal shield, the scutum, which may cover the entire dorsal surface of the male and approximately a third to a half of the anterior dorsal surface of larvae, nymphs and females (Cupp 1991, Walker 1994, Walker 1970).

In Africa, seven genera of ixodid ticks are most important in disease transmission (Andrew & Norval 1989, Bouwknegt et al. 2010, Bruce & Wilson 1998). These include *Amblyomma*, *Rhipicephalus*, *Hyalomma*, *Haemaphyalis*, *Demarcentor*, *Ixodes* and *Margaropus* (Sonenshine 1991, Walker 2003). Recent studies have placed the old genus *Boophilus* as a sub-genus of *Rhipicephalus* and it is now accepted to call these ticks *Rhipecephalus (Boophilus)* followed by species name, for example *Rhipecephalus (Boophilus) decoloratus* (Guglielmone et al. 2009). In this study, we are using *Rhipecephalus (Boophilus) decoloratus*.

1.7.2 Tick anatomy

The anatomy of ticks has been described by Sonenshine (1991), Balashov (1968) and Till et. al., (1961). Ticks are divided into two main regions, the capitulum (gnathosoma or false

head) and idiosoma or body. The capitulum comprises the basis capituli, a pair of four-segmented palps, a pair of two-segmented chelicerae and a hypostome.

The basis capituli provides anchorage to the flexible mouthparts and also contains the shaft for the chelicerae, the pharynx and salivary ducts. The paired chelicerae are located medial to the palps and on the dorsal aspect of the capitulum (Sonenshine 1991).

The hypostome forms the ventral aspect of capitulum. It is the primary organ of tick attachment is ventrally covered by regular rows of denticles (Sonenshine 1991, Zebrowski 1926). Dorsally, the hypostome is concave with a preoral canal through which blood is sucked by the pumping action of the pharynx (Till 1961). The four-segmented palps extend from antero- lateral aspects of the basis capituli (Sonenshine 1991).

Dorsally, in females and immature stages, the anterior part of the body is covered by a scutum, posterior to which is the alloscutum, which is characterised by innumerable superficial foldings of the cuticular surface. In males, the scutum covers the entire dorsal part of the body. The scutum bears simple eyes on the lateral margins (Balashov, Raikhel & Hoogstraal 1983, Sonenshine 1991, Till 1961).

On cross section, the body wall (integument) of the tick shows the cuticle and the epidermis. The epidermis lies below the cuticle and comprises a simple cuboidal epithelium resting on a basement membrane (Sonenshine 1991).

The body cavity of an ixodid tick is an open hemocoel, bathed with a body fluid, the hemolymph and has several loose connective tissues and membranes supporting various internal organs. Internal organ systems include the digestive, reproductive, respiratory (tracheal), nervous, and circulatory systems (Balashov, Raikhel & Hoogstraal 1983, Sonenshine 1991, Till 1961).

The digestive system organs include the preoral canal, the salivary glands, the pharynx, oesophagus, midgut and hindgut. Salivary glands comprise a pair of grape-like clusters extending antero-posteriorly along the lateral sides of the body (Balashov 1972, Sonenshine 1991, Till 1961, Walker, Fletcher & Gill 1985). The units of salivary glands are alveoli (acini) and each alveolus comprises 10-12 nucleated cells located around a central cavity (lumen), which flows into an alveolar duct. The lobular ducts flow into a short lobular duct and finally into a common duct (Zebrowski 1926).

The salivary glands comprise three types of alveoli in females and four in males (Sauer, Essenberg & Bowman 2000, Walker, Fletcher & Gill 1985). On the basis of types of cells contained, the salivary gland alveoli may be agranular (type 1) or granular (types 2, 3 & 4). Type 1 alveoli are made up of agranular cells and are confined to the main ducts (Till 1961, Walker, Fletcher & Gill 1985).

The foregut (mouth parts) comprises the preoral canal, pharynx and oesophagus. The preoral canal lies on the floor of the hypostome. It distally leads to the pharyngeal valve and pharynx proper. The pharynx is located about the centre of the basis capituli and it serves as a pump organ for the suction of blood (Sonenshine 1991, Zebrowski 1926). The mouthparts are lined by cuticle and a single layer of squamous cells. The oesophagus connects the pharynx and the midgut. It is a thin cuticle lined tube. It passes through the synganglion, entering it at the antero-ventral margin and emerging from the postero-dorsal edge. It forms a fold with muscle bundles, the proventricular valve, at its junction with the midgut, believed to prevent regurgitation of blood back from the midgut to the oesophagus (Balashov, Raikhel & Hoogstraal 1983, Balashov 1972, Sonenshine 1991, Till 1961, Zebrowski 1926).

The midgut is the largest organ in the tick. It comprises a central ventriculus, an equivalence of the stomach with paired tube-like diverticular or caeca with blind ends (Balashov 1972, Zebrowski 1926). The midgut comprises an epithelium whose cell composition varies according to stage of feeding. Unfed ticks have been described to contain undifferentiated (stem) cells and some digestive cells (Coons et al. 1986, Sonenshine 1991).

The hindgut comprises the rectal sac, a bulbous structure located in the posterior part of the body (Sonenshine 1991, Zebrowski 1926). The lumen normally contains masses of guanine crystals or blood cells (Balashov, Raikhel & Hoogstraal 1983, Balashov 1972).

The Malpighian tubules are a pair of long narrow tubules connected to the rectal sac at its junction to the rectum with a simple cuboidal epithelium and perform as are the excretory organs for nitrogenous products in ticks (Balashov, Raikhel & Hoogstraal 1983, Sonenshine 1991, Till 1961, Zebrowski 1926).

The central nervous system comprises the synganglion seen as a small rounded mass at the anterior bifurcation of the mid gut, in the antero- medial ventral region of the body (Balashov, Raikhel & Hoogstraal 1983, Sonenshine 1991, Till 1961, Zebrowski 1926). The oesophagus passes through synganglion, thereby dividing it into two un-equal parts (Marzouk

et al. 1987) and the nervous tissue is divided into the outer cortex, comprising the glial cells and neuronal cells and an inner neuropile. The entire mass is surrounded by a connective tissue, the neurilemma (El-Shoura 1989, Sonenshine 1991).

The female reproductive system comprises an ovary, paired oviducts, a common connecting tube, a seminal receptacle and vestibular and cervical vaginas. The ovary is a single tubular horse shoe shaped organ, the hind portion lies in the posterior third of the body in front of the rectal sac, ventral to the midgut diverticular. The lateral portions run anteriorly and bend sharply before forming the paired oviducts (Sonenshine 1991, Till 1961). The wall of ovary contains germinal cells (oocytes and oogonia), which have irregularly ovoid to round shapes, large nuclei (7-8µm in diameter) and dense cytoplasma. As the female feeds, their oocytes increase in size and the retarded oocytes form the dorsal groove (fold) that runs the longitudinal axis of the ovary (Balashov, Raikhel & Hoogstraal 1983, Brinton, Oliver & Jr. 1971, Sonenshine 1991, Till 1961).The oviducts are a pair of slender coiled tubes. Their wall is made up of cuboidal epithelial cells with large nuclei. Their lumen is filled with microvilli. After feeding, the wall becomes columnar. This is the site for spermatozoa attachment (Garcia-Fernandez et al. 2005, Till 1961).

The connecting tube is a single narrow duct with a wall made up of thin layer of epithelium and in virginal females, the lumen is collapsed and the walls are folded forming a labyrinth (Sonenshine 1991, Till 1961).

The seminal receptacle is a sac-like folded organ that lies dorsal to the cervical vagina and connective tube. They have multiplied epithelium with large nuclei and a thin layer of cuticle. The lumen of the RS, in mated females, is extended by the spermatophores (Till 1961).

The male reproductive system in ticks comprises paired testes, paired vas deferens, a single ejaculatory duct and a complex accessory gland. The testes are tubular with a small lumen. They occupy the lateral positions of the body, extending from the posterior margins of the synganglion to the level of the spiracles and lie ventral to the median part of the salivary glands. In unfed males, the testes comprise interstitial cells and germinal cells (spermatogonia) arranged in segments surrounded by delicate strands of connective tissue similar to tunica propria of ovaries. In feeding males, more advanced stages of spermatogonia including spermatocytes and eventually prosperms are encountered in the posterior parts of the testes (Balashov, Raikhel & Hoogstraal 1983, Sonenshine 1991, Till 1961).

The vas deferens is a pair of coiled flattened tubules that connect the testes to the ejaculatory duct and fuse into one tube anterior to the tip of the accessory gland with a They have a single epithelium layer of low columnar cells with small spherical to oval basally located nuclei. As the male tick feeds, the vas deferens becomes distended with spermatophores and the wall becomes so thin to the extent that the structure becomes difficult to distinguish (Balashov, Raikhel & Hoogstraal 1983, Till 1961).

The ejaculatory duct is a thin walled duct that receives the opening of the vas deferens and discharges into the genital opening. The wall contains a flattened epithelium and a folded cuticular lining (Till 1961).

The multi-lobed male accessory gland lies immediately postero-ventral to the synganglion and vas deferens. The wall comprises a multi-layered columnar epithelium with masses of secretory granules in fed males (Garcia-Fernandez et al. 1998, Sonenshine 1991, Till 1961).

The fat bodies constitute a network of single and branching strands in the hemocoel (Obenchai.Fd & Oliver 1973). The peripheral FBs are found around the tracheal trunks, hyaline tissues, below epidermal cells, on ventral surfaces and between organs. The FBs comprise trophocytes (acidophils), which are oval to cylindrical cells with minor diameters from 8 to 15 µm and major diameters up to 32 µm. The nucleus is approximately 4 to 7 µm wide. The other cell type, the nephrocytes, are basophilic and are found at the tips of the strands with diameters from 8 to 10 µm wide in unfed ticks while may reach up to 32 µm in fed ticks (Coons et al. 1990, El-Shoura 1989, Obenchai.Fd&Oliver 1973, Sonenshine 1991).

The circulatory system of ticks is open and comprises a heart, aorta and arterial vessels and sinuses, with hemolymph as circulatory fluid (Obenchain & Oliver 1976, Sonenshine 1991). Haemolymph comprises plasma and hemocyets. Plasma is a waterly fluid rich in proteins, amino acids, fat acids, hormones and other salts (Obenchain & Oliver 1976, Sonenshine 1991, Till 1961).

Ticks, like insects, utilise the tracheal system for gaseous exchange. The tracheal trunks originate from paired spiracles (stigmata) located within spiricular plates on the ventro-lateral surfaces, just prior to coxae IV. The trunks branch into smaller tracheae and smaller tracheae branch into much finer tracheoles which enmesh all internal organs (Balashov, Raikhel & Hoogstraal 1983, Sonenshine 1991, Till 1961).

1.7.3 Tick life cycle

Ticks undergo four development stages, namely, embryonated eggs, larvae, nymphs and adults and it is only in the adult stage that sexual dimorphism in ticks is evident (Cupp 1991, Kuhnert, Diehl & Guerin 1995). Larvae of Ixodid ticks have three pairs of legs, a scutum at the anterior end but are without a genital pore or spiracles. Nymphs have four pairs of legs, a scutum at the anterior end, spiracles but no genital pore (Walker 1994). As obligatory hematophagous parasites of vertebrate animals, ticks spend the active stages of the larvae, nymph and adult on a host. According to the number of vertebrate hosts a tick species parasitizes the ticks are described as a one-, two- or three-host ticks (Sonenshine 1991, Walker 2003, Walker 1994). A one-host tick such as *R. (B) decoloratus* spends its entire parasitic stages of its life cycle (i.e. from larvae through nymphs to adults) on a single host animal (Koshy, Rajavelu & Lalitha 1982, Vatsya et al. 2006). In two-host ticks such as *Hyalomma rufipes,* the larvae and nymph stages feed on one host and the adults on a second host (Chen et al. 2012, Sonenshine 1991). In three- host tick species, such as *Rhipicephalus appendiculatus* and *Amblyomma hebraeum,* each active stage (larvae, nymphs and adults) feeds on a different host (Pegram & Banda 1990, Rechav 1978). In all instances the males and females mate on the final host, except for *Ixodes* spp. which mates on the ground, and the engorged female drops to the ground, lays eggs and dies. The male remains on the host and is attracted to other feeding virgin females to attach and mate (Cupp 1991).

1.7.4 Seasonality of tick life cycle

In order to overcome adverse effects of weather, ticks have adapted seasonal variations. In southern Africa where there is a univoltine phenology, the moulting of *R. appendiculatus* and *A. hebraeum* nymphs to adults occurs towards the single rain season of the year and the peak of the larvae is towards the end of the wet season with the nymphal stages common in winter (May to July in South Africa) (Bryson et al. 2002b, Horak, Gallivan & Spickett 2011, Norval, Andrew & Meltzer 1991, Norval, Sutherst & Kerr 1996, Pegram & Banda 1990, Rechav 1978, Smith et al. 2009). *Rhipicephalus (Boophilus) decoloratus* larvae commonly quest early in summer months, such as in November and December. Females start to lay eggs at the end of summer and ticks overwinter as replete females or as eggs (Bryson et al. 2002b, Spickett & Heyne 1990). The synchronisation of seasonality in ticks seems to be controlled through diapause or quiescence, especially in the adult stage. Unfed *R. appendiculatus* adults have been shown to go through behavioural diapause (Madder & Berkvens 1997, Madder et al. 1999), which is manifested as suppression of host seeking in unfed adults or failure to

quest or feed (Belozerov 2008, Berkvens, Pegram & Brandt 1995, Rechav & Jager 1991). *Amblyomma variegatum* females dropping in August to October (Zambia), undergo morphogenic diapause, which postpones the oviposition until it is moist in November/December (Pegram et al. 1988). Seasonal synchronisation enables the larvae to hatch under humid and wet conditions hence protecting them from extreme drying (Walker 2003). Since overwintering of ticks does not reduce the viability of ticks (Dantas-Torres, Giannelli & Otranto 2012), it may have serious epidemiological implications for tick borne-diseases since during the time of dormancy, infected arthropods also maintain their pathogens (White et al. 2005).

1.7.5 Host finding and tick attachment

Ticks find their hosts by either questing or hunting. When questing, the ticks wait on the vegetation for a passing host. Questing is common among the *Rhipecephalus* species and the immature stages of *Amblyomma* species. The hunters, such as mature stages of *Amblyomma* and *Hyalomma* species on the other hand, actively seek the hosts. Both hunting and questing are stimulated by carbon dioxide, volatile host metabolites released by the hosts and other animal odours (Crooks & Randolph 2006, Donze, McMahon & Guerin 2004, Osterkamp et al. 1999, Wilkinson 1953).

1.7.6 Feeding and salivation

All active stages of the ticks are parasitic and require a blood meal to engorge before moulting to the next stage. Adult (male and female) ticks require the blood meal for sperm and egg production, respectively (Cupp 1991, Sonenshine 1991). Ticks are telmophagous, i.e., the chelicerae slash through the superficial layers of the skin and lacerate the capillary bed so that a pool of blood is formed subdermally and it is from this that the tick is able to feed. As the tick attaches, ixodid tick species secrete saliva, which contains pharmacological products that enable the ticks to successfully obtain their blood meal. The saliva, secreted on attachment, contains a white milky substance secreted by types II and III alveoli. This protein forms a cement cone that solidifies and adheres the tick mouth parts to the host skin (Bowman&Sauer 2004, Cupp 1991, Gregson 1960). Tick salivary gland secretions also include anti-coagulants, which inhibit thrombokinase, responsible for blood clotting. *Ixodes dammini* secretes enzyme apyrase, which prevents platelet aggregation. Tick salivary secretions also have vasoconstrictive mediators, prostaglandin E2 (PGE2) and prostacyclin. The combined effects of these agents is to prevent hemostasis, vasoconstriction and the

coagulation cascade which ensures a continuous flow of blood in a fluid form (Benavides Ortiz & Walker 1992, Ribeiro, Makoul & Robinson 1988, Ribeiro, Weis & Telford 1990, Sá-Nunes et al. 2007).

Saliva also contains proteins which cause immuno-modulation, which impairs host response to invasion (Chinery 1981, Marchal et al. 2011, Randolph 2009). The chemical agents in tick saliva include immunoglobulin G (IgG) binding proteins which remove host IgG that crosses the midgut and ultimately saving the haemocoel tissues from harm. Iinterleukin-2 (IL-2) binding protein, which binds to host cells with IL-2 receptors such as T-cells, B-cells, natural killer (NK) cells, cytotoxic T-cells, monocytes and macrophages is also present (Brossard & Wikel 1997, Brossard & Wikel 2004, Wikel, Ramachandra & Bergman 1994). *Rhipicephalus microplus* was seen to cause a reduction of peripheral blood lymphocytes (Inokuma et al. 1993). The ultimate effect is the suppression of both the classical and alternate complement pathways and failure of the host to recognise the presence of the ticks (Inokuma, Kemp & Willadsen 1994, Marchal et al. 2011, Randolph 2009).

Once feeding starts, the accumulation of blood in the midgut, creates a hypo-osmotic condition (Kaufman 1973). The excess water and ions are removed from the blood meal and move across the gut epithelium into the hemocoel from where they are transported to the salivary glands. The salivary glands produce watery saliva that is injected back into the host (Cupp 1991, Hsu & Sauer 1975). A 5- to 30s cyclic pattern of alternating sucking and salivation starts, with durations of blood sucking lasting longer than those of salivation, interrupted by periods of relative inactivity (Gregson 1960, Kaufman 1989). The process of constant removal of excess water and ions from the tick body helps in osmoregulation and enables the ticks, especially females, to concentrate their blood meal and take in large quantities of blood (Hsu & Sauer 1975, Kaufman 1973, Sonenshine 1991).

In addition to osmoregulation, saliva is used as a conduit of pathogens such as viruses, bacteria and protozoa (Balashov 1972, Sonenshine 1991). As the tick removes the excess water and ions, pathogens in the saliva are also passed into the mammalian host (Kaufman & Nuttall 1996). Saliva also potentiates pathogen establishment in the host through the immuno-modulatory mechanisms described above (Wikel, Ramachandra & Bergman 1994, Wikel 1999).

1.7.7 Infection of ticks

It has been generally accepted that ticks become infected from vertebrate animals following a blood meal on a viraemic host (Nuttall, Jones & Davies 1991, Sonenshine 1991, Tuppurainen et al. 2011). It has however been reported that uninfected ticks can also become infected by co-feeding with infected ticks on a non viraemic host, in what is termed "non-viraemic transmission" (Jones et al. 1989, Jones, Matthewson & Nuttall 1992, Labuda et al. 1993a). The presence of a factor in salivary glands of *R. appendiculatus* and *A. hebraeum* that enhanced transmission of Thogoto virus to ticks was also demonstrated (Jones et al. 1989). This factor, called saliva assisted transmission factor, was shown to be released after 6-8 days of feeding and, in females, it was shown to continue for the period of feeding (Jones et al. 1992).

1.7.8 Interrupted feeding

Under certain circumstances, feeding of ticks within a developmental stage may be interrupted. Male ticks whose female partners have fed to repletion and drop off to lay eggs, in response to sexual pheromones released by unmated feeding females, detach and move towards the females and attach (Andrew & Norval 1989, Leahy, Hajkova & Bouchalova 1981, Sonenshi.De et al. 1974, Sonenshine et al. 1976, Sonenshine, Silverstein & Homsher 1979). All tick stages may re-attach when their feeding is interrupted before they have taken enough blood meal to develop to the next stage (Wang, Henbest & Nuttall 1999). This is common following grooming or death of the host (Wang & Nuttall 2001).

1.7.9 Moulting

Following feeding to repletion of immature stages (larvae and nymphs), the engorged tick falls to the ground and because of their negative phototaxis and positive thigmotaxis, seek a sheltered habitat, such as leaves, soil and sand to moult. During moulting ticks metamorphose to the next stage. This process includes increase in body size and the generation of a new exoskeletone (endocuticle) while the old one is separated (apolysis) and lost (ecdysis) (Oliver&Dotson 1993, Sonenshine 1991). Internally, the epidermis underlining the endocuticle increases its mitotic activity as it secretes the new endocuticle. During moulting, ticks release enzymes that lead to histolysis of some internal organs such as the salivary glands (Balashov, Raikhel & Hoogstraal 1983). Organs that survive histolysis normally have increased activity. For example, in the tracheal system, there is increased epithelial cell division leading to replacement of cuticular intima. The haemocytes transform into cellular

and acellular connective tissues. Other organs that survive histolysis include the midgut, synganglion, the reproductive organs, the malpighian tubules and fat body (Balashov 1972, Till 1961). Moulting can only take place when a tick has fed a certain minimum amount of blood, approximately 30-35% of a normal complete blood meal (Balashov 1972). The size of the tick attained in the next stage is directly proportional to the amount of blood taken in the preceding stage (Balashov 1972, Sonenshine 1991). Events leading to moulting are controlled by moulting hormones, the ecdysteroids (Oliver & Dotson 1993). Temperature is another important factor for moulting. Moulting is delayed as temperatures are reduced and ceases at temperatures drop (Balashov 1972, Sonenshine 1991). Moulting failed at 13°C for *Heamaphysalis longicornis* (Yano, Shiraishi & Uchida 1987).

1.7.10 Oviposition

Mating in most ixodid ticks with exception of *Ixodes* spp. occurs on the host once feeding has commenced while feeding to engorgement follows mating (Balashov 1972, Leahy et al. 1983, Oliver 1986, Sonenshine 1991). The replete female drops to the ground and seeks shelter as described for moulting. Ixodid ticks undergo a single gonotrophic cycle, laying between 2000 and 20,000 eggs, depending on the species and the amount of blood taken. After laying eggs the female tick dies. The pre-oviposition period, i.e. period between engorgement and oviposition varies upon species and is also affected by diapause and temperature (Sonenshine 1991). Periods for pre-oviposition, ovipositing and hatching are increased as the temperature is lowered and ceases all together below certain temperatures e.g below 15°C for *H. longicornis* (Yano, Shiraishi & Uchida 1987). The number of eggs that hatch is also reduced while mortality rates increase at lower temperatures (Adejinmi & Akinboade 2011, Despins 1992, Yano, Shiraishi & Uchida 1987).

1.7.11 Mechanical persistence and transmission of viruses by ticks

This method of transmission occurs where there is no virus replication (biological) in the vector and happens when ticks are transferred between hosts following interrupted feeding. The pathogen may contaminate the mouth-parts and saliva along the pre-oral canal. In ticks, regurgitation of blood from the midgut has been reported and may also contribute to mechanical transmission (Brown 1988, Connat 1991, Gregson 1967).

1.7.12 Biological (intrastadial, transstadial and transovarial) passage of infection in ticks

Some pathogens in the blood meal survive the hostile "midgut barrier" and infect the tick cells. The pathogen undergoes a replication cycle within the tick. Some studies have shown viruses to cross the gut barrier and pass through the haemolymph to the ovaries, testes, synganglion and the salivary glands (Booth et al. 1989, Booth et al. 1991a, Booth et al. 1991b, Bouwknegt et al. 2010, Kaufman & Nuttall 2003).

Intrastadial passage of pathogens occurs when a pathogen reaches the salivary glands within the same tick development stage and the virus may be transmitted to another host following interrupted feeding (Andrew&Norval 1989, Wang & Nuttall 2001).

Under normal circumstances, a tick life cycle stage such as larvae, nymph and adult (female) completes its blood meal on one host (Cupp 1991, Norval 1974, Sonenshine 1991). In the case of larvae and nymphs, they moult to the next stage of nymph and adult, respectively. During moulting some tick tissues, such as the endocuticle, the salivary glands and cuticular intima of the tracheae undergo histiolysis (Balashov 1972, Sonenshine 1991). Viruses that infect tick tissues that survive histiolysis during moulting such as the fat body, synganglion, epithelial tissues and reproductive organs are able to persist transstadially (transstadial passage) in these tick organs and tissues while haemocytes and tick motile cells are important for dissemination of infection within the tick (Booth et al. 1989, Booth et al. 1991b, Jones et al. 1989). Infection extends to the salivary glands following their regeneration. Thogoto virus was reported to reach the salivary glands mechanically, i.e. without infection of the salivary gland epithelium, following intracoelomic infection (Kaufman & Nuttall 1996). Transstadial transmission may be effected as the tick takes the next blood meal following persistence of the virus from the previous stage (Bremer et al. 2005, Stich et al. 1989, Waal & Potgieter 1987).

In females, when the ovaries are infected, there may be transovarial passage of the pathogen to the eggs. Through the eggs infection may be passed on to the larvae. Transovarial transmission of a pathogen may be effected when larvae have their blood meal following persistence of the pathogen from the infected females of the previous generation (Connell & Hall 1972, Robinson 1982, Stich et al. 1989). Infection of sperm cells in males may also lead to sexual transmission to the female partner. This can further lead to infection of the oocytes and subsequent transovarial passage and transmission (Gonzalez et al. 1992).

1.7.13 Tick vector potential and competence

The first step in the vector competence of a tick relies on the ability of the pathogen to survive the tick midgut environment and cross to other organs (Booth et al. 1989, Jones et al. 1989, Nuttall et al. 1994, Zaher, Soliman & Diab 1977). During transstadial passage, the vector competence further depends on the tissue tropism of a pathogen to cell-lines that survive histiolysis during moulting such as synganglion, haemocytes, epithelial tissues and reproductive organs (Kaufman & Nuttall 2003, Nuttall et al. 1994). Vector capacity for transovarial transmission relies on the passage of infection to the eggs from the ovaries or through sexual transmission from the spermatids. The pathogen must persist up to the larval stage of the next generation (Dantas-Torres et al. 2010). It has not been determined if there is any barrier to transovarial passage in ticks. The passage of viruses to salivary glands may occur without necessarily infecting the salivary glands (Kaufman & Nuttall 1996).

1.8 Tick species used in this study

Rhipicephalus appendiculatus, often referred to as the brown ear tick is a three host tick infesting mainly ruminants such as cattle, goats, buffaloes and antelopes (Bryson et al. 2002a, Bryson et al. 2002b, Walker 2003). Adult ticks prefer to attach on the ears, around the eyes, the upper neck, the tail brush and around the anus. The immature stages attach on the ears, eyelids, muzzle, cheeks, the neck and ears (Sonenshine 1991, Walker 1970). It takes four to seven days for each parasitic stage to engorge. Engorged females lay between 3000 and 5000 eggs each, which hatch between 20 and 90 days (Walker 2003). *Rhipicephalus appendiculatus* has a single seasonal generation in southern Africa, where adults occur in the rain season (December to March), larvae in the late summer to winter and nymphs in the winter to early summer (Bryson et al. 2002b, Short & Norval 1981). The unfed adults regulate the pattern of seasonal occurrence through a behavioural diapause (Madder & Berkvens 1997, Madder et al. 1999). *Rhipicephalus appendiculatus* is the vector for *Theileria parva*, the agent for East Coast Fever, by transstadial transmission (Ochanda et al. 1998, Walker 2003) and also transmits viruses such as Nairobi sheep disease, thogoto virus and tick-borne encephalitis (Daubney & Hudson 1931, Davies & Mwakima 1982, Kaufman & Nuttall 2003, Labuda et al. 1993b). Although *R. appendiculatus* transmits pathogens mainly via transstadial transmission, transovarial transmission was reported in the transmission of Nairobi sheep disease (Daubney & Hudson 1931).

Amblyomma hebraeum, also known as the South African bont tick, is a three host tick and vector for *Ehrlichia ruminantium*, the cause of heartwater, Nairobi sheep disease and Q-fever (Norval 1977, Norval et al. 1997). Its hosts include cattle, goats, sheep and large wild angulates such buffaloes, giraffes, elands and rhinoceroses. Larvae find their hosts by questing while nymphs and adults are hunters. The larvae and nymphs take 7 to 14 days to engorge. Males that have been feeding for 4 to 6 days secrete pheromones, the aggregation and attachment pheromones (AAPs) which attract both the unfed adults and nymphs to attach. Females only attach in response to AAPs (Norval, Andrew & Yunker 1989, Norval et al. 1992, Norval et al. 1996) and after mating, they engorge within 7 to 9 days. The life cycle is also synchronised, with adults active during summer, the larvae during the late summer to autumn and nymphs during winter to early spring (Horak, Gallivan & Spickett 2011, Schroder, Uys & Reilly 2006, Walker 2003).

Rhipicephalus (Boophilus) decoloratus belongs to sub genus *Boophilus* which was formerly classified as a full genus, *Boophilus* (Guglielmone et al. 2009). It is commonly referred to as the blue tick because of the colour of the engorged ticks (Walker 2003, Walker 1994, Walker 1970). *Rhipicephalus (B) decoloratus* is a one-host tick. Cattle are the main hosts for *R. (B) decoloratus* although it may infest horses, donkeys, sheep, goats and wild angulates (Bryson et al. 2002a, Bryson et al. 2002b, Walker 2003). Larvae find their hosts by questing. After attachment, larvae take seven days to feed, engorge and moult to nymphs. Nymphs re-attach on the same host, feed, engorge and moult to adults after approximately seven days. After re-attachment, adults partially feed and mate, the females fully engorge and drop to the ground after .approximately a week. The whole period spent on a host is approximately three to four weeks and the entire life cycle including none parasitic stages, spent off the host, can be completed in two months (Walker 2003).

Rhipicephalus (Boophilus) decoloratus can complete several cycles in a year. In southern Africa, the life cycle is synchronised by over-wintering of eggs and hatching occurs as temperatures rise/increase after spring (Bryson et al. 2002b, Horak, Gallivan & Spickett 2011, Walker 2003). *Rhipicephalus (B) decoloratus* transmits pathogens mainly by transovarial passage (Sonenshine 1991, Walker 2003). It commonly transmits protozoan parasites such as Babesia species as well as Ricketssial organisms such as Anaplasma species (Heyne 1986, Meltzer, Norval & Donachie 1995, Tonnesen et al. 2006). Dugbe and Thogoto viruses were isolated from *R. (B) decoloratus* in Cameroon and the Central African Republic (Surea 1976).

1.9 Laboratory diagnosis of lumpy skin disease

Diagnosis is normally done by demonstrating the presence or effect of the presence of LSDV in specific host systems. These include:

1.9.1 Virus isolation

Lumpy skin disease virus can be isolated using a variety of cell cultures. The virus grows on primary lamb and calf adrenals, calf kidney and thyroid cells, chick embryo fibroblasts, foetal lamb and calf muscle cells, chicken embryo fibroblasts, adult velvet monkey kidney cells and lamb testis cells (Davies 1976). Bovine dermis cells have been used successfully to grow LSDV from blood, semen and other tissues (Bagla et al. 2006, Irons, Tuppurainen & Venter 2005, Tuppurainen, Venter & Coetzer 2005). Since capripoxviruses grow slowly in tissue culture, they may require several passages (Alexander 1957). Presence of the virus in cell cultures is demonstrated by cytopathic changes and intra-cytoplasmic inclusion bodies (Weiss 1968). Contamination of cell culture samples by bacteria, viruses and fungi is frequently encountered and could negatively affect growth of cell cultures (Castro et al. 1997, Hirayama, Minamoto & Kurata 1981).

1.9.2 Electron microscopy

Electron microscopy is used to identify the virus by its morphology (Gardner 1977, Kitching & Smale 1986, Solisch 1978). It's technically simple and provides a rapid method for detecting viruses in tissue samples (Gardner 1977). Negative contrast staining or positive staining of thin sections as well as immuno staining can be used (Gardner 1977, Hyatt 1989, Solisch 1978). The limit of detection for negative staining is 10^6 virus particles per ml and between 10^4 and 10^5 for ultra-thin sections (Laue & Bannert 2010). Advantages of electron microscopy include the high magnification of objects which allow visualising very small objects such as viruses, there is a wide range of application which provide high quality and detailed images. The major disadvantage is the high procurement and maintenance costs. Other disadvantages include the need for highly trained personnel to manage the facility and low sensitivity of this test (Gardner 1977, Solisch 1978).

1.9.3 Nucleic acid amplification

The polymerase chain reaction (PCR), has found wide use in veterinary diagnostics including the diagnosis of capripoxviruses (Awad et al. 2010, Belák & Ballagi-Pordány 1993, Ireland & Binepal 1998). Two real-time PCR assays utilising hydrolysis probes have been developed

and can be used to detect, without discriminating, capripoxviruses (Babiuk et al. 2008b, Bowden et al. 2008). A real-time PCR assay which discriminates the species of capripoxviruses has also been developed (Lamien et al. 2011). Real-time PCR is highly sensitive, i.e. can detect from as little as 10 DNA templates per reaction (Bowden et al. 2008) and combines the high sensitivity with high rapidity (Balinsky et al. 2008). Real time PCR allows the results to be read during the exponential phase of the reaction and does not require post reaction handling (Lamien et al. 2011). The real-time PCR developed by Bowden et al (2008) was used in this study

1.9.4 Serology

2.10.4.1. Serum viral neutralization

This assay is considered the gold standard sero-diagnostic method for detecting neutralising antibodies to LSDV (Babiuk et al. 2008a, OIE 2010). This method has been used as a screening test to confirm infection with LSDV (Chihota et al. 2001, Davies 1982, House et al. 1990, Tuppurainen, Venter & Coetzer 2005) and to carry out serological surveys on LSD (Gari et al. 2012, Hedger & Hamblin 1983). However, the test cannot differentiate between GPV, SPV and LSDV (Kitching & Taylor 1985a, Kitching, Hammond & Black 1986, World Organisation for Animal Health 2009, World Organisation for Animal Health 2012). Because immunity to LSDV is mostly cell-mediated, the sensitivity of this test is low and, therefore, cannot detect the circulating antibodies in animals where viraemia is very low (Chihota et al. 2001, OIE 2010). This usage of the test is also limited by the requirement of live virus which may not be available in countries where there disease does not exist (Bowden et al. 2009).

An enzyme linked immune-sorbent assay (ELISA) was developed based on the envelope protein P32 expressed in *Escherichia coli* (Carn et al. 1994, Heine et al. 1999) but was limited by the instability of the recombinant gene (Babiuk et al. 2009). An indirect ELISA has also been developed using inactivated sucrose gradient purified SPV as a coating antigen (Babiuk et al. 2009) but this method has a disadvantage of being too costly to produce in bulk (Bowden et al. 2009)**.** An indirect ELISA was developed using the virion core proteins ORF 095 and ORF 103, which were able to detect the capripoxvirus antibodies in both acute and convalescent phase sera (Bowden et al. 2009).

Other serological methods include an indirect immunofluorescent antibody test (IFAT) and an agar gel immune diffusion (World Organisation for Animal Health 2012). Serological tests

have been used singly or in combination with other techniques in the diagnosis of LSD (Awad et al. 2010, Barnard 1997, Bowden et al. 2009, Heine et al. 1999, Khadr, Aboul Soud & Khaliel 1999).

2.10.4.2. Histopathology and immunohistochemistry

Clinical histopathology has been used to determine changes in cells caused by LSDV using hematoxyline and eosin staining (Awad et al. 2010, Body et al. 2012, Prozesky & Barnard 1982) while immunohistochemistry confirms infection by demonstrating the viral antigen in the tissues (Annandale et al. 2010, Awadin et al. 2011). A monoclonal antibody was developed from the LSDV core protein (ORF 057) expressed in *E. coli* (Babiuk et al. 2008b). This assay demonstrates the pathogen's antigen in fixed tissues and is useful for studying the pathogenesis of the diseases and can be used as an additional diagnostic tool (Awad et al. 2010). Immuno histochemical method was used successfully in LSDV diagnosis during an outbreak in Egypt (Awadin et al. 2011) and to confirm the presence of LSDV in testes of experimentally infected bulls (Annandale et al. 2010). It was also used to demonstrate the presence of the virus in macrophages and hair follicle epithelium (Babiuk et al. 2008b). Immunohistochemistry has also been used in diagnosis of SPV (Gulbahar et al. 2000, Gulbahar et al. 2006) and to detect host antibodies in tick organs (Hlatshwayo et al. 2004, Kavitha et al. 2011).

1.10 Objectives of the study

The study had the following objectives:

To demonstrate transmission of LSDV to cattle by *A. hebraeum* and *R. appendiculatus* following interrupted feeding and transstadial development.

To demonstrate mechanical/ intrastadial and transstadial passage of LSDV by detecting the virus in tick saliva of *A. hebraeum* and *R. appendiculatus* adults.

To demonstrate tick organs infected by LSDV following interrupted feeding or transstadial persistence in *A. hebraeum* and *R. appendiculatus*.

To demonstrate transovarial passage of LSDV by *A. hebraeum*, *R. appendiculatus* and *R. (B) decoloratus* by detecting LSDV in larvae from eggs laid by females that fed on infected cattle.

To investigate over-wintering of LSDV in *A. hebraeum* during transstadial development and in *R. (B) decoloratus* during transovarial development.

1.11. References

Adejinmi, J.O. & Akinboade, O.A., 2011, 'Effect of temperature on the oviposition capacity of engorged adult females and hatchability of eggs of dog ticks: *Rhipicephalus sanguineus* and *Heamaphysalis leachi leachi* (Acari: Ixodidae).', *African Journal of Biomedical Research* 14(1), 35-42.

Ahmed, L.A., El-Desawy, O. & Mohamed, A.T., 2005, 'Studies on bovine field skin lesions in Fayoum Governorate', *Veterinary Medical Journal Giza* 53(1), 73-81.

Aiel, K., 2009, 'Lumpy skin disease', *Bovine & Ovine* (85), 22-24, 67.

Alexander, R.A., 1957, 'Cytopathogenic agents associated with lumpy-skin disease of cattle', *Bulletin of epizootic diseases of Africa. Bulletin des epizooties en Afrique* 5 489-492.

Ali, A.A., Esmat, M., Attia, H., Selim, A. & Abdel-Hamid, Y., 1990, 'Clinical and pathological studies of lumpy skin disease in Egypt', *Veterinary Record* 127(22), 549-550.

Ali, B.H. & Obeid, H.M., 1977, 'Investigation of the first outbreaks of lumpy skin disease in the Sudan', *British Veterinary Journal* 133(2), 184-189.

Andrew, H.R. & Norval, R.A.I., 1989, 'The role of males of the bont tick (*Amblyomma hebraeum*) in the transmission of *Cowdria ruminantium* (heartwater)', *Veterinary parasitology* 34(1-2), 15-23.

Annandale C.H, Holm D.E, Ebersohn K & Venter E.H, 2013, 'Seminal transfer of lumpy skin disease virus in heifers', *Transbound Emerg.Dis.* .

Annandale, C.H., Irons, P.C., Bagla, V.P., Osuagwuh, U.I. & Venter, E.H., 2010, 'Sites of persistence of lumpy skin disease virus in the genital tract of experimentally infected bulls', *Reproduction in Domestic Animals* 45(2), 250-255.

Awad, W.S., Ibrahim, A.K., Mahran, K., Fararh, K.M. & Abdel Moniem, M.I., 2010, 'Evaluation of different diagnostic methods for diagnosis of Lumpy skin disease in cows', *Tropical animal health and production* 42(4), 777-783.

Awadin, W., Hussein, H., Elseady, Y., Babiuk, S. & Furuoka, H., 2011, 'Detection of lumpy skin disease virus antigen and genomic DNA in formalin-fixed paraffin-embedded tissues from an Egyptian outbreak in 2006.', *Transboundary and Emerging Diseases* 58(5), 451-457.

Babiuk, S., Bowden, T.R., Boyle, D.B., Wallace, D.B. & Kitching, R.P., 2008a, 'Capripoxviruses: an emerging worldwide threat to sheep, goats and cattle', *Transboundary and Emerging Diseases* 55(7), 263-272.

Babiuk, S., Bowden, T.R., Parkyn, G., Dalman, B., Manning, L., Neufeld, J., Embury-Hyatt, C., Copps, J. & Boyle, D.B., 2008b, 'Quantification of lumpy skin disease virus

following experimental infection in cattle', *Transboundary and Emerging Diseases* 55(7), 299-307.

Babiuk, S., Wallace, D.B., Smith, S.J., Bowden, T.R., Dalman, B., Parkyn, G., Copps, J. & Boyle, D.B., 2009, 'Detection of antibodies against capripoxviruses using an inactivated sheeppox virus ELISA', *Transboundary and emerging diseases* 56(4), 132-141.

Bagla, V.P., Osuagwuh, U.I., Annandale, C.H., Irons, P.C. & Venter, E.H., 2006, 'Elimination of toxicity and enhanced detection of lumpy skin disease virus on cell culture from experimentally infected bovine semen samples', *The Onderstepoort journal of veterinary research* 73(4), 263-268.

Balashov, Y., S., Raikhel, A.S. & Hoogstraal, H., 1983, *An atlas of ixodid tick ultrastructure.* Entomological Society of America, College Park, MaryLand; USA.

Balashov, Y.S., 1972, *Blood-sucking ticks (Ixodoidea)-vectors of diseases of man and animals.* Entomological Society of America.

Balinsky, C.A., Delhon, G., Smoliga, G., Prarat, M., French, R.A., Geary, S.J., Rock, D.L. & Rodriguez, L.L., 2008, 'Rapid preclinical detection of sheeppox virus by a real-time PCR assay', *Journal of clinical microbiology* 46(2), 438-442.

Banadyga, L., Gerig, J., Stewart, T. & Barr, M., 2007, 'Fowlpox virus encodes a Bcl-2 homologue that protects cells from apoptotic death through interaction with the proapoptotic protein Bak', *Journal of virology* 81(20), 11032-11045.

Banadyga, L., Veugelers, K., Campbell, S. & Barry, M., 2009, 'The fowlpox virus BCL-2 homologue, FPV039, interacts with activated bax and a discrete subset of BH3-only proteins to inhibit apoptosis', *Journal of virology* 83(14), 7085-7098.

Barnard, B., 1997, 'Antibodies against some viruses of domestic animals in southern African wild animals', *Onderstepoort Journal of Veterinary Research* 64(2), 95-110.

Belák, S. & Ballagi-Pordány, A., 1993, 'Application of the polymerase chain reaction (PCR) in veterinary diagnostic virology', *Veterinary research communications* 17(1), 55-72.

Belozerov, V.N., 2008, 'Diapause and quiescence as two main kinds of dormancy and their significance in life cycles of mites and ticks (Chelicerata: Arachnida: Acari). Part 1. Acariformes.', *Acarina* 16(2), 79-130.

Benavides Ortiz, E. & Walker, A.R., 1992, 'Natural secretion of saliva by the tick *Rhipicephalus appendiculatus* (Neumann), fed artificially through capillary tubes', *Revista Colombiana de Entomología* 18(1), 1-7.

Berkvens, D.L., Pegram, R.G. & Brandt, J.R.A., 1995, 'A study of the diapausing behaviour of *Rhipicephalus appendiculatus* and *R. zambeziensis* under quasi-natural conditions in Zambia.', *Medical and veterinary entomology* 9(3), 307-315.

Body, M., Singh, K.P., Hussain, M.H., Al-Rawahi, A., Al-Maawali, M., Al-Lamki, K. & Al-Habsy, S., 2012, 'Clinico-histopathological findings and PCR based diagnosis of lumpy skin disease in the Sultanate of Oman.', *Pakistan Veterinary Journal* 32(2), 206-210.

Booth, T.F., Davies, C.R., Jones, L.D., Staunton, D. & Nuttall, P.A., 1989, 'Anatomical basis of Thogoto virus infection in BHK cell culture and in the ixodid tick vector, *Rhipicephalus appendiculatus*.', *Journal of General Virology* 70(5), 1093-1104.

Booth, T.F., Marriott, A.C., Steele, G.M. & Nuttall, P.A., 1991a, 'Dugbe virus in ticks: histological localization studies using light and electron microscopy', *Archives of Virology, Supplementum* (1), 207-218.

Booth, T.F., Steele, G.M., Marriott, A.C. & Nuttall, P.A., 1991b, 'Dissemination, replication, and trans-stadial persistence of Dugbe virus (Nairovirus, Bunyaviridae) in the tick vector *Amblyomma variegatum*', *American Journal of Tropical Medicine and Hygiene* 45(1), 146-157.

Bourdin, P., 1971, *[Lumpy skin disease of cattle]*, , L'Expansion Scientifique Francaise, Paris.

Bouwknegt, C., Rijn, P.A., Schipper, J.J.M., Hölzel, D., Boonstra, J., Nijhof, A.M., Rooij, E.M.A. & Jongejan, F., 2010, 'Potential role of ticks as vectors of bluetongue virus', *Experimental and Applied Acarology* 52(2), 183-192.

Bowden, T.R., Babiuk, S.L., Parkyn, G.R., Copps, J.S. & Boyle, D.B., 2008, 'Capripoxvirus tissue tropism and shedding: a quantitative study in experimentally infected sheep and goats', *Virology* 371(2), 380-393.

Bowden, T.R., Coupar, B.E., Babiuk, S.L., White, J.R., Boyd, V., Duch, C.J., Shiell, B.J., Ueda, N., Parkyn, G.R., Copps, J.S. & Boyle, D.B., 2009, 'Detection of antibodies specific for sheeppox and goatpox viruses using recombinant capripoxvirus antigens in an indirect enzyme-linked immunosorbent assay', *Journal of virological methods* 161(1), 19-29.

Bowman, A.S. & Sauer, J.R., 2004, 'Tick salivary glands: function, physiology and future.', *Parasitology* 129(Supplement), S67-S81.

Bremer, W.G., Schaefer, J.J., Wagner, E.R., Ewing, S.A., Rikihisa, Y., Needham, G.R., Jittapalapong, S., Moore, D.L. & Stich, R.W., 2005, 'Transstadial and intrastadial experimental transmission of *Ehrlichia canis* by male *Rhipicephalus sanguineus*.', *Veterinary parasitology* 131(1/2), 95-105.

Brenner, J., Haimovitz, M., Oren, E., Stram, Y., Fridgut, O., Bumbarov, V., Kuznetzova, L., Oved, Z., Waserman, A., Garazzi, S., Perl, S., Lahav, D., Edery, N. & Yadin, H., 2006, 'Lumpy skin disease (LSD) in a large dairy herd in Israel, June 2006', *Israel Journal of Veterinary Medicine* 61(3), 73-77.

Brinton, L.P., Oliver, J.H. & Jr., 1971, 'Gross anatomical, histological, and cytological aspects of ovarian development in *Dermacentor andersoni* Stiles (Acari : Ixodidae).', *Journal of Parasitology* 57(4), 708-719.

Brossard, M. & Wikel, S.K., 2004, 'Tick immunobiology.', *Parasitology* 129(Supplement), S161-S176.

Brossard, M. & Wikel, S.K., 1997, 'Immunology of interactions between ticks and hosts.', *Medical and veterinary entomology* 11(3), 270-276.

Brown, S.J., 1988, 'Evidence for regurgitation by Amblyomma americanum.', *Veterinary parasitology* 28(4), 335-342.

Bruce, D. & Wilson, A., 1998, *A trial to control or eradicate Amblyomma hebraeum ticks and heartwater on three ranches in Zimbabwe, .*

Bryson, N.R., Tice, G.A., Horak, I.G., Stewart, C.G. & Plessis, B.J.A., 2002a, 'Ixodid ticks on indigenous goats owned by small-scale farmers in four communal grazing areas in South Africa', *Journal of the South African Veterinary Association* 73(1), 26-30.

Bryson, N.R., Tice, G.A., Horak, I.G., Stewart, C.G. & Plessis, B.J.A.d., 2002b, 'Ixodid ticks on cattle belonging to small-scale farmers at 4 communal grazing areas in South Africa.', *Journal of the South African Veterinary Association* 73(3), 98-103.

Burdin, M.L. & Prydie, J., 1959, 'Observations on the first outbreak of lumpy skin disease in Kenya', *Bulletin of epizootic diseases of Africa. Bulletin des epizooties en Afrique* 7 21-26.

Capstick, P.B., Prydie, J., Coackley, W. & Burdin, M.L., 1959, 'Protection of cattle against the "Neethling" type virus of lumpy skin disease', *Veterinary Record* 71 422-423.

Carn, V.M. & Kitching, R.P., 1995a, 'The clinical response of cattle experimentally infected with lumpy skin disease (Neethling) virus', *Archives of Virology* 140(3), 503-513.

Carn, V.M. & Kitching, R.P., 1995b, 'An investigation of possible routes of transmission of lumpy skin disease virus (Neethling)', *Epidemiology and infection* 114(1), 219-226.

Carn, V.M., Kitching, R.P., Hammond, J.M. & Chand, P., 1994, 'Use of a recombinant antigen in an indirect ELISA for detecting bovine antibody to capripoxvirus', *Journal of virological methods* 49(3), 285-294.

Castro, M.D., Stoffregen, W.C., Brigman, G.P. & Hillard, K.A., 1997, 'A method to detect bovine viral diarrhea virus contamination in cell cultures using immunoperoxidase staining', *Journal of Veterinary Diagnostic Investigation* 9(4), 427-431.

Catroxo, M.H.B., Pongiluppi, T., Melo, N.A., Milanelo, L., Petrella, S., Martins, A.M.C.P.F. & Reboucas, M.M., 2009, 'Identification of Poxvirus Under Transmission Electron Microscopy During Outbreak Period in Wild Birds, in Sao Paulo, Brazil', *International Journal of Morphology* 27(2), 577-585.

Chen, Z., Li, Y., Liu, Z., Yang, J. & Yin, H., 2012, 'The life cycle of Hyalomma rufipes (Acari: Ixodidae) under laboratory conditions', *Experimental and Applied Acarology* 56(1), 85-92.

Chihota, C.M., Rennie, L.F., Kitching, R.P. & Mellor, P.S., 2003, 'Attempted mechanical transmission of lumpy skin disease virus by biting insects', *Medical and veterinary entomology* 17(3), 294-300.

Chihota, C.M., Rennie, L.F., Kitching, R.P. & Mellor, P.S., 2001, 'Mechanical transmission of lumpy skin disease virus by *Aedes aegypti* (Diptera: Culicidae)', *Epidemiology and infection* 126(2), 317-321.

Chinery, W.A., 1981, 'Observation on the saliva and salivary gland extract of Haemaphysalis spinigera and Rhipicephalus sanguineus sanguineus.', *Journal of Parasitology* 67(1), 15-19.

Coetzer, J.A.W., 2004, 'Lumpy skin disease' in pp. 1268-1276, Oxford University Press, Oxford.

Connat, J.L., 1991, 'Demonstration of regurgitation of gut content during blood meals of the tick *Ornithodoros moubata*. Possible role in the transmission of pathogenic agents.', *Parasitology research* 77(5), 452-454.

Connell, M. & Hall, W.T.K., 1972, 'Transmission of *Anaplasma marginale* by the cattle tick *Boophilus microplus*', *Australian Veterinary Journal* 48(8), 477.

Coons, L.B., L'Amoreaux, W.J., Rosell-Davis, R. & Starr-Spires, L., 1990, 'Fine structure of the fat body and nephrocytes in the life-stages of *Dermacentor variabilis*.', *Experimental & applied acarology* 8(1,2), 125-142.

Coons, L.B., Rosell-Davis, R., Tarnowski, B.I. & Davis, R.R., 1986, 'Bloodmeal digestion in ticks.', *Morphology, physiology, and behavioral biology of ticks* 248-279.

Crooks, E. & Randolph, S.E., 2006, 'Walking by Ixodes ricinus ticks: intrinsic and extrinsic factors determine the attraction of moisture or host odour', *Journal of Experimental Biology* 209(11), 2138-2142.

Cupp, E.W., 1991, 'Biology of ticks', *Veterinary Clinics of North America, Small Animal Practice* 21(1), 1-26.

Dantas-Torres, F., Giannelli, A. & Otranto, D., 2012, 'Starvation and overwinter do not affect the reproductive fitness of *Rhipicephalus sanguineus*.', *Veterinary parasitology* 185(2/4), 260-264.

Dantas-Torres, F., Martins, T.F., de Paiva-Cavalcanti, M., Figueredo, L.A., Lima, B.S. & Brandao-Filho, S.P., 2010, 'Transovarial passage of *Leishmania infantum* kDNA in artificially infected *Rhipicephalus sanguineus*', *Experimental parasitology* 125(2), 184-185.

Daubney, R. & Hudson, J.R., 1931, 'Nairobi Sheep Disease.', *Parasitology* 23(4), 507-524.

Davies, F.G., 1982, 'Observations on the epidemiology of lumpy skin disease in Kenya', *The Journal of hygiene* 88(1), 95-102.

Davies, F.G., 1976, 'Characteristics of a virus causing a pox disease in sheep and goats in Kenya, with observations on the epidemiology and control', *Journal of Hygiene* 76(2), 163-171.

Davies, F.G., Krauss, H., Lund, J. & Taylor, M., 1971, 'The laboratory diagnosis of lumpy skin disease', *Research in veterinary science* 12 123-127.

Davies, F.G. & Mwakima, F., 1982, 'Qualitative studies of the transmission of Nairobi sheep disease virus by *Rhipicephalus appendiculatus* (Ixodoidea, Ixodidae).', *Journal of comparative pathology* 92(1), 15-20.

Despins, J.L., 1992, 'Effects of Temperature and Humidity on Ovipositional Biology and Egg Development of the Tropical Horse Tick, *Dermacentor (Anocentor) Nitens*', *Journal of medical entomology* 29(2), 332-337.

Donze, G., McMahon, C. & Guerin, P.M., 2004, 'Rumen metabolites serve ticks to exploit large mammals', *Journal of Experimental Biology* 207(24), 4283-4289.

El-Shoura, S.M., 1989, 'Fine structure of the fat body in the female tick *Ornithodoros (Pavloskyella) erraticus* (Ixodoidea: Argasidae).', *Experimental & applied acarology* 7(3), 245-249.

Fenner, F.J. & White, O.D., 1976, *Medical Virology*, second edn., Academic Press, A subsidiary of Harcourt Brace Jovanovich, Publishers, New York.

Fenner, F., 1987, *Veterinary virology*, Academic Press, Orlando.

Garcia-Fernandez, C., Garcia, S.M.L.d., Garcia, R.N. & de Garcia, S.M.L., 1998, 'The male genital accessory gland complex of the cattle tick *Boophilus microplus* (Canestrini, 1887) (Acari: Ixodidae).', *Revista brasileira de biologia* 58(3), 453-462.

Garcia-Fernandez, C., Garcia, S.M.L.d., Garcia, R.N. & Valente, V.L.d.S., 2005, 'New histochemical and morphological findings in the female genital tract of *Boophilus microplus* (Acari, Ixodidae): an attempt toward the elucidation of fertilization in ticks.', *Iheringia, Serie Zoologia* 95(3), 295-303.

Gardner, P.S., 1977, 'Rapid virus diagnosis (review article)', *Journal of General Virology* 36(1), 1-29.

Gari, G., Bonnet, P., Roger, F. & Waret-Szkuta, A., 2011, 'Epidemiological aspects and financial impact of lumpy skin disease in Ethiopia.', *Preventive veterinary medicine* 102(4), 274-283.

Gari, G., Grosbois, V., Waret-Szkuta, A., Babiuk, S., Jacquiet, P. & Roger, F., 2012, 'Lumpy skin disease in Ethiopia: seroprevalence study across different agro-climate zones.', *Acta Tropica* 123(2), 101-106.

Gonzalez, J.P., Camicas, J.L., Cornet, J.P., Faye, O. & Wilson, M.L., 1992, 'Sexual and Transovarian Transmission of Crimean-Congo Hemorrhagic-Fever Virus in *Hyalomma-truncatum* Ticks', *Research in virology* 143(1), 23-28.

Green, H.F., 1960, 'Some hide and skin curiosities.', *Journal of the International Society of Leather Trades Chemists* 44 83-88.

Green, H.F., 1959, 'Lumpy skin disease-its effect on hides and leather and a comparison in this respect with some other skin diseases.', *Bull.Epizoot.Dis.Afr.* 7 63-94.

Gregson, J.D., 1967, 'Observations on Movement of Fluids in Vicinity of Mouthparts of Naturally Feeding Dermacentor Andersoni Stiles', *Parasitology* 57 1-&.

Gregson, J.D., 1960, 'Morphology and functioning of the mouthparts of *Dermacentor andersoni* Stiles.', *Acta Tropica* 17 48-79.

Greth, A., Calvez, D., Vassart, M. & Lefèvre, P.C., 1992a, 'Serological survey for bovine bacterial and viral pathogens in captive Arabian oryx (*Oryx leucoryx* Pallas, 1776)', *Revue Scientifique et Technique - Office International des Epizooties* 11(4), 1163-1168.

Greth, A., Gourreau, J.M., Vassart, M., Nguyen-Ba-Vy., Wyers, M. & Lefevre, P.C., 1992b, 'Capripoxvirus disease in an Arabian oryx (*Oryx leucoryx*) from Saudi Arabia', *Journal of wildlife diseases* 28(2), 295-300.

Guglielmone, A.A., Robbins, R.G., Apanaskevich, D.A., Petney, T.N., Estrada-Peña, A. & Horak, I.G., 2009, 'Comments on controversial tick (Acari: Ixodida) species names and species described or resurrected from 2003 to 2008', *Experimental and Applied Acarology* 48(4), 311-327.

Gulbahar, M.Y., Cabalar, M., Gul, Y. & Icen, H., 2000, 'Immunohistochemical detection of antigen in lamb tissues naturally infected with sheeppox virus', *Journal of veterinary medicine.B, Infectious diseases and veterinary public health* 47(3), 173-181.

Gulbahar, M.Y., Davis, W.C., Yuksel, H. & Cabalar, M., 2006, 'Immunohistochemical evaluation of inflammatory infiltrate in the skin and lung of lambs naturally infected with sheeppox virus', *Veterinary pathology* 43(1), 67-75.

Haig, D.A., 1957, 'Lumpy skin disease', *Bulletin of epizootic diseases of Africa. Bulletin des epizooties en Afrique* 5 421-430.

Hedger, R.S. & Hamblin, C., 1983, 'Neutralising antibodies to lumpy skin disease virus in African wildlife', *Comparative immunology, microbiology and infectious diseases* 6(3), 209-213.

Heine, H.G., Stevens, M.P., Foord, A.J. & Boyle, D.B., 1999, 'A capripoxvirus detection PCR and antibody ELISA based on the major antigen P32, the homolog of the vaccinia virus H3L gene', *Journal of immunological methods* 227(1-2), 187-196.

Heyne, H., 1986, 'Differentiation of *Boophilus decoloratus* and *Boophilus microplus*', *Journal of the South African Veterinary Association* 57(4), 251-252.

Hirayama, N., Minamoto, N. & Kurata, K., 1981, 'Effect of Mycoplasma on growth of canine distemper virus in Vero cells', *Japanese Journal of Veterinary Science* 43(5), 637-643.

Hlatshwayo, M., Szabo, M.J.P., Bechara, G.H. & Mbati, P.A., 2004, 'Cross-reactivity between antigens from *Amblyomma cajennense* and *A. hebraeum* (Acari : Ixodidae)', *Journal of the South African Veterinary Association-Tydskrif Van Die Suid-Afrikaanse Veterinere Vereniging* 75(1), 40-42.

Horak, I.G., Gallivan, G.J. & Spickett, A.M., 2011, 'The dynamics of questing ticks collected for 164 consecutive months off the vegetation of two landscape zones in the Kruger National Park (1988-2002). Part I. Total ticks, *Amblyomma hebraeum* and *Rhipicephalus decoloratus*.', *Onderstepoort Journal of Veterinary Research* 78(1), Arte2.

House, J.A., Wilson, T.M., el Nakashly, S., Karim, I.A., Ismail, I., el Danaf, N., Moussa, A.M. & Ayoub, N.N., 1990, 'The isolation of lumpy skin disease virus and bovine herpesvirus-4 from cattle in Egypt', *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc* 2(2), 111-115.

Hsu, M.H. & Sauer, J.R., 1975, 'Ion and water balance in the feeding lone star tick.', *Comparative Biochemistry and Physiology, A* 52(2), 269-276.

Hunter, P. & Wallace, D., 2001, 'Lumpy skin disease in southern Africa: a review of the disease and aspects of control', *Journal of the South African Veterinary Association* 72(2), 68-71.

Hyatt, A.D., 1989, 'The application of electron microscopy to veterinary virus diagnosis', *Australian Veterinary Journal* 66(12), 445-449.

Inokuma, H., Kemp, D.H. & Willadsen, P., 1994, 'Comparison of prostaglandin E2 (PGE2) in salivary gland of Boophilus microplus, Haemaphysalis longicornis and Ixodes holocyclus, and quantification of PGE2 in saliva, hemolymph, ovary and gut of B. microplus', *Journal of Veterinary Medical Science* 56(6), 1217-1218.

Inokuma, H., Kerlin, R.L., Kemp, D.H. & Willadsen, P., 1993, 'Effects of cattle tick (Boophilus microplus) infestation on the bovine immune system.', *Veterinary parasitology* 47(1-2), 107-118.

Ireland, D.C. & Binepal, Y.S., 1998, 'Improved detection of capripoxvirus in biopsy samples by PCR', *Journal of virological methods* 74(1), 1-7.

Irons, P.C., Tuppurainen, E.S. & Venter, E.H., 2005, 'Excretion of lumpy skin disease virus in bull semen', *Theriogenology* 63(5), 1290-1297.

Johnston, J.B. & McFadden, G., 2003, 'Poxvirus immunomodulatory strategies: current perspectives', *Journal of virology* 77(11), 6093-6100.

Jones, L.D., Davies, C.R., Steel, G.M. & Nuttall, P.A., 1989, 'Vector Capacity of Rhipicephalus-Appendiculatus and Amblyomma-Variegatum for Thogoto and Dhori Viruses', *Medical and veterinary entomology* 3(2), 195-202.

Jones, L.D., Hodgson, E., Williams, T., Higgs, S. & Nuttall, P.A., 1992, 'Saliva activated transmission (SAT) of Thogoto virus: relationship with vector potential of different haematophagous arthropods.', *Medical and veterinary entomology* 6(3), 261-265.

Jones, L.D., Matthewson, M. & Nuttall, P.A., 1992, 'Saliva-activated transmission (SAT) of Thogoto virus: dynamics of SAT factor activity in the salivary glands of *Rhipicephalus appendiculatus*, *Amblyomma variegatum*, and *Boophilus microplus* ticks.', *Experimental & applied acarology* 13(4), 241-248.

Kaufman, W.R., 1973, 'Ion and water balance in the ixodid tick *Dermacentor andersoni*. I. Routes of ion and water excretion', *Journal of Experimental Biology* 58(2), 523-536.

Kaufman, W.R., 1989, 'Tick-host interaction: a synthesis of current concepts.', *Parasitology Today* 5(2), 47-56.

Kaufman, W.R. & Nuttall, P.A., 2003, '*Rhipicephalus appendiculatus* (Acari: Ixodidae): dynamics of Thogoto virus infection in female ticks during feeding on guinea pigs.', *Experimental parasitology* 104(1/2), 20-25.

Kaufman, W.R. & Nuttall, P.A., 1996, '*Amblyomma variegatum* (Acari: Ixodidae): mechanism and control of arbovirus secretion in tick saliva', *Experimental parasitology* 82(3), 316-323.

Kavitha, K.T., Latha, B.R., Raj, G.D., Lalitha John & John, L., 2011, 'Immunolocalisation of midgut antigen of *Rhipicephalus haemaphysaloides* by indirect immunoperoxidase test using methylene green counter stain.', *Tamilnadu Journal of Veterinary and Animal Sciences* 7(2), 56-59.

Khadr, A.M., Aboul Soud, E.S. & Khaliel, S.A., 1999, 'A comparative study on the immune response of cattle to sheep pox and lumpy skin disease viral vaccines', *Veterinary Medical Journal Giza* 47(1), 29-37.

King, A., Lefkowitz, E., Adams, M.J. & Carstens, E.B. (eds.), 2011, 'Virus Taxonomy, 1st Edition. Ninth Report of the International Committee on Taxonomy Viruses'.

Kitching, R.P., 2003, 'Vaccines for lumpy skin disease, sheep pox and goat pox', *Developments in biologicals* 114 161-167.

Kitching, R.P., 1986, 'Passive protection of sheep against capripoxvirus', *Research in veterinary science* 41(2), 247-250.

Kitching, R.P., Hammond, J.M. & Black, D.N., 1986, 'Studies on the major common precipitating antigen of capripoxvirus', *The Journal of general virology* 67 (Pt 1)(Pt 1), 139-148.

Kitching, R.P. & Mellor, P.S., 1986, 'Insect transmission of capripoxvirus', *Research in veterinary science* 40(2), 255-258.

Kitching, R.P. & Smale, C., 1986, 'Comparison of the external dimensions of capripoxvirus isolates', *Research in veterinary science* 41(3), 425-427.

Kitching, R.P. & Taylor, W.P., 1985a, 'Clinical and antigenic relationship between isolates of sheep and goat pox viruses.', *Tropical animal health and production* 17(2), 64-74.

Kitching, R.P. & Taylor, W.P., 1985b, 'Transmission of capripoxvirus', *Research in veterinary science* 39(2), 196-199.

Koshy, T.J., Rajavelu, G. & Lalitha, C.M., 1982, 'Ecology and bionomics of the boophilids of Tamil Nadu', *Cheiron* 11(1), 25-30.

Kuhnert, F., Diehl, P.A. & Guerin, P.M., 1995, 'The life-cycle of the bont tick *Amblyomma hebraeum* in vitro', *International journal for parasitology* 25(8), 887-896.

Kumar, S.M., 2011, 'An outbreak of Lumpy Skin Disease in a Holstein dairy herd in Oman: a clinical report.', *Asian Journal of Animal and Veterinary Advances* 6(8), 851-859.

Labuda, M., Danielova, V., Jones, L.D. & Nuttall, P.A., 1993a, 'Amplification of tick-borne encephalitis virus infection during co-feeding of ticks.', *Medical and veterinary entomology* 7(4), 339-342.

Labuda, M., Jones, L.D., Williams, T., Danielova, V. & Nuttall, P.A., 1993b, 'Efficient transmission of tick-borne encephalitis virus between cofeeding ticks.', *Journal of medical entomology* 30(1), 295-299.

Lalanne, A., 1956, 'Lumpy skin disease in Madagascar in relation to the leather industry', *Bulletin de l'Office International des Epizootics* 46 596-611.

Lamien, C.E., Lelenta, M., Goger, W., Silber, R., Tuppurainen, E., Matijevic, M., Luckins, A.G. & Diallo, A., 2011, 'Real time PCR method for simultaneous detection, quantitation and differentiation of capripoxviruses', *Journal of virological methods* 171(1), 134-140.

Laue, M. & Bannert, N., 2010, 'Detection limit of negative staining electron microscopy for the diagnosis of bioterrorism-related micro-organisms', *Journal of applied microbiology* 109(4), 1159-1168.

Leahy, M., Kovacic, A., Mannion, C. & Schulze, L., 1983, 'Pheromone-induced aggregation of ixodid ticks before host contact.', *Experientia* 39(8), 859-860.

Leahy, M.G., Hajkova, Z. & Bouchalova, J., 1981, 'Two female pheromones in the metastriate tick *Hyalomma dromedarii* (Acarina, Ixodidae).', *Acta Entomologica Bohemoslovaca* 78(4), 224-230.

MacOwan, K.D.S., 1959, 'Observations on the epizootiology of lumpy skin disease during the first year of its occurrence in Kenya', *Bulletin of epizootic diseases of Africa. Bulletin des epizooties en Afrique* 7 7-20.

Madder, M. & Berkvens, D.L., 1997, 'Evaluation of an in vitro method to measure behavioural diapause in the tick *Rhipicephalus appendiculatus* (Acarina: Ixodidae) in the laboratory.', *Parasitology* 115(1), 97-100.

Madder, M., Speybroeck, N., Brandt, J. & Berkvens, D., 1999, 'Diapause induction in adults of three *Rhipicephalus appendiculatus* stocks.', *Experimental & applied acarology* 23(12), 961-968.

Marchal, C., Schramm, F., Kern, A., Luft, B.J., Yang, X.H., Schuijt, T., Hovius, J., Jaulhac, B. & Boulanger, N., 2011, 'Antialarmin effect of tick saliva during the transmission of Lyme disease.', *Infection and immunity* 79(2), 774-785.

Marzouk, A.S., Khalil, G.M., Mohamed, F.S.A. & Farid, N., 1987, '*Hyalomma dromedarii* (Acari, Ixodoidea, Ixodidae) - Central and Peripheral Nervous-System Anatomy', *Experimental & applied acarology* 3(2), 145-161.

Mebratu, G.Y., Kassa, B., Fikre, Y. & Berhanu, B., 1984, 'Observation on the outbreak of lumpy skin disease in Ethiopia', *Revue d'Élevage et de Médecine Vétérinaire des pays Tropicaux* 37(4), 395-399.

Mellor, P.S., Kitching, R.P. & Wilkinson, P.J., 1987, 'Mechanical transmission of capripox virus and African swine fever virus by Stomoxys calcitrans.', *Research in veterinary science* 43(1), 109-112.

Meltzer, M.I., Norval, R.A.I. & Donachie, P.L., 1995, 'Effects of tick infestation and tick-borne disease infections (heartwater, anaplasmosis and babesiosis) on the lactation and weight gain of Mashona cattle in south-eastern Zimbabwe', *Tropical animal health and production* 27(3), 129-144.

Mohamed, A.A., Hussen, H.A., Dini, A.H. & Ali, S.H., 1985, 'Lumpy skin like disease in Somalia', *Bollettino Scientifico della Facoltà di Zootecnia e Veterinaria, Università Nazionale Somala* 5 75-79.

Nawathe, D.R., Asagba, M.O., Abegunde, A., Ajayi, S.A. & Durkwa, L., 1982, 'Some observations on the occurrence of lumpy skin disease in Nigeria', *Zentralblatt fur Veterinarmedizin.Reihe B.Journal of veterinary medicine.Series B* 29(1), 31-36.

Norval, R.A.I., 1991, *Heartwater disease,* , Grosvenor Press International Ltd, London.

Norval, R.A.I., 1977, 'Ecology of the tick *Amblyomma hebraeum* Koch in the Eastern Cape Province of South Africa. I. Distribution and seasonal activity', *Journal of Parasitology* 63(4), 734-739.

Norval, R.A.I., 1974, 'The life cycle of *Amblyomma hebraeum* Koch, 1844 (Acarina: Ixodidae)', *Journal of the Entomological Society of Southern Africa* 37(2), 357-367.

Norval, R.A.I., Andrew, H.R. & Meltzer, M.I., 1991, 'Seasonal occurrence of the bont tick (*Amblyomma hebraeum*) in the southern lowveld of Zimbabwe', *Experimental & applied acarology* 13(2), 81-96.

Norval, R.A.I., Andrew, H.R. & Yunker, C.E., 1989, 'Pheromone-mediation of host-selection in bont ticks (*Amblyomma hebraeum* Koch).', *Science (Washington)* 243(4889), 364-365.

Norval, R.A.I., Perry, B.D., Meltzer, M.I., Kruska, R.L. & Booth, T.H., 1994, 'Factors affecting the distributions of the ticks *Amblyomma hebraeum* and *A. variegatum* in Zimbabwe: implications of reduced acaricide usage', *Experimental & applied acarology* 18(7), 383-407.

Norval, R.A.I., Peter, T., Sonenshine, D.E. & Burridge, M.J., 1992, 'Responses of the ticks *Amblyomma hebraeum* and *A. variegatum* to known or potential components of the aggregation-attachment pheromone. III. Aggregation', *Experimental & applied acarology* 16(3), 237-245.

Norval, R.A.I., Sonenshine, D.E., Allan, S.A. & Burridge, M.J., 1996, 'Efficacy of pheromone-acaricide-impregnated tail-tag decoys for controlling the bont tick, *Amblyomma hebraeum* (Acari: Ixodidae), on cattle in Zimbabwe', *Experimental & applied acarology* 20(1), 31-46.

Norval, R.A.I., Sutherst, R.W., Jorgensen, O.G. & Kerr, J.D., 1997, 'The effects of the bont tick, *Amblyomma hebraeum*, on milk production of Sanga and Sanga × Brahman cattle', *Medical and veterinary entomology* 11(2), 143-147.

Norval, R.A.I., Sutherst, R.W. & Kerr, J.D., 1996, 'Infestations of the bont tick *Amblyomma hebraeum* (Acari: Ixodidae) on different breeds of cattle in Zimbabwe', *Experimental & applied acarology* 20(10), 599-605.

Nuttall, P.A., Jones, L.D. & Davies, C.R., 1991, *The role of arthropod vectors in arbovirus evolution.* .

Nuttall, P.A., Jones, L.D., Labuda, M. & Kaufman, W.R., 1994, 'Adaptation of arboviruses to ticks.', *Journal of medical entomology* 31(1), 1-9.

Obenchai.Fd & Oliver, J.H., 1973, 'Qualitative Analysis of Form, Function and Interrelationships of Fat-Body and Associated Tissues in Adult Ticks (Acari-Ixodoidea)', *Journal of Experimental Zoology* 186(3), 217-235.

Obenchain, F. & Oliver, J.H.,Jr., 1976, 'The heart and arterial circulatory system of ticks (Acari: Ixodoidea)', *J.Arachnol* 3 57-74.

Ochanda, H., Young, A.S., Medley, G.F. & Perry, B.D., 1998, 'Vector competence of 7 rhipicephalid tick stocks in transmitting 2 Theileria parva parasite stocks from Kenya and Zimbabwe.', *Parasitology* 116(6), 539-545.

OIE, 2010, 'Lumpy skin disease' in May 2010, .

Oliver, J.H.,Jr., 1986, 'Induction of oogenesis and oviposition in ticks.', *Morphology, physiology, and behavioral biology of ticks* 233-247.

Oliver, J.H.,Jr. & Dotson, E.M., 1993, 'Hormonal control of molting and reproduction in ticks.', *American Zoologist* 33(3), 384-396.

Osterkamp, J., Wahl, U., Schmalfuss, G. & Haas, W., 1999, 'Host-odour recognition in two tick species is coded in a blend of vertebrate volatiles', *Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology* 185(1), 59-67.

Pegram, R.G. & Banda, D.S., 1990, 'Ecology and phenology of cattle ticks in Zambia: development and survival of free-living stages', *Experimental & applied acarology* 8(4), 291-301.

Pegram, R.G., Mwase, E.T., Zivkovic, D. & Jongejan, F., 1988, 'Morphogenetic diapause in *Amblyomma variegatum* (Acari: Ixodidae).', *Medical and veterinary entomology* 2(4), 301-307.

Peter, T.F., Bryson, N.R., Perry, B.D., O'Callaghan, C.J., Medley, G.F., Smith, G.E., Mlambo, G., Horak, I.G., Burridge, M.J. & Mahan, S.M., 1999, '*Cowdria ruminantium* infection in ticks in the Kruger National Park', *Veterinary Record* 145(11), 304-307.

Prozesky, L. & Barnard, B.J., 1982, 'A study of the pathology of lumpy skin disease in cattle', *The Onderstepoort journal of veterinary research* 49(3), 167-175.

Prozesky, L. & Plessis, J.L., 1987, 'Heartwater. The development and life cycle of *Cowdria ruminantium* in the vertebrate host, ticks and cultured endothelial cells', *Onderstepoort Journal of Veterinary Research* 54(3), 193-196.

Purcell, D.A., Mcferran, J.B., Clarke, J.K. & Hughes, D.A., 1972, 'Morphogenesis of Pigeonpox Virus', *Journal of General Virology* 15(APR), 79-&.

Randolph, S.E., 2009, 'Epidemiological consequences of the ecological physiology of ticks.', *Advances in Insect Physiology* 37 297-339.

Rechav, Y., 1978, 'Drop-off rhythms of engorged larvae and nymphs of the bont tick, *Amblyomma hebraeum* (Acari: Ixodidae), and the factors that regulate them', *Journal of medical entomology* 14(6), 677-687.

Rechav, Y. & Jager, C.d., 1991, 'Seasonal abundance of ticks associated with indigenous goats on a northern Transvaal farm.', *Journal of the South African Veterinary Association* 62(1), 10-11.

Ribeiro, J.M.C., Makoul, G.T. & Robinson, D.R., 1988, '*Ixodes dammini*: evidence for salivary prostacyclin secretion', *Journal of Parasitology* 74(6), 1068-1069.

Ribeiro, J.M.C., Weis, J.J. & Telford, S.R., 1990, 'Saliva of the tick *Ixodes dammini* inhibits neutrophil function', *Experimental parasitology* 70(4), 382-388.

Robinson, P.M., 1982, '*Theileria annulata* and its transmission -- a review', *Tropical animal health and production* 14(1), 3-12.

Salib, F.A. & Osman, A.H., 2011, 'Incidence of lumpy skin disease among Egyptian cattle in Giza governorate, Egypt', *Veterinary World* 4(4), 162-167.

Sá-Nunes, A., Bafica, A., Lucas, D.A., Conrads, T.P., Veenstra, T.D., Andersen, J.F., Mather, T.N., Ribeiro, J.M.C. & Francischetti, I.M.B., 2007, 'Prostaglandin E2 is a major inhibitor of dendritic cell maturation and function in *Ixodes scapularis* saliva', *Journal of Immunology* 179(3), 1497-1505.

Sauer, J.R., Essenberg, R.C. & Bowman, A.S., 2000, 'Salivary glands in ixodid ticks: control and mechanism of secretion.', *Journal of insect physiology* 46(7), 1069-1078.

Schroder, B., Uys, A.C. & Reilly, B.K., 2006, 'A survey of free-living ixodid ticks on a commercial game farm in the Thabazimbi District, Limpopo Province, South Africa', *Journal of the South African Veterinary Association* 77(3), 141-144.

Seet, B.T., Johnston, J.B., Brunetti, C.R., Barrett, J.W., Everett, H., Cameron, C., Sypula, J., Nazarian, S.H., Lucas, A. & McFadden, G., 2003, 'Poxviruses and immune evasion', *Annual Review of Immunology* 21 377-423.

Shipley, A.E., 1915, 'Stomoxys, the Stable Fly.', *British medical journal* 216-218.

Short, N.J. & Norval, R.A.I., 1981, 'Regulation of seasonal occurrence in the tick Rhipicephalus appendiculatus Neumann, 1901.', *Tropical animal health and production* 13(1), 19-26.

Smith, A., XiuYang, G., Fuente, J., Naranjo, V., Kocan, K.M. & Kaufman, W.R., 2009, 'The impact of RNA interference of the subolesin and voraxin genes in male *Amblyomma hebraeum* (Acari: Ixodidae) on female engorgement and oviposition', *Experimental and Applied Acarology* 47(1), 71-86.

Solisch, P., 1978, 'Morphological virus diagnosis - use of negative contrast technique for electron-microscopic study of animal viruses', *Archiv fur Experimentelle Veterinarmedizin* 32(4), 465-488.

Sonenshi.De, Silverst.Rm, Layton, E.C. & Homsher, P.J., 1974, 'Evidence for Existence of a Sex-Pheromone in 2 Species of Ixodid Ticks (Metastigmata-Ixodidae)', *Journal of medical entomology* 11(3), 307-315.

Sonenshine, D.E., Silverstein, R.M. & Homsher, P.J., 1979, 'Female produced pheromones of Ixodidae.', *Recent Advances in Acarology.Volume II.* 281-290.

Sonenshine, D.E., Silverstein, R.M., Plummer, E., West, J.R. & McCullough, T., 1976, '2,6-Dichlorophenol, the sex pheromone of the Rocky Mountain wood tick, Dermacentor andersoni Stiles and the American dog tick, *Dermacentor variabilis* (Say).', *Journal of chemical ecology* 2(2), 201-209.

Sonenshine, D.E., 1991, *Biology of ticks*, Oxford University Press, Oxford.

Spickett, A.M. & Heyne, H., 1990, 'The prehatch period and larval survival of Boophilus decoloratus (Koch, 1844) (Acarina: Ixodidae) under natural conditions in the Transvaal, South Africa.', *Onderstepoort Journal of Veterinary Research* 57(1), 95-98.

Stich, R.W., Kocan, K.M., Palmer, G.H., Ewing, S.A., Hair, J.A. & Barron, S.J., 1989, 'Transstadial and attempted transovarial transmission of *Anaplasma marginale* by *Dermacentor variabilis*', *American Journal of Veterinary Research* 50(8), 1377-1380.

Surea, P., 1976, 'Isolation of Thogoto virus from *Amblyomma* and *Boophilus* ticks in Central Africa', *Bulletin de la Societe de Pathologie Exotique* 69(3), 207-212.

Teesdale, C., 1955, 'Studies on the Bionomics of Aedcs aegypti (L.) in its natural Habitats in a coastal Region of Kenya.', *Bulletin of entomological research* 46(pt. 3), 711-742.

Thomas, A.D. & Mare, C., 1945, 'Knop-velsiekte', *Journal of the South African Veterinary Medical Association* 16 36-43.

Till, W.M., 1961, *A contribution to the anatomy and histology of the brown ear tick Rhipicephalus appendiculatus Neumann.* .

Tonnesen, M.H., Penzhorn, B.L., Bryson, N.R., Stoltsz, W.H. & Masibigiri, T., 2006, 'Seroprevalence of *Babesia bovis* and *Babesia bigemina* in cattle in the Soutpansberg region, Limpopo Province, South Africa, associated with changes in vector-tick populations', *Journal of the South African Veterinary Association* 77(2), 61-65.

Tulman, E.R., Afonso, C.L., Lu, Z., Zsak, L., Kutish, G.F. & Rock, D.L., 2001, 'Genome of lumpy skin disease virus', *Journal of virology* 75(15), 7122-7130.

Tulman, E.R., Afonso, C.L., Lu, Z., Zsak, L., Sur, J.H., Sandybaev, N.T., Kerembekova, U.Z., Zaitsev, V.L., Kutish, G.F. & Rock, D.L., 2002, 'The genomes of sheeppox and goatpox viruses', *Journal of virology* 76(12), 6054-6061.

Tuppurainen, E.S., Venter, E.H. & Coetzer, J.A., 2005, 'The detection of lumpy skin disease virus in samples of experimentally infected cattle using different diagnostic techniques', *The Onderstepoort journal of veterinary research* 72(2), 153-164.

Tuppurainen, E.S.M. & Oura, C.A.L., 2012, 'Review: lumpy skin disease: an emerging threat to Europe, the Middle East and Asia.', *Transboundary and Emerging Diseases* 59(1), 40-48.

Tuppurainen, E.S.M., Stoltsz, W.H., Troskie, M., Wallace, D.B., Oura, C.A.L., Mellor, P.S., Coetzer, J.A.W. & Venter, E.H., 2011, 'A potential role for ixodid (hard) tick vectors in the transmission of lumpy skin disease virus in cattle', *Transboundary and Emerging Diseases* 58(2), 93-104.

Vatsya, S., Banerjee, P.S., Yadav, C.L. & Kumar, R.R., 2006, 'Ivermectin pour-on against one-host cattle tick *Boophilus microplus*', *Indian Veterinary Journal* 83(11), 1154-1156.

Von Backström, U., 1945, 'Ngamiland cattle disease. Preliminary report on a new disease, the etiological agent being probably of an infectious nature', *Journal of the South African Veterinary Medical Association* 16 29-35.

Waal, D.T. & Potgieter, F.T., 1987, 'The transstadial transmission of *Babesia caballi* by *Rhipicephalus evertsi evertsi*', *Onderstepoort Journal of Veterinary Research* 54(4), 655-656.

Walker, A., 2003, *Ticks of domestic animals in Africa : a guide to identification of species*, Bioscience Reports, Edinburgh.

Walker, A.R., Fletcher, J.D. & Gill, H.S., 1985, 'Structural and Histochemical-Changes in the Salivary-Glands of Rhipicephalus-Appendiculatus during Feeding', *International journal for parasitology* 15(1), 81-100.

Walker, A., 1994, *The arthropods of humans and domestic animals : a guide to preliminary identification*, 1st edn., Chapman & Hall, London.

Walker, J.B., 1970, *Notes on the common tick species of East Africa*, 3 rev edn., Cooper, McDougall and Robertson, Nairobi.

Wang, H., Henbest, P.J. & Nuttall, P.A., 1999, 'Successful interrupted feeding of adult *Rhipicephalus appendiculatus* (Ixodidae) is accompanied by reprogramming of salivary gland protein expression.', *Parasitology* 119(2), 143-149.

Wang, H. & Nuttall, P.A., 2001, 'Intra-stadial tick-borne Thogoto virus (Orthomyxoviridae) transmission: accelerated arbovirus transmission triggered by host death.', *Parasitology* 122(4), 439-446.

Weiss, K.E., 1968, 'Lumpy skin disease virus', *Virology Monographs* 3 111-131.

Weiss, K.E., 1960, *The persistence of lumpy skin disease viruses in bovine tissues*, Muguga, Kenya: Inter-african Bureau of Animal Health. Paris: Office International des Epizootics.

Wernery, U., Kaaden, O.R. & Ali, M., 1997, 'Orthopox virus infections in dromedary camels in United Arab Emirates (U.A.E.) during winter season.', *Journal of Camel Practice and Research* 4(1), 51-55.

Wernery, U., Meyer, H. & Pfeffer, M., 1997, 'Camel pox in the United Arab Emirates and its prevention', *Journal of Camel Practice and Research* 4(2), 135-139.

White, D.M., Wilson, W.C., Blair, C.D. & Beaty, B.J., 2005, 'Studies on overwintering of bluetongue viruses in insects.', *Journal of General Virology* 86(2), 453-462.

Wikel, S.K., 1999, 'Tick modulation of host immunity: an important factor in pathogen transmission.', *International journal for parasitology* 29(6), 851-859.

Wikel, S.K., Ramachandra, R.N. & Bergman, D.K., 1994, 'Tick-induced modulation of the host immune response.', *International journal for parasitology* 24(1), 59-66.

Wilkinson, P.R., 1953, 'Observations on the sensory Physiology and Behaviour of Larvae of the Cattle Tick, *Boophilus microplus* (Can.) (Ixodidae).', *Australian Journal of Zoology* 1(3), 345-356.

Woods, J.A., 1988, 'Lumpy skin disease--a review', *Tropical animal health and production* 20(1), 11-17.

Woodson, B., 1968, 'Recent Progress in Poxvirus Research', *Bacteriological Reviews* 32(2), 127-&.

World Organisation for Animal Health, 2012, *Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees), Volume 1.* .

World Organisation for Animal Health 2009, viewed April/17 2013, from http://www.oie.int/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/LUMPY_SKIN_DISEASE_FINAL.pdf.

Yano, Y., Shiraishi, S. & Uchida, T.A., 1987, 'Effects of Temperature on Development and Growth in the Tick, *Haemaphysalis longicornis*', *Experimental & applied acarology* 3(1), 73-78.

Yeruham, I., Nir, O., Braverman, Y., Davidson, M., Grinstein, H., Haymovitch, M. & Zamir, O., 1995, 'Spread of lumpy skin disease in Israeli dairy herds', *The Veterinary record* 137(4), 91-93.

Young, E., Basson, P.A. & Weiss, K.E., 1970, 'Experimental infection of game animals with lumpy skin disease virus (prototype strain Neethling)', *Onderstepoort Journal of Veterinary Research* 37 79-88.

Zaher, M.A., Soliman, Z.R. & Diab, F.M., 1977, 'An experimental study of *Borrelia anserina* in four species of Argas ticks. 2. Transstadial survival and transovarial transmission.' *Zeitschrift fur Parasitenkunde* 53(2), 213-223.

Zebrowski, G., 1926, 'A Preliminary Report on the Morphology of the American Dog Tick.', *Transactions Amer.ent.Soc.* 51(4), 331-369.

Chapter 2

Materials and Methods

2.1. Cattle

Eighteen sero-negative Bonsmara cattle, about 13 months old and weighing about 250 kg, were purchased from a farm in the Pretoria area, South Africa from a herd where vaccination against LSDV is not practised. Before purchase the cattle were bled and tested negative for the presence of antibodies against LSDV using the SNT. They were brought to the Faculty of Veterinary Science, University of Pretoria (UP) 14 days before commencing the experiments in order for them to acclimatise to the environment and were kept in insect free stables at the University of Pretoria's Bioscience Research Centre (UPBRC).

2.2. Preparation of the virus inoculum

The virus used in the study was a South African virulent field isolate of LSDV (248/93). It was passaged 5 or 6 times on primary bovine dermis cells (BDC) at the Virology Section of the Department of Veterinary Tropical Diseases (DVTD),UP. The final titre of the stock virus suspension was 5.95 log $TCID_{50}$/ml.

2.3. Ticks

Laboratory-reared ticks were supplied by the Agricultural Research Council's Onderstepoort Veterinary Institute (ARC-OVI). Unfed nymphal and adult *Amblyomma hebraeum* and *Rhipicephalus appendiculatus* and unfed larval *Rhipicephalus (Boophilus) decoloratus* were used in this study.

2.4. Artificial infection of donor animals with LSDV

Seven donor animals were infected with the prepared LSDV inoculum. A volume of 2.5 ml of the virus was injected intravenously into the jugular vein and 0.25 ml intradermally at four sites on each side of the body on Day 0 post infection (pi). Six donor cattle were paired to form three groups and were used as source of infection for *Amblyomma hebraeum* (DA1 and DA2), *R. appendiculatus* (DR1 and DR2) and *R. (B) decoloratus* (DB1 and DB2),

respectively. The seventh donor animal (DA3) hosted additional unfed *A. hebraeum* and *R. appendiculatus* adults.

2.5. Testing for LSDV persistence and transmission by ticks

2.5.1. *Rhipicephalus (Boophilus) decoloratus*

About 2500 *R. (B) decoloratus* larvae (F1) were placed in containment body bags firmly attached with Genkem contact adhesive (Qualichem and Genkem, South Africa) on the lateral sides of the rib cage of donor animals (DB1 and DB2). The larvae were placed on the animal on Day 4 post infection (pi) when viraemia was expected to commence.

Semi-engorged ticks, especially males, were collected from the donor animals on Day 26 pi and placed on recipient animal (RB1) to test for mechanical transmission. Unfortunately, the ticks placed on RB1 failed to attach and this line of testing did not continue (Figure 2.1)

The rest of the ticks were left to complete their life cycle. Replete engorged females were collected after dropping, washed in running tap water, dried and incubated individually in tubes at 25°C and 85% relative humidity (RH) until they oviposited and the eggs hatched into F2 larvae. The emergent (F2) larvae were kept for two more months for complete maturation. Approximately 2500 emergent F2 larvae were there after placed in containment bags attached with Genkem contact adhesive on each of the recipient animals (RB2 and RB3) to test for transovarial transmission of LSDV. The day that ticks were placed on recipient animals was referred as Day 0 post attachment (pa). The remaining larvae were stored at -80°C to be tested for transovarial passage of LSDV by real-time PCR and virus isolation (VI).

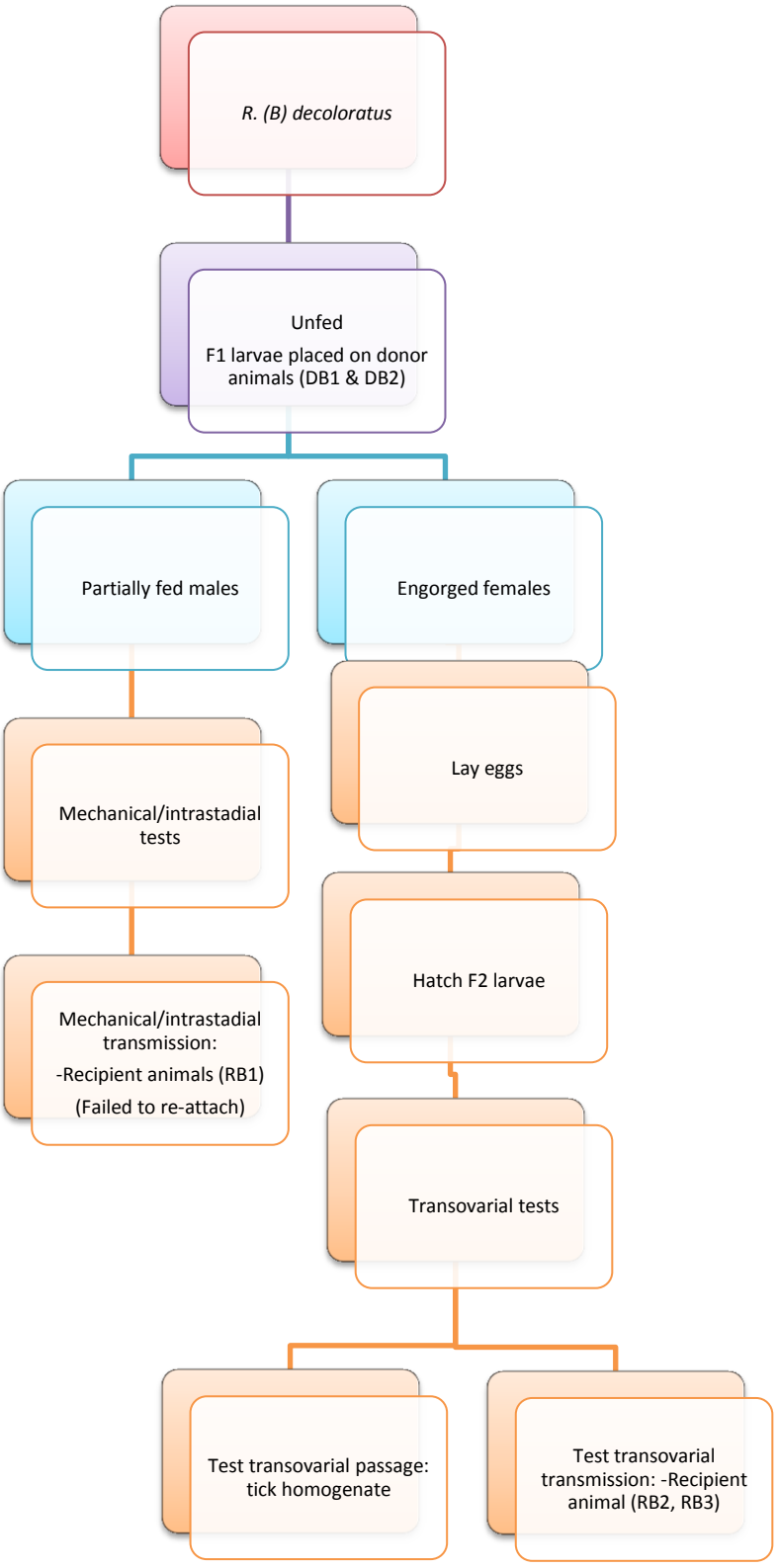

Figure 2.1: Outline of *Rhipicephalus (Boophilus) decoloratus* placement and testing for transovarial passage and transmission of LSDV. There was failure of re-attachment by *R. (B) decoloratus* males; therefore, mechanical/intrastadial and transstadial transmission could not be tested.

2.5.2. *Rhipicephalus appendiculatus*

Two hundred each of nymphal and adult (F1 adult) *R. appendiculatus* were placed in separate containment cotton bags firmly attached to the base of the ears of *Rhipicephalus* donor animals (DR1 and DR2) on Day 4 pi.

On Day 11 pi, 7 days after placing the ticks, 100 F1 adults (males) were collected from donor animals and placed into a containment bag attached to the base of recipient animal (RR1) to test for mechanical/intrastadial transmission. These ticks were later collected from RR1 and tested for mechanical/intrastadial passage of LSDV by real-time PCR and VI. Another 100 unfed *R. appendiculatus* adults were similarly placed in ear bags on donor animal DA3 (Day 4 pi) and were collected on Day 8 pi for testing of LSDV mechanical/intrastadial passage in saliva and tick organs (Figure 2.2).

F1 engorged female *R. appendiculatus* ticks were collected after they dropped off. They were washed running tap water, dried and incubated at 25°C and 85% RH until oviposition and eggs were allowed to hatch to F2 larvae. Approximately 2500 of the F2 larvae were placed on recipient animal (RR2) to test for transovarial transmission of LSDV (Figure 2.2). The remainder larvae were tested for transovarial passage by real-time PCR and VI.

The *R. appendiculatus* nymphs were allowed to feed on donor animals until repletion for approximately 5-6 days. The engorged nymphs were washed, dried and incubated at 25°C and 85% RH to moult into F2 adults. The emergent (F2) adults were incubated for another two months to allow for the mouthparts to harden after which 150 F2 adults were placed on recipient animal (RR3) to test for transstadial transmission and 100 were reserved to test for transstadial passage of LSDV by real-time PCR, VI and the detection of the virus in saliva and tick organs (Figure 2.2).

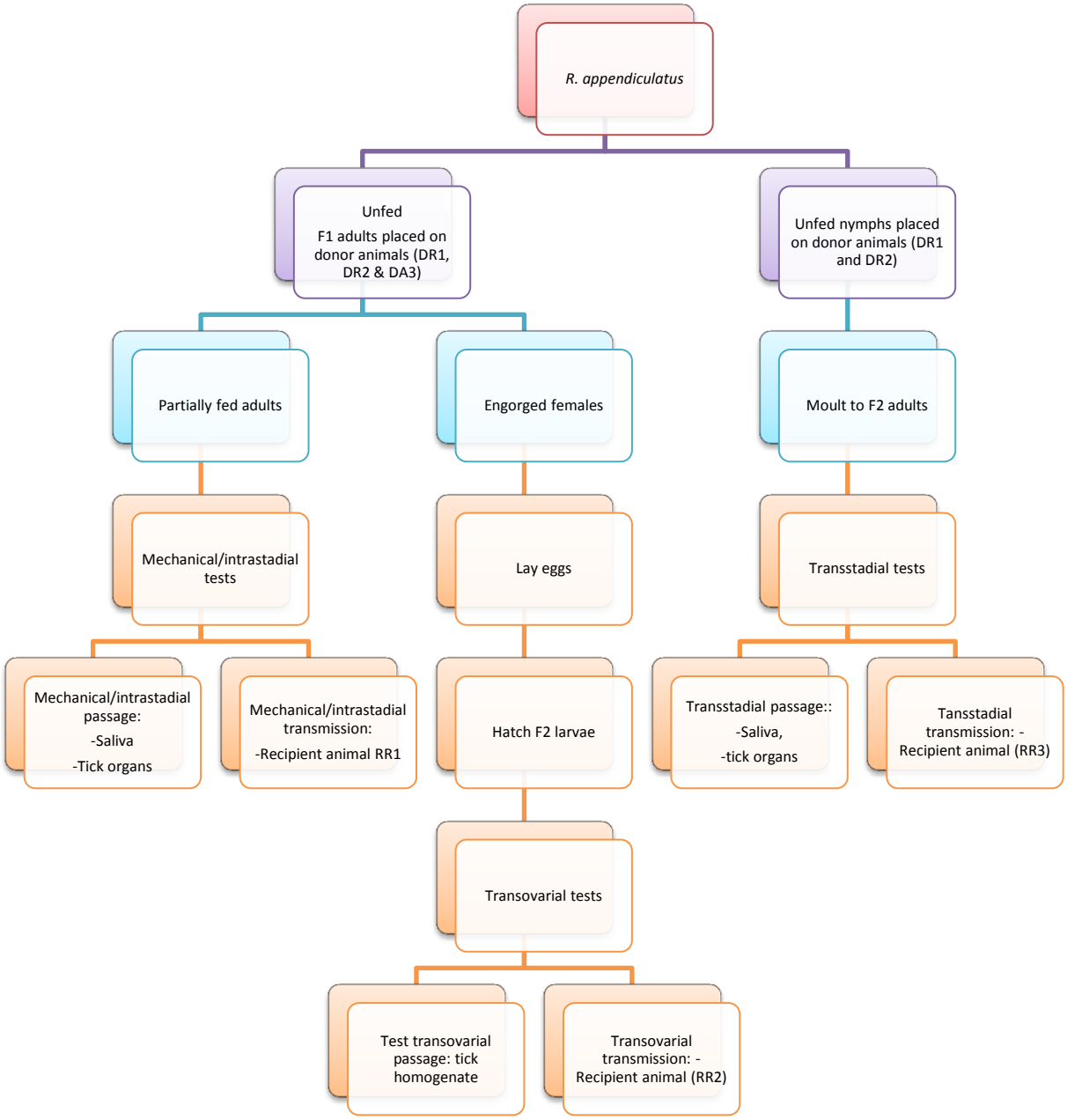

Figure 2.2: Outline of *R. appendiculatus* placement and processing for mechanical/intrastadial, transstadial and transovarial passage and transmission of LSDV

2.5.3. *Amblyomma hebraeum*

Male (F1 adult) *A. hebraeum* ticks were placed in containment bags attached to the body sides of donor animals (DA1 and DA2) on Day 4 pi, and since *A. hebraeum* females will only attach in response to aggregation and attachment pheromones secreted by males that have fed for at least four days (Norval, Andrew & Yunker 1989, Norval et al. 1992), 250 males were 4 days before the females (150) on each donor animal. On Day 12 pi, 200 *A. hebraeum* males from each of the two donor animals were transferred to recipient animals (RA1 and RA2) to test for intrasstadial/mechanical transmission. In addition, these ticks were tested by real-time PCR and VI for mechanical/intrastadial persistence of LSDV in the ticks. Another set of 75 F1 *A. hebraeum* males were placed on donor animal DA3 as above. Four days later (Day 8 pi), 25 females were added to the group of feeding males. All ticks were collected after another 4 days (Day 12 pi) to test for mechanical/intrastadial passage of LSDV by detecting the virus in tick saliva and organs.

Engorged F1 *A. hebraeum* females were collected, incubated and allowed to lay eggs as described for *R. appendiculatus* and *R. (B) decoloratus* females above. After a period of two months to allow for maturation, approximately 2,500 emergent (F2) *A. hebraeum* larvae were placed on recipient animal RA4 to test for transovarial transmission and approximately 500 larvae were tested for transovarial passage of LSDV by real-time PCR (Figure 2.3).

Amblyomma *hebraeum* nymphs were placed on the ear bags of donor animals (DA1 and DA2) (Figure 2.4) and were collected after repletion. As in *R. appendiculatus* above, they were incubated to moult into F2 adults. To test for transstadial transmission, 200 *A. hebraeum* F2 *A. hebraeum* males were placed on each of the recipient animals RA5 and RA6 on Day 0 pa and 4 days later (Day 4 pa), *A. hebraeum* females were added to the containment bags with males. On Day 8 pa, 100 males and partially fed females were collected to test for transstadial passage by detection of LSDV in tick saliva and organs.

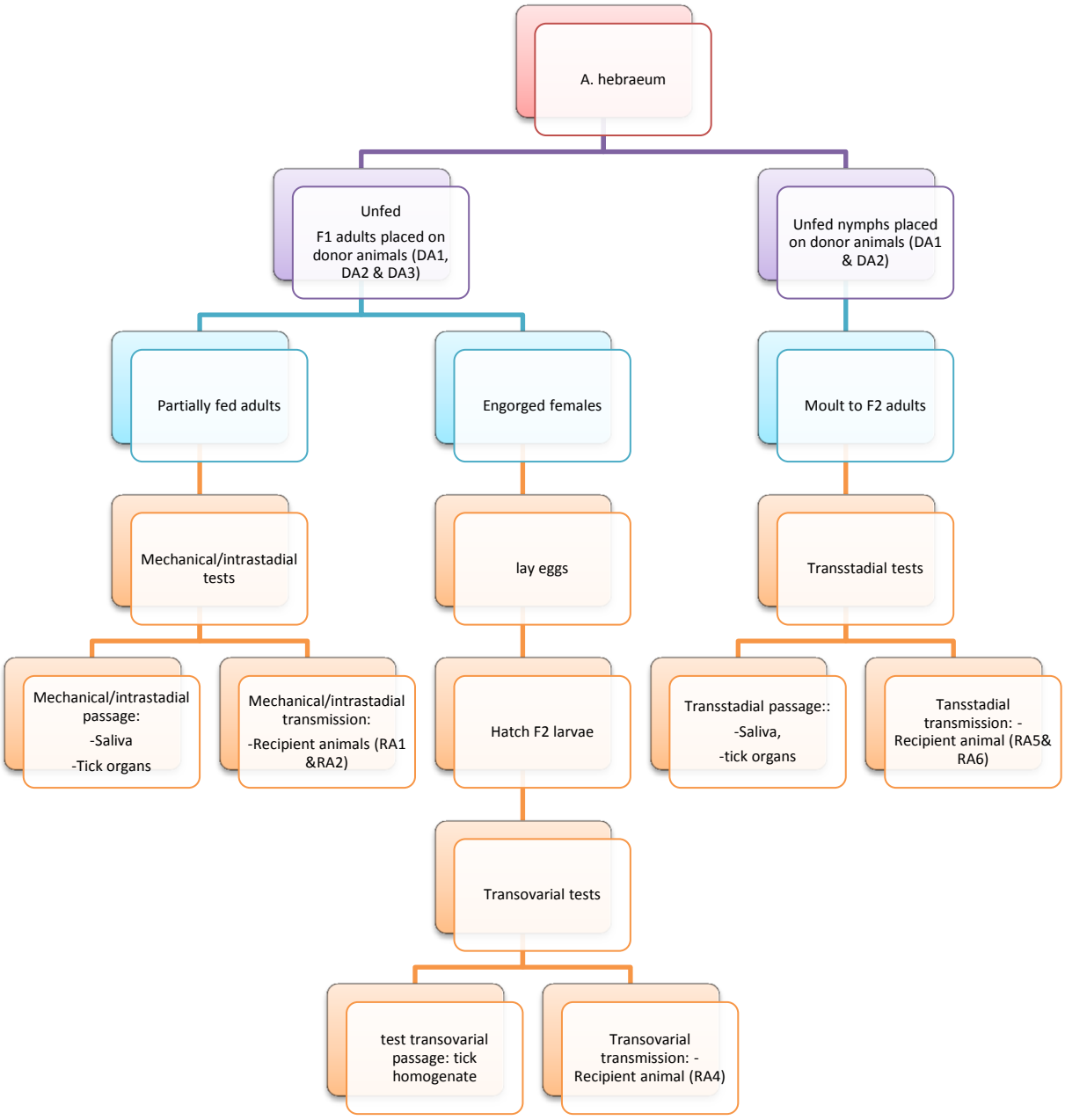

Figure 2.3: Outline of *A. hebraeum* placement and processing for mechanical/intrastadial, transstadial and transovarial passage and transmission of LSDV

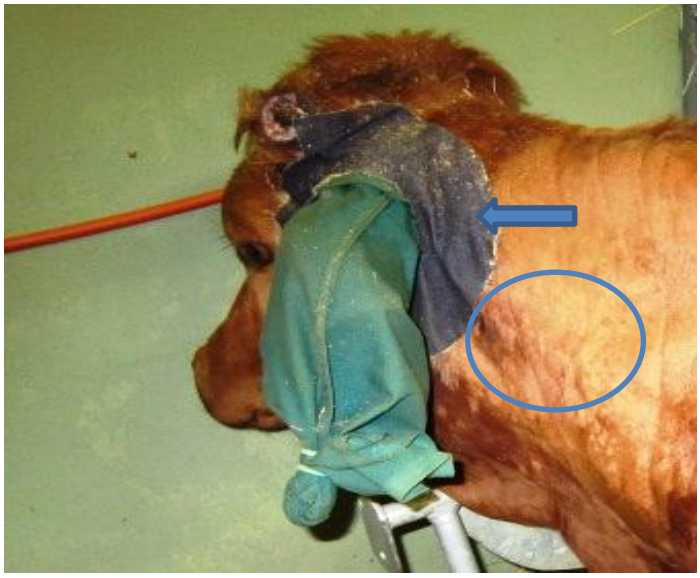

Figure 2.4:Donor animal (DA2) showing circumscribed skin lesions (Circled) on the neck and a containment bag attached at the base of the ear (Arrow) enclosing *A. hebraeum* nymphs

2.6. Collection of specimens from animals

Blood was collected from the jugular vein in EDTA tubes for PCR, heparin tubes for VI and whole blood in plain tubes for sera separation to be used for SNT. First collection of blood was done before inoculation of the virus on Day 0 and used as a negative control. Blood in EDTA and heparin was subsequently collected on Day 4 pi and on subsequent days, until the end of observation, mostly around Day 30 pi and stored at -20°C until testing. Unpreserved blood was collected from Day 14 pi from donor animals and from Day 10 pi from recipient animals. After collection, serum was separated and stored at -20°C until used.

2.7. Clinical monitoring of animals

Clinical monitoring of animals commenced from the day of arrival. The day of virus inoculation of donor animals was referred as Day 0 post inoculation (pi) while the day the potentially infected ticks were placed on recipient animals was referred to as Day 0 post attachment (pa). The clinical parameters monitored included body temperature, the presence of circumscribed skin lesions and enlargement of peripheral lymph nodes. These were recorded on a daily basis for the duration of the project.

Animals that developed severe clinical signs such as high fever (above 40°C) and eruptive lesions were treated with antibiotics to prevent secondary infections.

2.8. Collection of saliva from ticks

Adult ticks were induced to secrete saliva using previously published protocols (Chalaire et al. 2011, Ribeiro et al. 2004). Briefly; saliva samples were collected from *R. appendiculatus* and *A. hebraeum* F1 and F2 adults. These ticks were washed twice in phosphate buffered saline (PBS) to reduce surface contamination. After drying, 5 µl pilocarpine hydrochloride (Sigma-Aldrich, 50 mg/ml in 95% ethanol) was dropped onto the scutum of male ticks or alloscutum of female ticks. After the solution evaporated, the ticks were placed in clean 0.5 ml Eppendorf centrifuge tubes (Merck Millipore, South Africa) and incubated at 37°C for 3 hours. To collect the saliva, a small hole was made with a needle at the bottom of the tube and the tube was inserted into a 1.5 ml Eppendorf centrifuge tube. The saliva that was attached to the tick was washed off by placing 5 µl PBS on the tick in the 0.5 ml Eppendorf tube and centrifuged at 4400 rpm in an Eppendorf bench centrifuge (Wealtec, E-Centrifuge). Saliva was collected in the 1.5 ml Eppendorf tubes and then stored at -20°C until processed by either DNA extraction or VI.

Of the 85 saliva samples for *R. appendiculatus* F2 adults, 80 were pooled (n=16 pools of 5 samples each) and tested by real-time PCR and five saliva samples were individually tested by VI. The saliva samples (n=45) for *A. hebraeum* F2 adults (transstadial passage) were pooled (n=nine pools) and tested by real-time PCR. The other five samples were tested individually by VI. Fourteen saliva samples from each of *R. appendiculatus* and *A. hebraeum* F1 adults (mechanical/intrastadial passage) were tested by VI.

2.9. Tick dissection

Ticks were rinsed twice in sterile deionised water, dried on filter paper and then rinsed twice in PBS containing Ca^{2+} and Mg^{2+} (PBS^{+}). Adult ticks were dissected by removing the dorsal part of idiosoma (scutum or conscutum). Briefly, using a number 11 surgical blade, an incision was made along the lateral margins of the tick under a stereomicroscope (Nikon SMZ 800, Japan). The scutum or conscutum was lifted and separated from the rest of the body parts using pointed surgical forceps. The salivary glands were identified as white grape-like structures on the antero-lateral aspects of the tick. The midgut was identified as black tubes with branches (diverticulae) extending in several directions. The synganglion was identified as a white mass in the midline just antero-ventral to the midgut and between the anterior parts of the salivary ducts. The salivary glands, midguts and synganglia were put in

separate 1.5 ml tubes and fixed in 2.5% gluteraldehyde (Sigma-Aldrich Products) before submission to the Electron Microscope (EM) Unit (Department of Anatomy, FVS, UP).

2.10. Homogenisation of tick samples

Homogenisation of *A. hebraeum* adult ticks was performed by combining techniques used by Bell-Sakyi and co-workers (Bell-Sakyi, Ruzek & Gould 2009) and Sang and co-workers (Sang et al. 2006). Briefly; ticks were rinsed twice in sterile deionised water and dried on filter paper after which they were rinsed twice in PBS^{+} with double the normal dose (0.2%) of Gentamycin (stock: 50 mg/ml, Genta 50, Virbac Animal Health). The ticks were cut into 4 parts and placed into tubes containing beads (Roche Diagnostics, Mannheim, Germany) and 1 ml of Modified Eagle's Medium (MEM) (Highveld Biological, SA) with 0.2 mg/ml Gentamycin (stock: 50 mg/ml). The tubes were then cooled at -80°C for 5 minutes and crushed using a Magnalyser (Roche Diagnostics) at 6500 rpm for 1 minute. The supernatant was collected in 1.5 ml Eppendorf tubes (Eppendorf, South Africa) and stored at -80°C for further use in VI and real-time PCR.

Tick eggs and hatched larvae (approximately 5 g) were collected in 1.5 ml micro tubes and rinsed three times in PBS^{+} with 0.2% Gentamycin and 0.5% Amphotericin B. and homogenised as above.

Samples containing tick egg homogenates were stored for testing by real-time PCR while larval homogenate samples were stored for testing by real-time PCR and VI.

2.11. Virus isolation

Virus isolation was carried out according to standard operation procedures of the DVTD, UP based on OIE guidelines (OIE, 2010). Briefly; 500 µl heparinised blood was inoculated onto bovine dermis cells in 6 well-plates and incubated at 37°C for an hour. After washing three times with PBS^{+}, the medium was replaced with 3 ml MEM containing 5% foetal calf serum and 0.1% Gentamycin and incubated at 37°C. The cells were observed daily for cytopathic effects (CPE). To minimise cytotoxicity of bovine dermis cells, a 1:10 dilution (in MEM containing 5% foetal calf serum and 2% Gentamycin) of the tick homogenate was used for VI. Bovine dermis cells in 24-well-plates were inoculated with 300 µl of homogenate and incubated at 37°C for an hour. After incubation, the cells were washed with PBS^{+} followed by addition of fresh medium, (3 ml MEM containing 5% foetal calf serum) (Highveld

Biological, SA) and 0.2% Gentamycin (Genta 50, Virbac Animal Health) and incubated at 37°C. The cells were observed daily for CPE. In the absence of CPE a second and sometimes a third passage were done. The used cell culture media were stored at -80°C and was tested by real-time PCR to confirm that the CPE was caused by LSDV.

2.12. Serum neutralisation test

This test was done according to protocols of the DVTD, UP , based on the OIE guideline (OIE 2010) (Beard et al. 2010). The tests were performed on 96-well plates. Test sera were diluted 1:5 in PBS^{+} (200 µl sera: 800 µl PBS^{+}) and inactivated at 56°C for 30 minutes. To each of the 96 wells, 100 µl MEM containing 5% foetal calf serum (FCS) and 0.1% (0.05 mg/ml) Gentamycin (Genta 50, Virbac Animal Health) was added. Using 100 µl per well, a series of 2-fold dilutions of each serum was made from 1:5 to 1:320. An equal volume (100 µl) of the South African field isolate (LSDV 248/93) at a concentration of $100TCID_{50}$ was added. A virus control on each plate was set up in 5 wells of 6 columns, where only virus and no serum were added. In the well of the first two columns, stock virus was added. Ten-fold serially diluted virus was added in the next 4 wells. No virus was added in the wells used as cell controls. The plates were then incubated at 37°C for 1hr, after which 80 µl bovine dermis cells at a concentration of 4.8 $x10^{5}$/ml were added per well. The plates were incubated at 37°C in 5% CO_2 for 14 days. The monolayers were examined daily for CPE and the end-point titre was determined from the last dilution where the virus/serum mixture inhibited CPE.

2.13. Real-time Polymerase chain reaction

2.13.1. DNA extraction

Extraction of DNA was based on the protocol by Tuppurainen *et al.*(2005). Briefly; 200 µl of specimen was added to 100 µl of lysis buffer containing 0.378 g KCl, 1 ml Tris (1.0 M, pH8), 0.5 ml Tween 20 and 60% guanidine thiocyanate (Roche products, Mannheim, German). To digest protein bands, 10 µl of proteinase K (Roche products, Mannheim, Germany) were added to the mixture in 1.5 ml tubes. The mixture was incubated at 56°C for 2 hours for blood, saliva and VI supernatants or overnight for tick homogenate and then heated to 90°C for 10 minutes to stop enzyme activity. Then 300 µl of Trizol, a solution of phenol:chloroform:isoamylalcohol (Roche products, Mannheim, Germany), was added,

mixed by vortexing and incubated at room temperature for 10 minutes before centrifuging at 13,000 rpm (Centrifuge 5417R, Eppendorph international, Germany) at 4°C for 15 minutes. The supernatant (200 µl) was collected in a 2 ml tube and DNA precipitated in two volumes (400 µl) of ice cold absolute ethanol mixed with 20 µl (1/10) of 3M sodium acetate. DNA was collected by centrifugation at 13,000 rpm for 15 minutes and the pellet washed with 70% ethanol, centrifuged and the pellet dissolved in 30 µl dilution buffer AE and stored at -20°C until used.

2.13.2. Reaction parameters

The extracted DNA was tested by real-time PCR using a Taqman assay. This assay amplifies an 89 bp region within the capripoxviruses ORF 74 region, which encodes the intracellular mature protein, P32 (Bowden et al. 2008). Capripoxvirus-specific primers and probes with the following sequences were used: Forward primer-CaPV074F1 5'-AAA ACG GTA TAT GGA ATA GAGTT GAA-3', Reverse primer- CaPV074R1 5'-AAA TGA AAC CAA TGG ATG GGA TA-3' and Probe CaPV074P1 5'FAM-TGG CTC ATA GAT TTC CT-MGB/NFQ -3' (Bowden et al. 2008).

The reaction was done using a Light Cycler®, 2.0 machine (Roche Products, Mannheim, Germany). The reaction mixture comprised 4 µl of 10X FastStart DNA Master^{plus} HybProbe kit (Roche Products), 1 µl (0.1 µM) each of the primers, 1 µl (0.2 µM) of hybridization probe, 0.5 µl of Uracil-DNA Glycosylase (UDG) (Roche Products) 8.5 µl of water and 4 µl of DNA template (up to 500 ng). The reaction was done according to the procedures described by Bowden *et al.* (2008). Briefly; UDG was activated by raising the temperature to 40°C for 10 minutes followed by a FastStart *Taq* Polymerase enzyme activation step of 10 minutes at 95°C. The amplification comprised 45 cycles of denaturation at 95°C for 10 seconds (s), annealing at 60°C for 60s and extension at 72°C for 1s (single acquisition mode). DNA extracted from LSDV-infected cell cultures was used as positive control. Negative controls included a water sample processed through the DNA extraction procedures and a no-DNA template water control.

2.14. Immunohistochemistry

To determine the presence of LSDV in specific tick organs following interrupted feeding (intrastadial passage of the virus), nine F1 *A. hebraeum* and five *R. appendiculatus* adults were collected from recipient animal RR3. They were sectioned in the sagittal plane and

fixed overnight in 10% phosphate buffered formalin. Both halves of each tick were then embedded in paraffin and processed according to standard protocols of the Pathology Section in the Department of Paraclinical Sciences, FVS, UP.

To determine the presence of LSDV antigens in specific tick organs following transstadial passage of the virus, five unfed F2 and 10 partially fed F2 adults from recipient animals (RA5 and RA6) were processed as above. Similarly, to demonstrate the presence of the virus during transstadial infection in *R. appendiculatus*, four unfed F2 adults and 17 partially fed F2 adults on recipient animal RR3 were processed as above. Four un-inoculated ticks were processed in the same way and used as negative controls.

Pilot studies were conducted to optimise the immunoperoxidase labelling technique. Briefly; three 3 to 4 µm-thick tick sections were mounted on positively charged microscope slides and dried overnight in an oven at 38°C. After de-waxing in xylene for 10 minutes, the specimens were rehydrated through a graded ethanol and distilled water series (three minutes each in 100%, 96% and 70% ethanol). Endogenous peroxidase activity was quenched by incubating the tick sections in 3% hydrogen peroxide (in methanol) for 15 minutes at room temperature (22-25°C) and rinsed three times in distilled water. For the purpose of antigen retrieval, slides were incubated in citrate buffer (pH of 6) at 96°C for 14 minutes, followed by cooling for 15 minutes at room temperature and then rinsed twice in distilled water and in PBS buffer for five minutes. To block non-specific immunoglobulin binding, the slides were incubated with normal horse serum (1:10 dilution) for 20 minutes at room temperature. The blocking serum was decanted and replaced with the primary F80G5 monoclonal antibody (anti–S057) (Babiuk et al. 2008), diluted 1:1000 and incubated overnight with the sections. The slides were then rinsed three times in distilled water and then in PBS for 10 minutes. The secondary antibody, a biotinylated polyclonal rabbit anti-mouse antibody (Catalogue no: EO354, DakoCytomation, Denmark), was incubated with the tick sections for 30 minutes in a humidified chamber at room temperature. The slides were rinsed in distilled water for three minutes before rinsing in PBS for 10 minutes. The sections were incubated with the peroxidase conjugated avidin-biotinylated complex (Catalogue no: PK6100, Vector laboratories, USA) for 30 minutes at room temperature and rinsed twice as before. A Vector® Nova red substrate (catalogue no: SK-4800, Vector laboratories, USA) was reconstituted according to manufacturer's instructions and incubated with the sections at room temperature. During this time, the positive-tissue control, a section of skin with characteristic lesions from a cow with confirmed lumpy skin disease (PCR, EM and IHC-positive on a skin sample) was

monitored at 100X magnification for positive labelling, using light microscopy. As soon as there was evidence of clear, specific positive labelling in the positive-tissue control section, all of the tick sections were immediately rinsed in a distilled water bath to halt the substrate reaction. The sections were then counterstained with Mayer's haematoxylin for 20 seconds and rinsed under running tap water for 10 minutes. The sections were routinely dehydrated through 70%, 96% and 100% alcohol, cleared in xylene and mounted in Entellan® (Merck Chemicals, Darmstadt, Germany). Specific positive labelling was confined to cytoplasmic granular labelling in tick cells and tissues, comparable with the labelling in target cells in the positive-tissue control.

To determine the limit of detection for the IMPS, bovine dermal cell monolayers were cultured on 8-well glass-slides (AEC-Amersham, LTD, South Africa). In each well, 400 µl of MEM containing 480,000 bovine dermis cells per ml was aliquoted and incubated for a day to allow the cells to form a confluent monolayer. Ten-fold serial dilutions of the virus (titre 4.5 Log $TCID_{50}$/ml) were performed and 10 µl of the respective virus dilution was inoculated into individual wells. After four days incubation, the wells were examined for CPE. The supernatant was removed for testing by real-time PCR to confirm presence of LSDV. The monolayers were air-dried and went through the process of immunoperoxidase staining according to protocols of the Section of Pathology as a modification of above. The stained slides were examined for the presence of LSDV. The viral titre for the highest dilution to show LSDV antigen staining was taken as the limit of detection.

2.15. Transmission electron microscopy

Tick organs, i.e. the salivary glands, midguts and synganglions fixed in 2.5% gluteraldehyde and submitted to the EM Unit (Department of Anatomy, FVS, UP) were processed according to standard protocols of the unit and were examined under transmission electron microscopy for the presence of LSDV particles.

2.16. References

Babiuk, S., Bowden, T.R., Parkyn, G., Dalman, B., Manning, L., Neufeld, J., Embury-Hyatt, C., Copps, J. & Boyle, D.B., 2008, 'Quantification of lumpy skin disease virus following experimental infection in cattle', *Transboundary and Emerging Diseases* 55(7), 299-307.

Beard, P.M., Sugar, S., Bazarragchaa, E., Gerelmaa, U., Tserendorj, S., Tuppurainen, E. & Sodnomdarjaa, R., 2010, 'A description of two outbreaks of capripoxvirus disease in Mongolia', *Veterinary microbiology* 142(3), 427-431.

Bell-Sakyi, L., Ruzek, D. & Gould, E.A., 2009, 'Cell lines from the soft tick Ornithodoros moubata', *Experimental and Applied Acarology* 49(3), 209-219.

Bowden, T.R., Babiuk, S.L., Parkyn, G.R., Copps, J.S. & Boyle, D.B., 2008, 'Capripoxvirus tissue tropism and shedding: a quantitative study in experimentally infected sheep and goats', *Virology* 371(2), 380-393.

Chalaire, K.C., Kim, T.K., Garcia-Rodriguez, H. & Mulenga, A., 2011, '*Amblyomma americanum* (L.) (Acari: Ixodidae) tick salivary gland serine protease inhibitor (serpin) 6 is secreted into tick saliva during tick feeding', *Journal of Experimental Biology* 214(4), 665-673.

Norval, R.A.I., Andrew, H.R. & Yunker, C.E., 1989, 'Pheromone-mediation of host-selection in bont ticks (*Amblyomma hebraeum* Koch).', *Science (Washington)* 243(4889), 364-365.

Norval, R.A.I., Peter, T., Sonenshine, D.E. & Burridge, M.J., 1992, 'Responses of the ticks *Amblyomma hebraeum* and *A. variegatum* to known or potential components of the aggregation-attachment pheromone. III. Aggregation', *Experimental & applied acarology* 16(3), 237-245.

OIE, 2010, 'Lumpy skin disease' in May 2010, .

Ribeiro, J.M.C., Zeidner, N.S., Ledin, K., Dolan, M.C. & Mather, T.N., 2004, 'How much pilocarpine contaminates pilocarpine-induced tick saliva?', *Medical and veterinary entomology* 18(1), 20-24.

Sang, R., Onyango, C., Gachoya, J., Mabinda, E., Konongoi, S., Ofula, V., Dunster, L., Okoth, F., Coldren, R., Tesh, R., Rosa, A.T., Finkbeiner, S., Wang, D., Crabtree, M. & Miller, B., 2006, 'Tickborne arbovirus surveillance in market livestock, Nairobi, Kenya', *Emerging Infectious Diseases* 12(7), 1074-1080.

Chapter 3

Evidence of transstadial and mechanical transmission of lumpy skin disease virus by *Amblyomma hebraeum* ticks

Published online by Transboundary and Emerging Diseases Journal

doi: 10.1111/tbed. 12102

3.1 Summary

Lumpy skin disease is an economically important disease caused by lumpy skin disease virus (LSDV), a *Capripoxvirus*, characterised by fever and circumscribed skin lesions. It is suspected to be transmitted mechanically by biting flies. To assess the vector potential of *Amblyomma hebraeum* in transmission of LSDV, mechanical/ intrastadial and transstadial modes of transmission of the virus by this tick species were investigated. Two cattle were artificially infected as sources (donors) of infection to ticks. Ticks were infected as either nymphs or adults. Male *A. hebraeum* ticks were partially fed on donor animals and transferred to recipient animals to test for mechanical/ intrastadial transmission. Nymphal *A. hebraeum* were fed to repletion on donor animals. The emergent adult ticks were placed on recipient animals to test for transstadial transmission of the virus. Successful transmission of LSDV infection was determined in recipient animals by monitoring development of clinical signs, testing of blood for the presence LSDV by real-time PCR, virus isolation and the serum neutralisation test. This report provides further evidence of mechanical/ intrastadial and, for the first time, transstadial transmission of LSDV by *A. hebraeum.* These findings implicate *A. hebraeum* as a possible maintenance host in the epidemiology of the disease.

Key words: *Amblyomma hebraeum*, ticks, lumpy skin disease virus, poxvirus, transmission

3.1 Introduction

Lumpy skin disease (LSD) is an acute, sub-acute or inapparent disease characterised by fever, multiple circumscribed firm skin nodules and generalised lymphadenitis that affects all age groups and breeds of cattle. This disease is of economic importance due to damage to the skin, the reduced milk and meat production, lowered fertility and animal movement and trade restrictions imposed on infected areas (Davies 1981, Prozesky & Barnard 1982, Woods 1988).

Lumpy skin disease is caused by the lumpy skin disease virus (LSDV), a member of the *Capripoxvirus*, a genus it shares with sheep pox virus (SPV) and goat pox virus (GPV) in the family *Poxviridae* (Fenner & White 1976, Fenner 1987, House et al. 1990, Plowright & Ferris 1958, Plowright & Witcomb 1959, Van Rooyen et al. 1959, Weiss 1968).

Cattle are the major hosts for LSDV. Severity of clinical signs depends on various factors. For instance, *Bos taurus* breeds are more susceptible than *Bos indicus* breeds, lactating and young cattle appear more susceptible (Ali et al. 1990, Awadin et al. 2011, Thomas & Mare 1945, Weiss 1968). Clinical cases of LSD may be severe, mild or inapparent (Salib & Osman 2011). Fever (up to 41.5°C) is usually the first clinical sign observed, and may persist for 6 days (Tuppurainen, Venter & Coetzer 2005) Viraemia has been detected between Day 4 and 21 post infection (pi) (Awad et al. 2010, Tuppurainen, Venter & Coetzer 2005, Tuppurainen et al. 2011). Nodules on the skin and mucous membranes follow the onset of fever. Skin lesions are common on the head, neck, udder, genitalia, perineum and legs. Superficial lymph nodes are enlarged and oedematous (Prozesky & Barnard 1982).

Most outbreaks of LSD have been reported during the wet and warm season, coinciding with high activity of biting flies (Chihota et al. 2001, Chihota et al. 2003, Hunter & Wallace 2001). The outbreak in Kenya in 1957 was associated with a high incidence of *Aedes natronius* and *Culex mirificus* mosquitoes (Burdin 1959) and the 1989 outbreak in Israel was attributed to *Stomoxys calcitrans* (stable fly) carried by wind from Ismailiya in Egypt (Yeruham et al. 1995). It has also been shown that *S. calcitrans* can transmit capripoxviruses between sheep under experimental conditions (Kitching & Mellor 1986). The experimental transmission of LSDV by *Aedes aegypti* to cattle suggests that *Ae. aegypti* and other biting insects have the ability to transmit LSDV under natural conditions (Chihota et al. 2001). In all of the above mentioned studies, mechanical transmission was suspected since virus

replication was not demonstrated in the potential vectors (Chihota et al. 2001, Hunter & Wallace 2001).

During the outbreak of LSD in Sudan in 1971, affected animals were observed to be heavily infested with ticks, mainly of *Amblyomma* spp. (Ali & Obeid 1977). *Rhipicephalus appendiculatus, R. (B) decoloratus* and *A. hebraeum* species of ticks have been experimentally implicated in the transmission of the virus, where LSDV DNA was detected from feeding sites for ticks that had earlier fed on infected animals (Tuppurainen et al. 2011, Tuppurainen & Oura 2012). Mechanical/ intrastadial transmission of LSDV by *R. appendiculatus* has recently been demonstrated (Tuppurainen et al. 2013)

Ticks are known to transmit a wide range of pathogens (Jongejan et al. 1980, Kaufman 1989). The life cycle of ixodid (hard) ticks, where each active stage from larvae, nymphs to adults requires a blood meal from a vertebrate host for their development, enables them to transmit pathogens between different life cycle stages by transstadial transmission and through eggs by transovarial transmission. Interrupted tick feeding within a stage, common but not restricted to males, may cause mechanical transmission (Little, Hostetler & Kocan 2007). *A. hebraeum* is a 3-host tick that is common in south eastern Africa. The larvae are most active in late summer to autumn. *Amblyomma* spp. over-winter as nymphs, while adults become active during early summer. It is the principle vector of *Erhlichia ruminantium*, the causative agent of heartwater in ruminants (Bryson et al. 2002, Neitz 1937, Walker & Olwage 1987). It has also been demonstrated to transmit Crimean Congo haemorrhagic fever virus (CCHFV) (Shepherd et al. 1989), the bacteria *Rickettsia africae* and *R. conori* as well as *Theileria mutans* (Walker & Olwage 1987).

In this experiment, the potential role of *A. hebraeum* ticks in mechanical/ intrastadial and transstadial transmission of LSDV was investigated. Ticks were fed on two cattle artificially infected with LSDV (donor cattle) and subsequently transferred to naïve recipient cattle. Successful transmission of LSDV to recipient animals was determined through monitoring of clinical signs and laboratory detection of LSDV by real-time polymerase chain reaction (PCR), the serum neutralisation test (SNT) and virus isolation (VI).

3.2. Materials and Methods

3.2.1. Cattle

Seven seronegative Bonsmara cattle (a breed originating from crossbreeding *Bos indicus* and *Bos taurus* cattle) 18 months old and weighing between 190 and 250 kg were purchased from a farm in the Pretoria area, South Africa, where vaccination against LSDV is not practised.. The cattle were allowed to acclimatize for 14 days before onset of the trial. Experimentally infected donor animals were kept separately from naïve recipient animals in insect-free stables at the University of Pretoria's Bioscience Research Centre (UPBRC). The experimental procedures for the animals were approved by the Animal Use and Care Committee (AUCC) of the University of Pretoria, South Africa.

3.2.2. Preparation of the virus

The virus used in the study to artificially infect donor animals was a virulent South African LSDV field isolate (248/93) that was propagated for five to six passages on primary bovine dermis cells at the Virology Section of the Department of Veterinary Tropical Diseases (DVTD), UP.

3.2.3. Artificial infection of donor animals

Two donor animals (DA1 and DA2) were infected with the prepared LSDV inoculum. The titre of the virus was 5.95 log $TCID_{50}$/ml. On Day 0, a volume of 2.5 ml of the virus suspension was injected intravenously in the jugular vein and 0.25 ml was injected intradermally at each of four sites on the back of the animals (Tuppurainen et al. 2013).

3.2.4. Ticks transmission studies:

Unfed *A. hebraeum* nymphs and F1 adult ticks were reared at the Agricultural Research Council Onderstepoort Veterinary Institute (ARC-OVI). The ticks were placed to feed on donor and recipient animals inside cotton cloth bags which were fixed using Genkem Contact Adhesive glue. For attachment of nymphs, the bags were attached at the base ears while for attachment of adult ticks, the bags were placed on the back, on top of the inoculation site of the challenge virus for donor cattle. Nymphs and adult ticks were orally challenged to LSDV infection by feeding them on donor animals during viraemia and on skin lesions.

To test for mechanical/ transstadial transmission, seventy five (F1 adult) *A. hebraeum* males were placed in each of two body bags and 100 males in a third body bag attached to the sides

of the donor animals on Day 4 pi. After feeding for 12 to 14 days (Days 16 and 18 pi), 150 males were transferred from donor animals to the recipient animals (RA1 and RA2) within 24 hours of collection. This day is referred to as Day 0 pi for recipient animals RA1 and RA2.

To test for transstadial transmission, 400 to 500 *A. hebraeum* nymphs were placed on the ears of each donor animal. They were collected after feeding to repletion for 7-9 days, on Days 14 to 16 pi. They were incubated for up to 35 days to moult to F2 adults in an acaridarium at 25°C and relative humidity (RH) 85%. The emergent F2 adults were kept under the same conditions for two months. Since *A. hebraeum* females only attach in response to attraction, aggregation and attachment pheromones released by male ticks after feeding (Norval, Andrew & Yunker 1989, Norval et al. 1992, Norval et al. 1996), 200 males were placed on each of the recipient animals RA5 and RA6, on Day 0 pi while 200 females were added to each animal four days later.

3.2.5. Collection of specimens from animals

Blood was collected from the jugular vein in vacutainer tubes containing EDTA for testing by PCR (all animals), heparin containing tubes for virus isolation (recipient animals RA5 and RA6) and tubes without anticoagulant for collection of serum from all animals for SNT. Blood samples were collected from donor animals on the following days pi: 0, 4, 7- 10, 13-17, 20 - 24, 27 - 30. Samples on Day 0 pi were collected before inoculation of the virus as negative control. From recipient animal RA1, blood samples were collected on days 0, - 3, 6 - 10, 13 - 17, 20 and 22, pi and from animal RA2 on days 0, 4 - 8, 11 - 14, 17 and 19 pi. Blood from recipient animals RA5 and RA6 was collected on days 0, 3, 6 - 10, 13 - 17, 20 - 24, 27 - 30 pi. Serum was collected and stored at -20°C. Blood samples collected in EDTA and heparin tubes were stored at -20°C until processed.

3.2.6. Monitoring of clinical signs in animals

Clinical monitoring of animals commenced from the day of arrival. The day of virus inoculation or placement of ticks was designated as Day 0 pi. Each animal was monitored for the following signs: increased temperature, circumscribed skin lesions and enlargement of peripheral lymph nodes. These were recorded daily from Day 0 to Day 30 pi. Depending on the severity of these signs, the disease was classified as severe, i.e. with high fever (body temperature above 40°C) and skin lesions more than 6mm diameter; mild where there was mild fever (39.1°C to 40°C) and small or transient skin lesions (less than 6mm); no disease or inapparent where no clinical signs were detected. Animals that developed severe clinical

signs (such as high fever, above 40°C) and eruptive skin lesions were treated with antibiotics to prevent secondary bacterial infections.

3.2.7. Real-time polymerase chain reaction

Extraction of DNA from blood preserved in EDTA was done according to protocol of Tuppurainen *et al.* (2005). Proteins were digested by adding 2- 4 IU of proteinase K (Roche products, Mannheim, Germany) (10mg/ml) to the samples which were then incubated at 56°C for 2 hours or overnight. The DNA was precipitated in two volumes of ice cold absolute ethanol with 1/10 of 3M sodium acetate.

The extracted DNA was tested by a real-time PCR Taqman assay. This assay amplifies an 89 bp region within the *Capripoxvirus* ORF 74 region (Tulman et al. 2001), which encodes the intracellular mature protein P32 (Bowden et al. 2008). Capripoxvirus-specific primers and probes with the following sequences were used: Forward primer-CaPV074F1 5'-AAA ACG GTA TAT GGA ATA GAGTT GAA-3', Reverse primer- CaPV074R1 5'-AAA TGA AAC CAA TGG ATG GGA TA-3' and Probe CaPV074P1 5'FAM-TGG CTC ATA GAT TTC CT-MGB/NFQ -3' (Bowden et al. 2008).

The reaction was done using a Light Cycler®, 2.0 machine (Roche products, Mannheim, Germany). The reaction mixture comprised 4 µl of 10X FastStart DNA Master^{plus} HybProbe kit (Roche products, Mannheim, Germany), 1 µl (0.1 µM) each of the primers, 1 µl (0.2µM) of hybridization probe, 0.5 µl of UDG, 8.5 µl of water and 4 µl of DNA template (up to 500 ng). The reaction was done according to the procedures described by Bowden *et al.* (2008). Briefly, UDG was activated by raising the temperature to 40°C for 10 minutes followed by a FastStart *Taq* Polymerase enzyme activation step of 10 minutes at 95°C. The amplification comprised 45 cycles of denaturation at 95°C for 10 seconds (s), annealing at 60°C for 60s and extension at 72°C for 1s (single acquisition mode). DNA extracted from known infected cell cultures was used as positive control. Negative controls included a water sample processed through the DNA extraction procedures and a non-DNA template water control. The C_t value 39.36, was established as the highest positive C_t value following the determination of the limit of detection (unpublished data), using a quantitive real-time PCR on 10-fold serially diluted LSDV (4.5 log TCID50/ml) isolated from cell cultures in triplicates based on protocol followed by Polci et al (2007).

3.2.8. Serum neutralisation test

This test was performed on 96-well microtitre plates according to standard protocols (SOP) of the Virology Section of the DVTD, UP, based on guidelines of OIE (2010). Neutralizing antibodies were measured using a constant-virus/varying-serum method and bovine dermis cells. Negative sera controls were collected from research animals before LSDV inoculation or attachment of ticks. Sera from artificially infected (donor) animals were used as positive sera control. Titres were determined as the last dilution that gave a 50% end point.

3.2.9. Virus isolation

Virus isolation was carried out according to a SOP of the Virology Section of the DVTD, UP based on guidelines of OIE (2010). Briefly: 500 µl heparinised blood was inoculated onto bovine dermis cells in 6-well plates and incubated at 37°C for an hour. After washing 3 times with phosphate buffered saline with Ca^{2+} and Mg^{2+} (PBS^{+}) the medium was replaced with 3 ml MEM containing 5% foetal calf serum and 0.1% gentamycin and incubated at 37°C. The cells were observed daily for cytopathic effects (CPE). In the absence of CPE a second or third passage was performed. After the third passage, the cell culture supernatants for each animal were pooled and the virus concentrated using the PEG precipitation kit (Biovision Research Products, Mountain view, USA). A fourth passage of the concentrated virus was performed on bovine dermis cells in 6-well plates. **C**ell culture media were stored at -80°C until used. Cytopathic effects caused by LSDV were confirmed by testing cell culture supernatants of the third and fourth passage with a real-time PCR.

3.3. Results

3.3.1. Donor animals

Donor animal DA1 manifested mild LSD with a fever (40°C) on Day 4 pi and enlarged prescapular and precrural lymph nodes. Donor animal DA2 developed a moderate form of LSD with a high fever (41°C) on Day 3 pi, which persisted at 40°C up to Day 13. DA2 also developed erosive circumscribed skin lesions on the body on Day 7 pi as well as in the nostrils and mouth that led to increased salivation and there was enlargement of precrural lymph nodes on Day 8 pi. Both animals had diarrhoea, from Day 3 until they were treated with Baycox®5% (Bayer pty LTD, Isando, South Africa) (Oral suspension: Toltrazuril 50mg/ml) for possible coccidial infection on Day 8 pi.

Blood samples of both donor animals tested negative by real-time PCR on Day 0 pi. DA1 tested positive between Day 4 and 23 pi. Cycle threshold (C_t) values varied between 35 and 39. Blood samples collected from donor animal DA2 tested positive between Day 4 and 24 pi and C_t values varied between 32 and 38.

Donor animal DA2 started to seroconvert on Day 14 pi while donor animal DA1 started on Day 16 pi, with antibody titres of 1:10 and 1:20 respectively. By Day 23 pi the antibody titre for DA1 was 1:20 and for DA2 was 1:320.

3.3.2. Recipient animals

3.3.2.1. Recipient animals for mechanical/ intrastadial transmission (RA1 and RA2)

Recipient animal RA1 showed a mild raise in body temperature of 39.0°C on Day 2 pi and 39.3°C on Day 11 pi. Enlargement of prescapular and precrural lymph nodes was detected from Day 8 pi onwards. Nodular swellings of the skin (4 mm in diameter) on two feeding sites of *A. hebraeum* males were detected on Day 9 pi, while the skin on the neck area became uneven with small intradermal swellings on Day 10 pi (Table 3.1). Similarly, RA2

Table3.1: Clinical signs observed for recipient animals RA1, RA2, RA5 and RA6

Animal identity	Mode of transmission	Number of ticks attached	Body temp	Pox lesions	Lymph node (LN) swelling	Wet stool	Clinical diagnosis
RA1	Mechanical	150 males	Up to 39.3°C (Day 11 pi)	Small swellings at feeding sites (Day 9 pi), on neck (Day 10 pi)	Mild enlargement: Precrural LN (Day 8 pi), Prescapular (Day 10 pi)	None seen	Mild LSD suspected
RA2	Mechanical	150 males	Up to 39.5°C (Day 6 pi)	Small skin swelling on left side feeding site (Day 17 pi)	Mild enlargement: Precrural and Prescapular LNs (Day 6 pi)	None seen	Mild LSD suspected
RA5	Transstadial	400 ticks (200 males + 200 females)	39.4°C (Day 6 pi), 39.7°C (Day 7 pi), 39.3°C (Day 15, 16 & 21 pi)	Small skin nodules and erosions at site of feeding (start Day 14 pi)	Mild enlargement: Precural LN (Day 2 pi)	From Day 6 to 14 pi	Mild LSD suspected
RA6	Transstadial	400 ticks (200 males + 200 females)	Up to 39.5°C (day 6 pi), 39.3 (Days 7 & 8 pi)	Small skin nodules between feeding sites from Day 8 pi. Necrotic and erosive lesions at feeding site	Severe enlargement: Precrural LN (Day 2 pi)	From Day 6 to 16 pi	Mild LSD

showed a slight raise in body temperature of 39.5°C (Day 6 pi), 39.3°C (Day 7 pi) and 39.2 (Day 12 pi). (Figure 3.1, Table 3.1). The feeding sites developed small skin swellings and there was mild enlargement of the prescapular and precrural lymph nodes (Table 3.1).

Blood samples collected from RA1 tested positive for LSDV by real-time PCR with C_t value 37.6 on Day 8 pi. RA2 blood also tested positive by real-time PCR on Day 6 pi with C_t value of 36.04 (Table 3.2). Neither RA1 nor RA2 seroconverted by SNT and the tick feeding sites tested negative.

Table 3.2: Summary of test results obtained from recipient animals Ra1, RA2, RA5 and RA6

Animal #	**Transmission**	**Real-time PCR**	**SNT**	**Virus isolation (2nd passage)**	**Conclusion**
RA1	Mechanical/ intrastadial	Pos (days 8 pi)	Neg.	Not done	Infected
RA2	Mechanical/ intrastadial	Pos (days 6 pi)	Neg.	Not done	Infected
RA5	Transstadial	Pos (days 10, 13, 17 pi)	Neg.	Pos (days 13, 14, 20, 23, 29)	Infected
RA6	Transstadial	Pos (days 10, 13, 16pi)	Neg.	Pos (days 13, 20, 21, 23, 24, 27, 29 pi)	Infected

Post infection (pi)

3.3.2.2. Recipient animals (RA5 and RA6) for transstadial transmission

Recipient animals RA5 and RA6 exhibited mild clinical signs of LSD. The body temperature for RA5 was raised to 39.4°C on Day 6 pi and to 39.7°C the following day and remained mostly above 39.0°C up to Day 22 (Figure 3.1, Table 3.1). RA5 developed small skin lesions in the area between tick feeding sites on Day 14 pi as well as on the tick feeding sites (Table 3.1). One of the lesions on the tick feeding sites subsequently became necrotic, sloughed off and left a raw wound, which healed following parenteral and topical treatments with antibiotics.

The body temperature for RA6 was 39.5°C on Day 6 pi and remained mostly above 39.0°C up to Day 13 pi and began rising again on Day 19 pi up to Day 28 pi (Table 3.1, Figure 3.1).

Table 3.3: Real time PCR results (and their C_t values) for DNA extracted from EDTA blood samples and Virus isolation (VI) materials from blood of recipient animals RA1, RA2, RA5 and RA6

Day post infection	EDTA blood results				VI media results	
	RA1	RA2	RA5	RA6	RA5	RA6
5						
6		36.04				
7						
8	37.06					
9						
10			36.04	35.84		
11						
12						
13			37.07	37.26	37.8	35.41
14					38.01	
15						
16				35.5		
17			37.06			
18						
19						
20					36.85	36.28
21						36.05
22						
23					34.63	39.09
24						36.27
25						
26						
27						37.33
28						
29					34.6	38.98
30						
31						

Shaded areas show real-time PCR positive samples. C_t values are included in the positive results. VI= virus isolation

Recipient animal RA6 developed small skin lesions in the area adjacent to the tick feeding sites on Day 6 pi. Animals RA5 and RA6 showed swelling of precrural lymph nodes (Figure 3.2) from Day 7 pi. Significant enlargement of this lymph node on RA6 was observed on Day 20 pi. Diarrhoea was also observed in both recipient animals for transstadial transmission (Table 3.1) from Day 3 pi (RA5) and Day 6 pi (RA6). Both animals were treated with Baycox to control the diarrhoea.

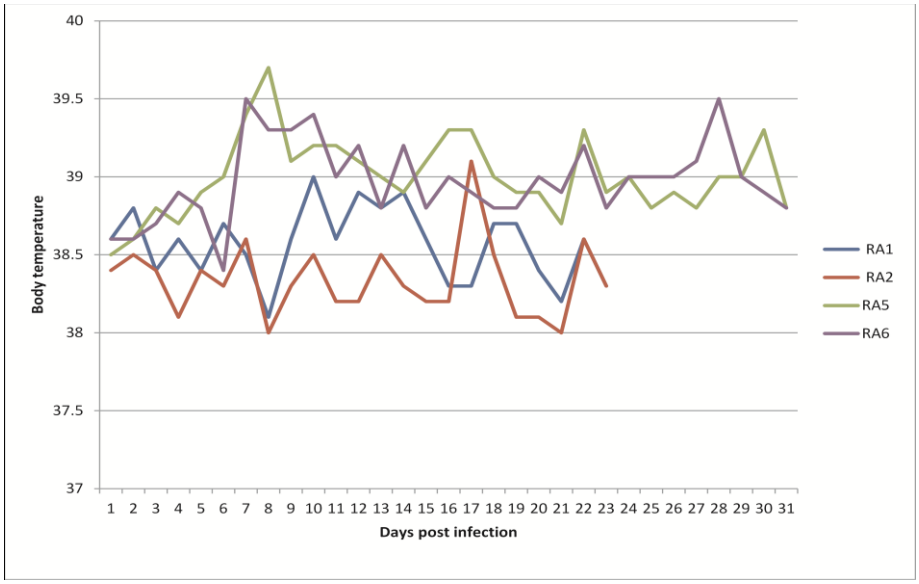

Figure 3.1: Daily body temperature readings for recipient animals RA1, RA2, RA5 AND RA6.

The blood samples collected from RA5 tested positive by real-time PCR on days 10 13 and 17 while RA6 was positive on days 10, 13 and 16 (Table 3).

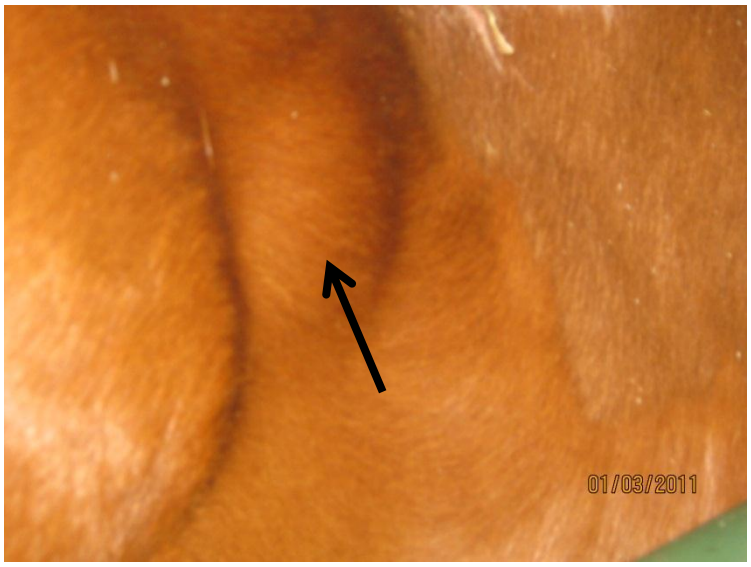

Figure 3.2: Enlarfged precrural lymph node (arrow) on recipient animal RA6 (transstadial transmission)

None of the recipient animals showed detectable seroconversion by SNT. However, LSDV DNA was detected in cell cultures of blood samples for Days 13, 14, 20, 23 and 29 pi for RA5 and Days 13, 20, 21,23, 24, 27 and 29 pi for RA6 (Table 3.3). Cytopathic effect was observed after concentration and a fourth passage of the virus in cell cultures. C_t values by real-time PCR of 21.86 for RA6 and 30.33 for RA5 were obtained for cell culture material after the fourth passaged.

3.4. Discussion

In this study mechanical and transstadial methods of transmission of the virus by *A. hebraeum* ticks are described. Most clinical signs observed in donor and recipient animals such as fever, enlargement of lymph nodes and circumscribed lesions are consistent with those reported by other authors (Carn & Kitching 1995, Coetzer 2004, Kumar 2011, Salib & Osman 2011, Tuppurainen, Venter & Coetzer 2005, Weiss 1960, Weiss 1968). However, no diarrhoea in animals has been reported during outbreaks of the disease. The cause of the diarrhoea, observed in the experimental animals in this study is uncertain. The immediate suspicion was coccidiosis while, on the other hand, its occurrence after LSDV challenge associates it with LSD. Nodules have been reported in the mucosa of the alimentary tract, especially the abomasum, of LSDV infected cattle (Prozesky & Barnard 1982, Weiss 1968). The presence of such nodules could possibly lead to gastro-enteritis and ultimately, inflammatory type of diarrhoea.

Mechanical/ intrastadial transmission of LSDV to recipient animals RA1 and RA2 was indicated by mild clinical signs for LSD such as transient skin swellings, mild lymph node enlargement and mild fever. It was confirmed by the detection of LSDV in the blood samples by real-time PCR, despite not showing detectable antibodies by SNT (Table 3.2). Manifestation of mild or inapparent disease without seroconversion in LSDV infected animals has been described previously (Annandale et al. 2010, Chihota et al. 2001, Irons, Tuppurainen & Venter 2005, Tuppurainen, Venter & Coetzer 2005). The synchronisation in the onset of clinical signs such as temperature rise, enlargement of superficial lymph nodes and skin nodules with the detection of viral DNA from blood samples by real-time PCR between Day 6 and 8 pi suggests that these clinical signs were caused by LSDV infection.

Previously, it has been reported that feeding *A. hebraeum* males were able to transfer viral DNA from the skin of experimentally infected animals to the skin of naïve recipient cattle (Tuppurainen *et al.*, 2011). Mechanical/ intrastadial transmission by ticks occurs when feeding of ticks is interrupted and they attach to another host within the same stage of their life cycle (Kaufman & Nuttall 1996). Interrupted feeding commonly occurs in males in search of females to mate. After attaching and mating, females continue their blood meal until they are engorged and drop off the host. Males reattach after mating, feed and seek other females to mate (Sonenshine 1991). Movement of male *Rhipicephalus sanguineus* between dogs housed in close proximity has been demonstrated (Little, Hostetler & Kocan 2007). The

movement of *Dermacentor andersoni* males has also been observed in cattle (Stiller&Coan 1995). The movement of male ticks towards females may be influenced by sex pheromones released by females (Andrew & Norval 1989, Rechav, Norval & Oliver 1982).

Interrupted feeding of all stages of ticks may occur when they are dislodged because of grooming or death of the host (Wang & Nuttall 2001, Andrew & Norval 1989). Andrew and Norval (1989) showed that dislodged female *A. hebraem* ticks reattach and transmit disease within the same stage successfully. The salivary gland protein profile in partially fed female ticks, whose feeding was interrupted, has been shown to revert back to the none-parasitic stage, which enabled them to successfully reattach and feed (Wang, Henbest & Nuttall 1999).

The developments of clinical signs, characteristic for LSD such as the rise in body temperature, accompanied by enlargement of lymph nodes and skin lesions indicate that transstadial transmission of LSDV to recipient animals RA5 and RA6 may have occurred. The erosive lesions on the tick feeding sites on RA5 and enlargement of the precrural lymph node, especially in RA6 (Figure 3.2), are also indications of the LSD, although ticks may also cause physical damage to the skin (Rajput et al. 2006); especially *A. hebraeum* causes damage to the skin due to deep penetrating mouth parts (Walker 2003, Walker 1994). Therefore, the erosive lesions on the tick feeding sites and superficial lymph node enlargement may have been, in part, caused by feeding of *A. hebraeum*. Although RA5 and RA6 like RA1 and RA2, did not show detectable SNT seroconversion, the positive results obtained by real-time PCR and virus isolation confirm the presence of the virus in these animals (Table 3.2).

Transstadial transmission of pathogens is common among 2 and 3- host ticks, where ticks infected as either larvae or nymphs transmit pathogens to new hosts that they feed on after moulting to nymphs or adults, respectively. *A. hebraeum* has been reported to transstadially transmit *E. ruminantium*, the cause of heartwater (Andrew & Norval 1989, Bryson et al. 2002, Kocan, Norval & Donovan 1993, Neitz 1937, Norval 1991). The transstadial transmission of viruses such as CCHFV and Thogoto virus by *A. hebraeum* has also been demonstrated (Kaufman & Nuttall 1996, Shepherd et al. 1989).

Transstadial transmission of LSDV by *A. hebraeum* may have important epidemiological implications for LSD. Since *A. hebraeum* ticks over-winter as nymphs (Walker 2003), the virus may over-winter in engorged nymphs and the emergent unfed adults and be a source of outbreaks when the adults attach to animals in summer. In the Eastern Cape Province of

South Africa, the life cycle of *A. hebraeum* has been shown to extend up to 3 years (Norval 1977). Such long life cycles may permit the ticks to act as reservoirs of LSDV. Infected ticks may be spread over large areas by movement of domestic and wild animals and in this way may help to disseminate the virus over long distances.

In all cases where recipient animals tested positive for LSDV by real-time PCR, the C_t values obtained ranged from 35 to 39 indicating that the DNA copy number was low. This range of C_t values was also obtained from the tests of donor animals, which varied from 35 to 39 for DA1 and 32 to 38 for DA2. On developing this assay, Bowden *et al.* (2008) did quantification of 10-fold serial dilutions of plasmid template. They designated all real-time PCR reactions with C_t values greater than 37 and less than 45 as positive with a template copy number of less than 10. In this study, the limit of detection for real-time PCR, based on 10-fold serial dilutions of LSDV (4.5 log TCID 50/ml) was found at 10^{-3}TCID50/ml with C_t value of 39.36 (unpublished data). The detection of low viraemia by both PCR and VI, as well as the absence of SNT detectable antibodies, accompanied by mild clinical signs, suggests that a very low copy number of the LSDV is transmitted by *A. hebraeum* ticks under natural conditions and this may cause only inapparent or transient infections. The development of LSDV DNA from blood samples and the presence of skin lesions in areas remote to the site of inoculation suggest that the infection was generalised. Carn and Kitching (1995) reported that generalised infections were mostly caused by parenteral inoculations. The long mouthparts of *A. hebraeum* (Walker 2003)**,** allows for deep penetration into the vascular-enriched dermis and subcutis.

Blood obtained from recipient animals had virus titres between 10^{-1}log $TCID_{50}$/ml and 10^{-3}log $TCID_{50.}$ High C_t values by real-time PCR were also obtained from cell culture material of these samples (Table 3.3). These results suggest poor infectivity to the mammalian cells by LSDV following passaging in the tick and could have been influenced by variations in the genetic determinants for viral replication and pathogenicity between the tick cells and mammalian host cells as demonstrated for tick borne flaviviruses following exposure to *Ixodes ricinus* (Labuda et al. 1994, Mitzel et al. 2007, Mitzel et al. 2008).

This study shows the potential for LSDV transmission by *A. hebraeum* and introduces the possible roles of *A. hebraeum* in the epidemiology of the disease. The low viraemia detected in recipient animals suggests that transmission by *A. hebraeum* requires contribution of other vectors to increase the infective doses in order to result in severe disease during outbreaks. It

has been shown that adult *A. hebraeum* ticks attach in summer (Norval 1977, Rechav, Norval & Oliver 1982), during wet conditions when flying insects are abundant to cause LSD outbreaks (Ali & Obeid 1977, MacOwan 1959, Von Backström 1945, Weiss 1968). Ticks may therefore play a maintenance role, i.e. help to introduce the virus. Amongst the arthropod vectors, ticks are better suited to play the maintenance role because they can survive for long periods off the host using stored nutrients from previous blood meals (Agbede, Kemp & Hoyte 1986, Coons et al. 1986, Sonenshine 1991, Tarnowski & Coons 1989). Ticks can remain hydrated by absorbing atmospheric water vapour with the aid of hygroscopic chemicals released by type 1 acini of their salivary glands (Bowman & Sauer 2004, Sonenshine 1991) and have capacity to diapause during winter until weather conditions are favourable in summer (Belozerov 2008, Madder et al. 1999, Speybroeck et al. 2003). This may explain where LSDV survives in-between outbreaks.

This report demonstrates further evidence of mechanical transmission of LSDV by *A. hebraeum*. It strengthens observations of mechanical transmission by *R. appendiculatus* previously reported (Tuppurainen et al. 2013). It also provides the first evidence of biological transmission by *A. hebraeum* ticks. Further work is required to investigate in detail the vector competence of *A. hebraeum* for LSDV and other biological factors that may affect host (cattle) response to tick-borne LSDV infection.

3.5. References

Agbede, R.I.S., Kemp, D.H. & Hoyte, H.M.D., 1986, 'Secretory and digest cells of female *Boophilus microplus*: invasion and development of *Babesia bovis*; light and electron microscope studies.', *Morphology, physiology, and behavioral biology of ticks* 457-471.

Ali, A.A., Esmat, M., Attia, H., Selim, A. & Abdel-Hamid, Y., 1990, 'Clinical and pathological studies of lumpy skin disease in Egypt', *Veterinary Record* 127(22), 549-550.

Ali, B.H. & Obeid, H.M., 1977, 'Investigation of the first outbreaks of lumpy skin disease in the Sudan', *British Veterinary Journal* 133(2), 184-189.

Andrew, H.R. & Norval, R.A.I., 1989, 'The role of males of the bont tick (*Amblyomma hebraeum*) in the transmission of *Cowdria ruminantium* (heartwater)', *Veterinary parasitology* 34(1-2), 15-23.

Annandale, C.H., Irons, P.C., Bagla, V.P., Osuagwuh, U.I. & Venter, E.H., 2010, 'Sites of persistence of lumpy skin disease virus in the genital tract of experimentally infected bulls', *Reproduction in Domestic Animals* 45(2), 250-255.

Awad, W.S., Ibrahim, A.K., Mahran, K., Fararh, K.M. & Abdel Moniem, M.I., 2010, 'Evaluation of different diagnostic methods for diagnosis of Lumpy skin disease in cows', *Tropical animal health and production* 42(4), 777-783.

Awadin, W., Hussein, H., Elseady, Y., Babiuk, S. & Furuoka, H., 2011, 'Detection of lumpy skin disease virus antigen and genomic DNA in formalin-fixed paraffin-embedded tissues from an Egyptian outbreak in 2006.', *Transboundary and Emerging Diseases* 58(5), 451-457.

Belozerov, V.N., 2008, 'Diapause and quiescence as two main kinds of dormancy and their significance in life cycles of mites and ticks (Chelicerata: Arachnida: Acari). Part 1. Acariformes.', *Acarina* 16(2), 79-130.

Bowden, T.R., Babiuk, S.L., Parkyn, G.R., Copps, J.S. & Boyle, D.B., 2008, 'Capripoxvirus tissue tropism and shedding: a quantitative study in experimentally infected sheep and goats', *Virology* 371(2), 380-393.

Bowman, A.S. & Sauer, J.R., 2004, 'Tick salivary glands: function, physiology and future.', *Parasitology* 129(Supplement), S67-S81.

Bryson, N.R., Horak, I.G., Venter, E.H., Mahan, S.M., Simbi, B. & Peter, T.F., 2002, 'The prevalence of *Cowdria ruminantium* in free-living adult *Amblyomma hebraeum* collected at a communal grazing area and in 2 wildlife reserves in South Africa.', *Journal of the South African Veterinary Association* 73(3), 131-132.

Burdin, M.L., 1959, 'The use of histo-pathological examinations of skin material for the diagnosis of lumpy skin disease in Kenya', *Bulletin of epizootic diseases of Africa. Bulletin des epizooties en Afrique* 7 27-36.

Carn, V.M. & Kitching, R.P., 1995, 'The clinical response of cattle experimentally infected with lumpy skin disease (Neethling) virus', *Archives of Virology* 140(3), 503-513.

Chihota, C.M., Rennie, L.F., Kitching, R.P. & Mellor, P.S., 2003, 'Attempted mechanical transmission of lumpy skin disease virus by biting insects', *Medical and veterinary entomology* 17(3), 294-300.

Chihota, C.M., Rennie, L.F., Kitching, R.P. & Mellor, P.S., 2001, 'Mechanical transmission of lumpy skin disease virus by *Aedes aegypti* (Diptera: Culicidae)', *Epidemiology and infection* 126(2), 317-321.

Coetzer, J.A.W., 2004, 'Lumpy skin disease' in pp. 1268-1276, Oxford University Press, Oxford.

Coons, L.B., Rosell-Davis, R., Tarnowski, B.I. & Davis, R.R., 1986, 'Bloodmeal digestion in ticks.', *Morphology, physiology, and behavioral biology of ticks* 248-279.

Davies, F.G., 1981, *Lumpyskin disease*, , Academic Press, London.

Fenner, F.J. & White, O.D., 1976, *Medical Virology*, second edn., Academic Press, A subsidiary of Harcourt Brace Jovanovich, Publishers, New York.

Fenner, F., 1987, *Veterinary virology*, Academic Press, Orlando.

House, J.A., Wilson, T.M., el Nakashly, S., Karim, I.A., Ismail, I., el Danaf, N., Moussa, A.M. & Ayoub, N.N., 1990, 'The isolation of lumpy skin disease virus and bovine herpesvirus-4 from cattle in Egypt', *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc* 2(2), 111-115.

Hunter, P. & Wallace, D., 2001, 'Lumpy skin disease in southern Africa: a review of the disease and aspects of control', *Journal of the South African Veterinary Association* 72(2), 68-71.

Irons, P.C., Tuppurainen, E.S. & Venter, E.H., 2005, 'Excretion of lumpy skin disease virus in bull semen', *Theriogenology* 63(5), 1290-1297.

Jongejan, F., Perie, N.M., Franssen, F.F.J. & Uilenberg, G., 1980, 'Artificial infection of Rhipicephalus appendiculatus with Theileria parva by percutaneous injection', *Research in veterinary science* 29(3), 320-324.

Kaufman, W.R., 1989, 'Tick-host interaction: a synthesis of current concepts.', *Parasitology Today* 5(2), 47-56.

Kaufman, W.R. & Nuttall, P.A., 1996, '*Amblyomma variegatum* (Acari: Ixodidae): mechanism and control of arbovirus secretion in tick saliva', *Experimental parasitology* 82(3), 316-323.

Kitching, R.P. & Mellor, P.S., 1986, 'Insect transmission of capripoxvirus', *Research in veterinary science* 40(2), 255-258.

Kocan, K.M., Norval, R.A.I. & Donovan, P.L., 1993, 'Development and transmission of *Cowdria ruminantium* by *Amblyomma* males transferred from infected to susceptible sheep.', *Revue d'elevage et de medecine veterinaire des pays tropicaux* 46(1/2), 183-188.

Kumar, S.M., 2011, 'An outbreak of lumpy skin disease in a Holstein dairy herd in Oman: a clinical report.', *Asian Journal of Animal and Veterinary Advances* 6(8), 851-859.

Labuda, M., Jiang, W.R., Kaluzova, M., Kozuch, O., Nuttall, P.A., Weismann, P., Eleckova, E., Zuffova, E. & Gould, E.A., 1994, 'Change in phenotype of tick-borne encephalitis-virus following passage in *Ixodes ricinus* Ticks and Associated Amino acid substitution in the envelope protein', *Virus research* 31(3), 305-315.

Little, S.E., Hostetler, J. & Kocan, K.M., 2007, 'Movement of Rhipicephalus sanguineus adults between co-housed dogs during active feeding.', *Veterinary parasitology* 150(1/2), 139-145.

MacOwan, K.D.S., 1959, 'Observations on the epizootiology of lumpy skin disease during the first year of its occurrence in Kenya', *Bulletin of epizootic diseases of Africa. Bulletin des epizooties en Afrique* 7 7-20.

Madder, M., Speybroeck, N., Brandt, J. & Berkvens, D., 1999, 'Diapause induction in adults of three *Rhipicephalus appendiculatus* stocks.', *Experimental & applied acarology* 23(12), 961-968.

Mitzel, D.N., Best, S.M., Masnick, M.F., Porcella, S.F., Wolfinbarger, J.B. & Bloom, M.E., 2008, 'Identification of genetic determinants of a tick-borne flavivirus associated with host-specific adaptation and pathogenicity', *Virology* 381(2), 268-276.

Mitzel, D.N., Wolfinbarger, J.B., Long, R.D., Masnick, M., Best, S.M. & Bloom, M.E., 2007, 'Tick-borne flavivirus infection in Ixodes scapularis larvae: development of a novel method for synchronous viral infection of ticks.', *Virology* 365(2), 410-418.

Neitz, W.O., 1937, 'The Transmission of Heartwater to and from Biesbuck (Damaliscus albifrons) by means of the Bont-Tick (*Amblyomma hebraeum*).', *Onderstepoort Journal of Veterinary Science* 9 87-46.

Norval, R.A.I., 1991, *Heartwater disease,* , Grosvenor Press International Ltd, London.

Norval, R.A.I., 1977, 'Ecology of the tick *Amblyomma hebraeum* Koch in the Eastern Cape Province of South Africa. I. Distribution and seasonal activity', *Journal of Parasitology* 63(4), 734-739.

Norval, R.A.I., Andrew, H.R. & Yunker, C.E., 1989, 'Pheromone-mediation of host-selection in bont ticks (*Amblyomma hebraeum* Koch).', *Science (Washington)* 243(4889), 364-365.

Norval, R.A.I., Peter, T., Sonenshine, D.E. & Burridge, M.J., 1992, 'Responses of the ticks *Amblyomma hebraeum* and *A. variegatum* to known or potential components of the aggregation-attachment pheromone. III. Aggregation', *Experimental & applied acarology* 16(3), 237-245.

Norval, R.A.I., Sonenshine, D.E., Allan, S.A. & Burridge, M.J., 1996, 'Efficacy of pheromone-acaricide-impregnated tail-tag decoys for controlling the bont tick, *Amblyomma hebraeum* (Acari: Ixodidae), on cattle in Zimbabwe', *Experimental & applied acarology* 20(1), 31-46.

Plowright, W. & Ferris, R.D., 1958, 'The growth and cytopathogenicity of sheep-pox virus in tissue cultures.', *Brit.J.Exper.Path.* 39 424-435.

Plowright, W. & Witcomb, M.A., 1959, 'The growth in tissue cultures of a virus derived from lumpy-skin disease of cattle.', *Journal of Pathology and Bacteriology* 78 397-407.

Prozesky, L. & Barnard, B.J., 1982, 'A study of the pathology of lumpy skin disease in cattle', *The Onderstepoort journal of veterinary research* 49(3), 167-175.

Rajput, Z.I., Hu SongHua, Chen WanJun, Arijo, A.G. & Xiao ChenWen, 2006, 'Importance of ticks and their chemical and immunological control in livestock.', *Journal of Zhejiang University (Science B)* 7(11), 912-921.

Rechav, Y., Norval, R.A.I. & Oliver, J.H.,Jr., 1982, 'Interspecific mating of *Amblyomma hebraeum* and *Amblyomma variegatum* (Acari: Ixodidae).', *Journal of medical entomology* 19(2), 139-142.

Salib, F.A. & Osman, A.H., 2011, 'Incidence of lumpy skin disease among Egyptian cattle in Giza governorate, Egypt', *Veterinary World* 4(4), 162-167.

Shepherd, A.J., Swanepoel, R., Cornel, A.J. & Mathee, O., 1989, 'Experimental studies on the replication and transmission of Crimean-Congo hemorrhagic fever virus in some African tick species.', *American Journal of Tropical Medicine and Hygiene* 40(3), 326-331.

Sonenshine, D.E., 1991, *Biology of ticks*, Oxford University Press, Oxford.

Speybroeck, N., Madder, M., Brandt, J., Chungu, H., Bossche, P.v.d., Mbao, V. & Berkvens, D., 2003, 'Questing activity of *Rhipicephalus appendiculatus* (Acari: Ixodidae) nymphs: a random process?', *Physiological Entomology* 28(4), 356-361.

Stiller, D. & Coan, M.E., 1995, 'Recent developments in elucidating tick vector relationships for anaplasmosis and equine piroplasmosis.', *Veterinary parasitology* 57(1/3), 97-108.

Tarnowski, B.I. & Coons, L.B., 1989, 'Ultrastructure of the Midgut and Blood Meal Digestion in the Adult Tick *Dermacentor variabilis*', *Experimental & applied acarology* 6(4), 263-289.

Thomas, A.D. & Mare, C., 1945, 'Knop-velsiekte', *Journal of the South African Veterinary Medical Association* 16 36-43.

Tulman, E.R., Afonso, C.L., Lu, Z., Zsak, L., Kutish, G.F. & Rock, D.L., 2001, 'Genome of lumpy skin disease virus', *Journal of virology* 75(15), 7122-7130.

Tuppurainen, E.S.M., Lubinga, J.C., Stoltsz, W.H., Troskie, M., Carpenter, S.T., Coetzer, J.A.W., Venter, E.H. & Oura, C.A.L., 2013, 'Mechanical transmission of lumpy skin

disease virus by *Rhipicephalus appendiculatus* male ticks', *Epidemiology and infection, Epidemiol.Infect.* 141, 2 425-430.

Tuppurainen, E.S., Venter, E.H. & Coetzer, J.A., 2005, 'The detection of lumpy skin disease virus in samples of experimentally infected cattle using different diagnostic techniques', *The Onderstepoort journal of veterinary research* 72(2), 153-164.

Tuppurainen, E.S.M. & Oura, C.A.L., 2012, 'Review: lumpy skin disease: an emerging threat to Europe, the Middle East and Asia.', *Transboundary and Emerging Diseases* 59(1), 40-48.

Tuppurainen, E.S.M., Stoltsz, W.H., Troskie, M., Wallace, D.B., Oura, C.A.L., Mellor, P.S., Coetzer, J.A.W. & Venter, E.H., 2011, 'A potential role for ixodid (hard) tick vectors in the transmission of lumpy skin disease virus in cattle', *Transboundary and Emerging Diseases* 58(2), 93-104.

Van Rooyen, P.J., Kumm, N.A.L., Weiss, K.E. & Alexander, R.A., 1959, 'A preliminary note on the adaptation of a strain of lumpy skin disease virus to propagation in embryonated eggs.', *Bull.Epizoot.Dis.Afr.* 7 79-85.

Von Backström, U., 1945, 'Ngamiland cattle disease. Preliminary report on a new disease, the etiological agent being probably of an infectious nature', *Journal of the South African Veterinary Medical Association* 16 29-35.

Walker, A., 2003, *Ticks of domestic animals in Africa : a guide to identification of species*, Bioscience Reports, Edinburgh.

Walker, A., 1994, *The arthropods of humans and domestic animals : a guide to preliminary identification*, 1st edn., Chapman & Hall, London.

Walker, J.B. & Olwage, A., 1987, 'The tick vectors of Cowdria ruminantium (Ixodoidea, Ixodidae, genus Amblyomma) and their distribution.', *Onderstepoort Journal of Veterinary Research* 54(3), 353-379.

Wang, H., Henbest, P.J. & Nuttall, P.A., 1999, 'Successful interrupted feeding of adult *Rhipicephalus appendiculatus* (Ixodidae) is accompanied by reprogramming of salivary gland protein expression.', *Parasitology* 119(2), 143-149.

Wang, H. & Nuttall, P.A., 2001, 'Intra-stadial tick-borne Thogoto virus (Orthomyxoviridae) transmission: accelerated arbovirus transmission triggered by host death.', *Parasitology* 122(4), 439-446.

Weiss, K.E., 1968, 'Lumpy skin disease virus', *Virology Monographs* 3 111-131.

Weiss, K.E., 1960, *The persistence of lumpy skin disease viruses in bovine tissues*, Muguga, Kenya: Inter-african Bureau of Animal Health. Paris: Office International des Epizootics.

Woods, J.A., 1988, 'Lumpy skin disease--a review', *Tropical animal health and production* 20(1), 11-17.

Yeruham, I., Nir, O., Braverman, Y., Davidson, M., Grinstein, H., Haymovitch, M. & Zamir, O., 1995, 'Spread of lumpy skin disease in Israeli dairy herds', *The Veterinary record* 137(4), 91-93.

Chapter 4

Detection of lumpy skin disease virus in saliva of ticks fed on lumpy skin disease virus-infected cattle

Published online by Experimental and Applied Acarology: ISSN 0168-8162

Doi: 10.1007/s 10493-013-9679-5

4.1. Abstract

Lumpy skin disease is an economically important disease of cattle that is caused by the lumpy skin disease virus (LSDV), which belongs to the genus *Capripoxvirus*. It is endemic in Africa and outbreaks have also been reported in the Middle-East. Transmission has mostly been associated with blood-feeding insects but recently, the authors have demonstrated mechanical transmission by *Rhipicephalus appendiculatus* as well as mechanical/ intrastadial and transstadial transmission by *Amblyomma hebraeum*. Saliva is the medium of transmission of pathogens transmitted by biting arthropods and, simultaneously, it potentiates infection in the vertebrate host. This study aimed to detect LSDV in saliva of *A. hebraeum* and *R. appendiculatus* adult ticks fed, as nymphs or as adults, on LSDV-infected animals, thereby also demonstrating transstadial or mechanical / intrastadial passage of the virus in these ticks. Saliva samples were tested for LSDV by real-time PCR and virus isolation. Supernatants obtained from virus isolation were further tested by real-time PCR to confirm that the cytopathic effects observed were due to LSDV. Lumpy skin disease virus was detected, for the first time, in saliva samples of both *A. hebraeum* and *R. appendiculatus* ticks. At the same time, mechanical / intrastadial and transstadial passage of the virus was demonstrated and confirmed in *R. appendiculatus* and *A. hebraeum*.

Key words: Lumpy skin disease virus, ticks, saliva, transstadial, intrastadial transmission

4.2. Introduction

Lumpy skin disease virus is a member of the genus *Capripoxvirus* in the family *Poxviridae* (Weiss 1968, Woods 1988). It is the causative agent of lumpy skin disease (LSD), an economically important disease of cattle. The disease is characterised by high fever (up to 41.5°C), lachrymation, salivation, development of circumscribed skin nodules and enlarged lymph nodes (Von Backström 1945, Weiss 1968). Currently, it is widely accepted that LSD is transmitted mechanically by blood-feeding insects such mosquitoes and stable flies (Carn & Kitching 1995, Chihota et al. 2001, Chihota et al. 2003). This is supported by earlier observations that associated most outbreaks with high abundance of biting flies such as in areas along water courses and during wet seasons (Von Backström 1945, Weiss 1968). Transmission of LSDV by *Aedes aegypti* mosquitoes has been demonstrated (Chihota et al. 2001).

Recent studies have shown mechanical and transstadial transmission by the three-host ticks *Rhipicephalus appendiculatus* and *Amblyomma hebraeum* as well as transovarial transmission by *Rhipicephalus (Boophilus) decoloratus* (Tuppurainen et al. 2013); Tuppurainen *et al*, submitted; Lubinga *et al*. submitted).

Saliva is a medium of transmission of pathogens in biting arthropods (Jones et al. 1992, Titus & Ribeiro 1990) and the salivarian route of transmission of pathogens has been supported by the detection of pathogens in arthropod salivary glands (Kaufman, Bowman & Nuttall 2001, Kaufman & Nuttall 2003) and confirmed by demonstration of pathogens in arthropod saliva (Kaufman & Nuttall 1996, Kaufman, Bowman & Nuttall 2001).

Tick saliva is secreted by paired salivary glands that are situated antero-laterally in the hemocoele of the tick (Gill & Walker 1987, Gill & Walker 1988). Saliva functions to help the tick successfully obtain its blood meal over protracted periods. It contains pharmacological substances that enable the tick to adhere firmly to the host. The pharmacological substances in tick saliva also counter the vertebrate host mechanisms that respond to injury created by the tick mouth parts, they ensure a copious flow of blood and counter or evade the immune and inflammatory response by the host that would lead to rejection of the tick (Jones, Kaufman & Nuttall 1992, Mans & Neitz 2004, Wikel 1984).

Tick saliva may contain pathogens that were taken up during a previous blood meal. These pathogens reach the saliva in the salivary glands from the hemocoele after passing through

and/or infecting the midgut and other organs in transstadial and intrastadial transmission (Kocan & Bezuidenhout 1987, Kocan & Stiller 1992). Mechanical passage of pathogens in ticks is indicated to occur where the virus is passed to the host by the tick without multiplying in the vector (Chihota et al. 2001). For example, when blood is regurgitated from the midgut back, through mouthparts, to the host (Brown 1988, Connat 1991) and possibly when the pathogen present in the tick mouth parts (preoral cavity) contaminates the saliva as saliva is passed into the host.

The immuno-modulatory effect of tick saliva on the vertebrate host lowers the host immune response to infections and potentiates transmission of pathogens to the vertebrate host during blood meals (Jones et al. 1992, Jones, Kaufman & Nuttall 1992, Wikel 1999).

In order to support observations that hard (ixodid) ticks play a role in the transmission of LSDV (Tuppurainen et al. 2011) and further investigate the mechanical / intrastadial and transstadial transmission of the virus by *R. appendiculatus* males and *A. hebraeum* ticks (Tuppurainen et al. 2013, Tuppurainen et al. 2011), this study was designed to demonstrate the presence of the virus in saliva of adult *A. hebraeum* and *R. appendiculatus* ticks fed on LSDV-infected animals by the use of virus isolation (VI) and real-time PCR.

4.3. Materials and methods

4.3.1. Experimentally infected host animals

Four sero-negative Bonsmara cattle, purchased in areas around Pretoria from LSD free herds, which had never been vaccinated against LSDV, were used as host animals to infect the ticks. These cattle tested negative for antibodies to LSDV by the serum neutralisation test (SNT). The cattle were kept in insect-free stables at the University of Pretoria's Bioscience Research Centre (UPBRC). They were brought to the facility 14 days before commencement of the experiment to acclimatise to the environment. The cattle were infected with a known virulent strain of LSDV (V248/93) that has been passaged five or six times in bovine dermis cells. The final titre of the virus suspension was 5.95 log $TCID_{50}$/ml. Each animal was injected intravenously into the jugular vein with 2.5ml virus suspension and 0.25 ml intradermally at each of four sites on the back of the animals (Tuppurainen et al. 2011). This was designated as Day 0 post infection (pi). The experimental procedures for the animals were approved by the Animal Use and Care committee (AUCC) of the University of Pretoria, South Africa

4.3.2. Infection of ticks

Two cattle (DA1 and DA2) were used to host *A. hebraeum* nymphs and adults, the other two (DR1 and DR2) for *R. appendiculatus* nymphs and adults.

On Day 4 pi, 100 *R. appendiculatus* nymphs were placed on one ear of each of the host animals (DR1 and DR2) and 100 *A. hebraeum* nymphs were similarly placed on ears of DA1 and DA2 inside cotton cloth bags, which were attached to shaven areas at the base of the ears with Genkem Contact Adhessive glue. Engorged nymphs were collected after feeding to repletion for approximately 5-6 days (*R. appendiculatus*) and 7-8 days (*A. hebraeum*). The engorged nymphs were incubated at 25°C, relative humidity (RH) 85% to moult into F2 adults between 17 and 24 days (*R. appendiculatus*) and 30 to 35 days (*A. hebraeum*). They were stored under these conditions until testing, for approximately a month. The *R. appendiculatus* F2 adults (n=85) and *A. hebraeum* F2 adults (n=50) were induced to release saliva for LSDV testing as described for the collection of saliva below. The presence of LSDV in saliva of these adult ticks would demonstrate transstadial passage of the virus in the ticks.

In order to test for mechanical / intrastadial passage of LSDV, laboratory-reared unfed *R. appendiculatus* F1 adults were placed on one ear of each of the host animals (DR1 and DR2) on Day 4 pi. After feeding for 4 days, 14 F1 adults were collected and then induced to salivate 3 to 5 days after collection. Similarly, 50 *A. hebraeum* F1 males were placed in body bags attached to the back of the host animals (DA1 and DA2) over the virus inoculation sites on Day 4 pi. Fifty female ticks were placed into the body bags 4 days after males (Day 8 pi) since females only attach to hosts in response to attachment pheromones released by feeding male ticks (Norval, Andrew & Yunker 1989, Norval et al. 1992, Stachurski 2006). Fourteen partially fed F1 adults were collected and induced to salivate 4 days after attachment of female ticks. All ticks were washed and dried after collection.

4.3.3. Collection of saliva from ticks

The adult ticks were induced to secrete saliva using previously published protocols (Chalaire et al. 2011, Ribeiro et al. 2004). Briefly, the F1 and F2 adult ticks (*R. appendiculatus* and *A. hebraeum*), collected for saliva testing as described above, were washed twice in phosphate buffered saline (PBS) to reduce surface contamination. After drying, 5 µl pilocarpine hydrochloride (Sigma-Aldrich, 50 mg/ml in 95% ethanol) was dropped on to the scutum of male ticks or alloscutum of female ticks. After the solution evaporated, the ticks were placed

in clean 0.5 ml Eppendorf centrifuge tubes (Merck Millipore, South Africa) and incubated at 37°C for 3 hours. To collect the saliva, a small hole was made with a needle at the bottom of the tube and the tube was inserted into a 1.5 ml Eppendorf centrifuge tube. The saliva that was attached to the tick was washed off by placing 5 µl phosphate buffered saline (PBS) on the tick in the 0.5 ml Eppendorf tube and centrifuged at about 4400 rpm (Wealtec, E-Centrifuge). Saliva was collected in the 1.5 ml Eppendorf tubes and then stored at -20°C until processed by either DNA extraction or virus isolation.

Of the 85 saliva samples for *R. appendiculatus* F2 adults, 80 were pooled (n=16 pools of 5 samples each) and tested by real-time PCR and five saliva samples were individually tested by VI. The saliva samples (n=45) for *A. hebraeum* F2 adults (transstadial passage) were pooled (n=9 pools) and tested by real-time PCR. The other 5 samples were tested individually by virus isolation. Fourteen saliva samples from each of *R. appendiculatus* and *A. hebraeum* F1 adults (mechanical / intrastadial passage) were tested by virus isolation.

4.3.4. DNA extraction from tick saliva

DNA extraction from saliva of tick pooled samples was carried out according to a protocol used for blood (Tuppurainen, Venter & Coetzer 2005). Since the pooled samples of saliva amounted to approximately 40 µl each, 160 µl of phosphate buffered saline (PBS) was added to make up to a volume of 200 µl. Proteins were digested with 2-4 IU of proteinase K (Roche Products, Mannheim, Germany) and incubated with lysis buffer at 56°C for 2 hours. DNA was precipitated in two volumes of ice cold absolute ethanol with 1/10 volumes of 3 M sodium acetate. DNA was collected and stored at -20°C until analysed.

4.3.5. Real-time PCR

The extracted DNA was tested by real-time PCR using a Taqman assay. This assay amplifies an 89 bp region within the ORF 74 region of capripoxviruses, which encodes the intracellular mature protein P32 (Bowden et al. 2008). Capripoxvirus-specific primers and probes with the following sequences were used: forward primer-CaPV074F1 5'-AAA ACG GTA TAT GGA ATA GAG TTG GAA-3', reverse primer- CaPV074R1 5'-AAA TGA AAC CAA TGG ATG GGA TA-3' and probe CaPV074P1 5'-6FAM-TGG CTC ATA GAT TTC CT-MGBNFQ -3' (Bowden et al. 2008). The reaction was carried out using a Light Cycler®, 2.0 instrument (Roche Diagnostics, Mannheim, Germany). The reaction mixture comprised 4 µl of 10X Faststart DNA Master^{plus} Hybridization Probes Mix (Roche Products), 1 µl (0.1 µM) each of the primers, 1 µl (0.2 µM) hybridization probes, 0.5 µl Uracil-DNA Glycosylase (UDG)

(Roche Products), 8.5 µl water and 4 µl DNA (up to 500 ng). The reaction was performed according to procedures described by Bowden et al. (Bowden et al. 2008). Briefly, UDG was activated by raising the temperature to 40°C for 10 minutes, followed by a FastStart *Taq* Polymerase enzyme activation step of 10 minutes at 95°C. The amplification comprised 45 cycles of denaturation at 95°C for 10s, annealing at 60°C for 60s and extension at 72°C for 1s (single acquisition mode).

4.3.6. Virus isolation

Virus isolation was carried out according to a standard procedure of the Virology Section of the Department of Veterinary Tropical Diseases (DVTD), University of Pretoria. Since the tick saliva volumes collected were very low, the first passage was done on a 96-well plate. Briefly, each individual tick saliva sample was mixed with Minimum Essential Medium (MEM) to a final volume of 10 µl. The toxic effects of saliva on cells were minimised by dispensing 80 µl MEM with 5% foetal calf serum and 0.2% gentamycin (Genta 50, Virbac Animal Health, 50 mg/ml) to each well with a monolayer of bovine dermis cells on a 96-well plate before the saliva sample (10 µl) was inoculated. The monolayers were then incubated at 37°C for 1 hour at 5% CO2. The supernatant was then removed and the plate washed 3 times with PBS containing Ca2+ and Mg2+ (PBS+). Then 80 µl MEM with 5% foetal calf serum and 0.2% gentamycin was added to the wells and incubated at 37°C for 14 days. The cells were observed after 24 hours for cytoxicity and daily thereafter for cytopathic effects (CPE). Two more blind passages were done using 24-well plates when CPE was not observed. The supernatant for the last passage was collected and tested by real-time PCR for LSDV. Positive DNA samples were sequenced to confirm the presence of LSDV (Inqaba Biotechnology, Pretoria, South Africa).

4.4. Results

The results for host animals (DR1 and DR2) were reported by Tuppurainen et al (2012) and those of DA1 and DA2 by Lubinga et al (submitted). Briefly, all host animals became febrile. DA2 and DR1developed multiple skin lesions while DA1 and DR2 had only mild clinical disease with only a few or no skin lesions others than on the intradermal virus inoculation sites. None of the animals developed skin lesions on the ear lobes where the nymphs were feeding. All animals developed viraemia between Day 4 and 7 pi. They remained viremic until Day 24, by real-time PCR results. The host animals began to sero-convert between Days 14 and 16.

Seven of nine pooled samples collected from *A. hebraeum* F2 adults tested positive by real-time PCR (Table 1). Six of the 16 pooled saliva samples for *R. appendiculatus* F2 adults tested positive.

Table 4.1: Real-time PCR results for pooled saliva samples collected from *A. hebraeum* and *R. appendiculatus* adults fed on experimentally infected host cattle as nymphs.

Sample #	Tick species	Real-time PCR result	Ct Value
1 Long Space?	*A. hebraeum*	Negative	
2	*A. hebraeum*	Negative	
3	*A. hebraeum*	Positive	>40
4	*A. hebraeum*	Positive	>40
5	*A. hebraeum*	Positive	>40
6	*A. hebraeum*	Positive	>40
7	*A. hebraeum*	Positive	>40
8	*A. hebraeum*	Positive	36.1
9	*A. hebraeum*	Positive	35.5
10	*R. appendiculatus*	Negative	
11	*R. appendiculatus*	Negative	
12	*R. appendiculatus*	Negative	
13	*R. appendiculatus*	Negative	
14	*R. appendiculatus*	Negative	
15	*R. appendiculatus*	Positive	>40
16	*R. appendiculatus*	Negative	
17	*R. appendiculatus*	Negative	
18	*R. appendiculatus*	Positive	>40
19	*R. appendiculatus*	Positive	38.7
20	*R. appendiculatus*	Negative	
21	*R. appendiculatus*	Positive	37.55
22	*R. appendiculatus*	Negative	
23	*R. appendiculatus*	Positive	>40
24	*R. appendiculatus*	Positive	>40
25	*R. appendiculatus*	Positive	>40

The saliva samples from *R. appendiculatus* F1 adults showed CPE in 13 of 14 (92.8%) wells (Table 2). The samples for *R. appendiculatus* F2 adults showed CPE in four out of five (80%)

wells. Samples for *A hebraeum* F1 adults showed CPE in 11 out of 14 (78.6%) wells while those tested for transstadial passage (F2 adults) showed CPE in four out of five (80%) wells.

Real-time PCR on virus isolation (VI) media for *R. appendiculatus* saliva detected LSDV in six of the 14 (42.8%) from mechanical / intrastadial passage (F1 adults) samples and two of five (40%) from transstadial passage (F2 adults) samples. The real-time PCR of virus isolation media for *A. hebraeum* saliva samples detected LSDV in six out of 14 (35.7%) for mechanical / intrastadial passage (F1 adults) and two out of five (40%) from transstadial passage (F2 adults) samples. In all cases, material from wells where there was no CPE also tested negative by PCR.

Table 4.2: Virus isolation results for tick saliva samples from adult *A. hebraeum* and *R. appendiculatus* fed as either adults or nymphs

Tick Species	Virus passage method	Number tested	Number showing CPE (3rd passage)	Number supernatants PCR positive
R. appendiculatus	Mechanical/ intrastadial	14	13 (92.8%)	6 (42.9%)
R. appendiculatus	Transstadial	5	4 (80%)	4 (80%)
A. hebraeum	Mechanical/ intrastadial	14	11 (78.6%)	6 (42.9%)
A. hebraeum	Transstadial	5	4 (80%)	2 (40%)

4.5. Discussion

Lumpy skin disease virus was demonstrated in saliva of *A. hebraeum* and *R. appendiculatus* adult ticks previously fed on LSDV-infected animals. Saliva samples inoculated directly on to cell cultures produced cytotoxicity (unpublished results). The cytotoxicity was reduced by dilution of saliva samples with MEM. Real-time PCR of VI media confirmed that the CPE was due to LSDV.

However, the rate of real-time PCR positive results on cell cultures was low, i.e. between 40% and 80% (Table 2). The presence of other infectious agents (other viruses, bacteria and protozoa) in the tick saliva was not determined in this study. While typical CPE such as clumping of cells, spindle shaped cells, separation and detachment was manifested in some cell cultures as described by other authors (Plowright & Ferris 1958, Plowright & Witcomb

1959), other cell cultures manifested general signs of cell death, i.e. rounding and detachment (unpublished results). Most negative results of real-time PCR on cell culture supernatants were obtained from samples where non-typical CPE for LSDV were observed. It is possible that some of such cell deaths were caused by other infectious agents alone or in combination with LSDV. Also, the possible presence of other infectious organisms in saliva may have reduced the opportunity for LSDV to multiply adequately in the cells and, given the slow rate of LSDV multiplication in cell cultures, the multiplication of LSDV may have been further reduced instead of being amplified with each passage.

Both VI and real-time PCR indicated the presence of LSDV in tick saliva. Although all efforts to reduce surface contamination were made, there is still a remote possibility of the saliva being contaminated with virus from the surface of the ticks obtained as the ticks were feeding on infected host animals. This is more likely for the F1 adults and less likely for F2 adults. During moulting of larvae to nymphs and nymphs to adults, the outer cuticle is shed off and replaced by a new inner coat (Balashov 1967). The surface contaminating virus is therefore expected to remain attached to the discarded old cuticle. Contamination of saliva from blood cannot be ruled out as this may constitute part of mechanical passage (transmission) of the virus.

Cytopathic effects were mostly detected after the third passage of cell culture indicating a low copy number of virus in tick saliva. The low copy numbers could also be due to the small volumes of saliva collected for the test and, possibly, compounded by the dilution factor to minimise cytotoxicity. High Ct values of positive saliva samples obtained by real-time PCR, i.e. above 35 (Table 1), are also an indication of low copy number of virus in the samples. Similar high Ct values were also obtained in the detection of LSDV in recipient animals on which infected *R. appendiculatus* males (Tuppurainen et al. 2013) and *A. hebraeum* ticks (Lubinga *et al*, submitted) fed. When developing this assay, Bowden and co-workers (2008) performed quantifications of 10-fold serial dilutions of the plasmid virus. They designated all real-time reactions with Ct values between 37 and 45 as positive and estimated the DNA copy number of such values to be less than 10. The low copy number of LSDV in tick saliva is suggestive of a low copy number of virus in the ticks.

This is the first report demonstrating LSDV in tick saliva. It corroborates with the previous findings that implicated *R. appendiculatus* (Tuppurainen et al. 2012) and *A. hebraeum* (Lubinga *et. al.* submitted) as competent vectors of LSDV. It also demonstrates the

persistence of LSDV in ticks between tick developmental stages (transstadial) and within the same stage (mechanical / intrastadial). It further confirms findings by Tuppurainen and co-workers (2011) that ticks may play a role in transmission of LSDV in the field.

Ixodid ticks normally take one blood meal per stage of their life cycle. Since *A. hebraeum* and *R. appendiculatus* are 3-host ticks (Cupp 1991, Sonenshine 1991, Walker 2003, Walker 1970), it is most ideal for these ticks to transmit the pathogens to the vertebrate host during their next life cycle stage (transstadial). However, this way of transmission is altered when the feeding of the tick is interrupted such as when male ticks detach and re-attach (as they seek females to mate), in all tick stages when the vertebrate host dies or the tick is groomed off the host, before the tick has taken enough blood to moult or lay eggs (Bremer et al. 2005, Wang, Henbest & Nuttall 1999, Wang & Nuttall 2001).

The tick organs where LSDV persists have not been determined. During transstadial passage, protozoan and rickettsial pathogens have been shown to replicate in the tick midgut epithelial cells before crossing into the hemocoele, from where the pathogens cross to infect the salivary glands. These pathogens survive the moulting process between tick life cycle stages (Cowdry & Ham 1932, Kocan & Bezuidenhout 1987, Kocan, Bezuidenhout & Hart 1987, Martin, Barnett & Vidler 1964). Thogoto virus was shown to multiply in the synganglion and the salivary gland (Booth et al. 1989).

The demonstration of LSDV in tick saliva in adult *R. appendiculatus* and *A. hebraeum* F1 ticks shows that the virus can reach the salivary glands before repletion and hence can be transmitted to another host following interruption of feeding. While it is possible that the virus will reach the salivary glands after multiplying in some other tick organs (intrastadial passage), it is also possible that mechanical passage may take place. For example, Thogoto virus intracoelomically inoculated into ticks, was shown to cross into salivary glands without multiplying in (i.e. infecting) the salivary gland epithelium (Kaufman & Nuttall 1996). It will be important to determine the organs where LSDV persists in ticks this will have an impact on the understanding of the epidemiological role these tick species play in the transmission of LSDV.

It has also been shown for thogoto virus and looping ill virus that ticks infected with viruses can pass the infection to clean co-feeding ticks in the absence of viraemia in the vertebrate host, through non-viraemic saliva activated transmission (SAT) (Gilbert et al. 2000, Jones, Hodgson & Nuttall 1989, Jones et al. 1992, Jones, Matthewson & Nuttall 1992, Jones et al.

1997). The demonstration of LSDV in tick saliva, therefore, implies a potential for LSDV infected ticks to pass the virus to other co-feeding clean ticks through SAT, which can lead to amplification of virus transmission.

Because of interrupted feeding and the potential for intrastadial / mechanical transmission of LSDV, ticks on dead animals (including slaughtered), should be considered a potential threat for spreading disease especially in areas where ticks can readily obtain an alternative host to continue their blood meal. It is therefore imperative that the control of LSD in endemic countries should include tick control on cattle.

4.6. Reference

Balashov, Y.S., 1967, *Blood-sucking ticks (Ixodoidea)-vectors of diseases of man and animals.*

Booth, T.F., Davies, C.R., Jones, L.D., Staunton, D. & Nuttall, P.A., 1989, 'Anatomical basis of Thogoto virus infection in BHK cell culture and in the ixodid tick vector, Rhipicephalus appendiculatus.', *Journal of General Virology* 70(5), 1093-1104.

Bowden, T.R., Babiuk, S.L., Parkyn, G.R., Copps, J.S. & Boyle, D.B., 2008, 'Capripoxvirus tissue tropism and shedding: a quantitative study in experimentally infected sheep and goats', *Virology* 371(2), 380-393.

Bremer, W.G., Schaefer, J.J., Wagner, E.R., Ewing, S.A., Rikihisa, Y., Needham, G.R., Jittapalapong, S., Moore, D.L. & Stich, R.W., 2005, 'Transstadial and intrastadial experimental transmission of *Ehrlichia canis* by male *Rhipicephalus sanguineus*.', *Veterinary parasitology* 131(1/2), 95-105.

Brown, S.J., 1988, 'Evidence for regurgitation by *Amblyomma americanum*.', *Veterinary parasitology* 28(4), 335-342.

Carn, V.M. & Kitching, R.P., 1995, 'An investigation of possible routes of transmission of lumpy skin disease virus (Neethling)', *Epidemiology and infection* 114(1), 219-226.

Chalaire, K.C., Kim, T.K., Garcia-Rodriguez, H. & Mulenga, A., 2011, '*Amblyomma americanum* (L.) (Acari: Ixodidae) tick salivary gland serine protease inhibitor (serpin) 6 is secreted into tick saliva during tick feeding', *Journal of Experimental Biology* 214(4), 665-673.

Chihota, C.M., Rennie, L.F., Kitching, R.P. & Mellor, P.S., 2003, 'Attempted mechanical transmission of lumpy skin disease virus by biting insects', *Medical and veterinary entomology* 17(3), 294-300.

Chihota, C.M., Rennie, L.F., Kitching, R.P. & Mellor, P.S., 2001, 'Mechanical transmission of lumpy skin disease virus by *Aedes aegypti* (Diptera: Culicidae)', *Epidemiology and infection* 126(2), 317-321.

Connat, J.L., 1991, 'Demonstration of regurgitation of gut content during blood meals of the tick Ornithodoros moubata. Possible role in the transmission of pathogenic agents.', *Parasitology research* 77(5), 452-454.

Cowdry, E.V. & Ham, A.W., 1932, 'Studies on East Coast Fever. I. The Life Cycle of the Parasite in Ticks.', *Parasitology* 24(1), 1-49.

Cupp, E.W., 1991, 'Biology of ticks', *Veterinary Clinics of North America, Small Animal Practice* 21(1), 1-26.

Gilbert, L., Jones, L.D., Hudson, P.J., Gould, E.A. & Reid, H.W., 2000, 'Role of small mammals in the persistence of Louping-ill virus: field survey and tick co-feeding studies.', *Medical and veterinary entomology* 14(3), 277-282.

Gill, H.S. & Walker, A.R., 1988, 'The salivary glands of *Hyalomma anatolicum anatolicum*: nature of salivary gland components and their role in tick attachment and feeding.', *International journal for parasitology* 18(1), 83-93.

Gill, H.S. & Walker, A.R., 1987, 'The salivary glands of *Hyalomma anatolicum anatolicum*: structural changes during attachment and feeding.', *International journal for parasitology* 17(8), 1381-1392.

Jones, L.D., Gaunt, M., Hails, R.S., Laurenson, K., Hudson, P.J., Reid, H., Henbest, P. & Gould, E.A., 1997, 'Transmission of louping ill virus between infected and uninfected ticks co-feeding on mountain hares.', *Medical and veterinary entomology* 11(2), 172-176.

Jones, L.D., Hodgson, E. & Nuttall, P.A., 1989, 'Enhancement of virus transmission by tick salivary glands', *Journal of General Virology* 70(7), 1895-1898.

Jones, L.D., Hodgson, E., Williams, T., Higgs, S. & Nuttall, P.A., 1992, 'Saliva activated transmission (SAT) of Thogoto virus: relationship with vector potential of different haematophagous arthropods.', *Medical and veterinary entomology* 6(3), 261-265.

Jones, L.D., Kaufman, W.R. & Nuttall, P.A., 1992, 'Modification of the skin feeding site by tick saliva mediates virus transmission.', *Experientia* 48(8), 779-782.

Jones, L.D., Matthewson, M. & Nuttall, P.A., 1992, 'Saliva-activated transmission (SAT) of Thogoto virus: dynamics of SAT factor activity in the salivary glands of *Rhipicephalus appendiculatus*, *Amblyomma variegatum*, and *Boophilus microplus* ticks.', *Experimental & applied acarology* 13(4), 241-248.

Kaufman, W.R., Bowman, A.S. & Nuttall, P.A., 2001, 'Salivary fluid secretion in the ixodid tick *Rhipicephalus appendiculatus* is inhibited by Thogoto virus infection.', *Experimental and Applied Acarology* 25(8), 661-674.

Kaufman, W.R. & Nuttall, P.A., 2003, '*Rhipicephalus appendiculatus* (Acari: Ixodidae): dynamics of Thogoto virus infection in female ticks during feeding on guinea pigs.', *Experimental parasitology* 104(1/2), 20-25.

Kaufman, W.R. & Nuttall, P.A., 1996, '*Amblyomma variegatum* (Acari: Ixodidae): mechanism and control of arbovirus secretion in tick saliva', *Experimental parasitology* 82(3), 316-323.

Kocan, K.M. & Bezuidenhout, J.D., 1987, 'Morphology and development of *Cowdria ruminantium* in *Amblyomma* ticks.', *Onderstepoort Journal of Veterinary Research* 54(3), 177-182.

Kocan, K.M., Bezuidenhout, J.D. & Hart, A., 1987, 'Ultrastructural features of *Cowdria ruminantium* in midgut epithelial cells and salivary glands of nymphal *Amblyomma hebraeum*.', *Onderstepoort Journal of Veterinary Research* 54(1), 87-92.

Kocan, K.M. & Stiller, D., 1992, 'Development of *Anaplasma ovis* (Rickettsiales: Anaplasmataceae) in male *Dermacentor andersoni* (Acari: Ixodidae) transferred from infected to susceptible sheep.', *Journal of medical entomology* 29(1), 98-107.

Mans, B.J. & Neitz, A.W.H., 2004, 'Adaptation of ticks to a blood-feeding environment: evolution from a functional perspective.', *Insect biochemistry and molecular biology* 34(1), 1-17.

Martin, H.M., Barnett, S.F. & Vidler, B.O., 1964, 'Cyclic development and longevity of *Theileria parva* in the tick *Rhipicephalus appendiculatus*.', *Experimental parasitology* 15 527-555.

Norval, R.A.I., Andrew, H.R. & Yunker, C.E., 1989, 'Pheromone-mediation of host-selection in bont ticks (*Amblyomma hebraeum* Koch).', *Science (Washington)* 243(4889), 364-365.

Norval, R.A.I., Peter, T., Sonenshine, D.E. & Burridge, M.J., 1992, 'Responses of the ticks *Amblyomma hebraeum* and *A. variegatum* to known or potential components of the aggregation-attachment pheromone. III. Aggregation', *Experimental & applied acarology* 16(3), 237-245.

Plowright, W. & Ferris, R.D., 1958, 'The growth and cytopathogenicity of sheep-pox virus in tissue cultures.', *Brit.J.Exper.Path.* 39 424-435.

Plowright, W. & Witcomb, M.A., 1959, 'The growth in tissue cultures of a virus derived from lumpy-skin disease of cattle.', *Journal of Pathology and Bacteriology* 78 397-407.

Ribeiro, J.M.C., Zeidner, N.S., Ledin, K., Dolan, M.C. & Mather, T.N., 2004, 'How much pilocarpine contaminates pilocarpine-induced tick saliva?', *Medical and veterinary entomology* 18(1), 20-24.

Sonenshine, D.E., 1991, *Biology of ticks*, Oxford University Press, Oxford.

Stachurski, F., 2006, 'Attachment kinetics of the adult tick *Amblyomma variegatum* to cattle.', *Medical and veterinary entomology* 20(3), 317-324.

Titus, R.G. & Ribeiro, J.M.C., 1990, 'The role of vector saliva in transmission of arthropod-borne disease.', *Parasitology Today* 6(5), 157-160.

Tuppurainen, E.S.M., Lubinga, J.C., Stoltsz, W.H., Troskie, M., Carpenter, S.T., Coetzer, J.A.W., Venter, E.H. & Oura, C.A.L., 2013, 'Mechanical transmission of lumpy skin disease virus by *Rhipicephalus appendiculatus* male ticks', *Epidemiology and infection, Epidemiol.Infect.* 141, 2 425-430.

Tuppurainen, E.S., Venter, E.H. & Coetzer, J.A., 2005, 'The detection of lumpy skin disease virus in samples of experimentally infected cattle using different diagnostic techniques', *The Onderstepoort journal of veterinary research* 72(2), 153-164.

Tuppurainen, E.S.M., Stoltsz, W.H., Troskie, M., Wallace, D.B., Oura, C.A.L., Mellor, P.S., Coetzer, J.A.W. & Venter, E.H., 2011, 'A potential role for ixodid (hard) tick vectors in the transmission of lumpy skin disease virus in cattle', *Transboundary and Emerging Diseases* 58(2), 93-104.

Von Backström, U., 1945, 'Ngamiland cattle disease. Preliminary report on a new disease, the etiological agent being probably of an infectious nature', *Journal of the South African Veterinary Medical Association* 16 29-35.

Walker, A., 2003, *Ticks of domestic animals in Africa : a guide to identification of species*, Bioscience Reports, Edinburgh.

Walker, J.B., 1970, *Notes on the common tick species of East Africa*, 3 rev edn., Cooper, McDougall and Robertson, Nairobi.

Wang, H., Henbest, P.J. & Nuttall, P.A., 1999, 'Successful interrupted feeding of adult *Rhipicephalus appendiculatus* (Ixodidae) is accompanied by reprogramming of salivary gland protein expression.', *Parasitology* 119(2), 143-149.

Wang, H. & Nuttall, P.A., 2001, 'Intra-stadial tick-borne Thogoto virus (Orthomyxoviridae) transmission: accelerated arbovirus transmission triggered by host death.', *Parasitology* 122(4), 439-446.

Weiss, K.E., 1968, 'Lumpy skin disease virus', *Virology Monographs* 3 111-131.

Wikel, S.K., 1999, 'Tick modulation of host immunity: an important factor in pathogen transmission.', *International journal for parasitology* 29(6), 851-859.

Wikel, S.K., 1984, 'Immunomodulation of host responses to ectoparasite infestation - an overview.', *Veterinary parasitology* 14(3/4), 321-339.

Woods, J.A., 1988, 'Lumpy skin disease--a review', *Tropical animal health and production* 20(1), 11-17.

Chapter 5

Transovarial passage and transmission of LSDV by *Amblyomma hebraeum*, *Rhipicephalus appendiculatus* and *Rhipicephalus (Boophilus) decoloratus*

Submitted to Experimental and Applied Acarology Journal

Manuscript number: APPA-D-13-00046

5.1. Abstract

Lumpy skin disease (LSD), an acute, sub-acute or innapparent disease of cattle, is caused by lumpy skin disease virus (LSDV), a member of the genus *Capripoxvirus* in the family *Poxviridae.* Lumpy skin disease is characterised by high fever, formation of circumscribed skin lesions and ulcerative lesions on the mucous membranes of the mouth, respiratory and digestive tracts. It is an economically important disease due to the permanent damage to hides, the reduction in productivity and trade restrictions imposed on affected areas.

Transmission has been associated with blood-feeding insects such as stable flies (*Stomoxysis calcitrans*) and mosquitoes (*Aedes aegypti*). Mechanical (intrastadial) and transstadial transmission by *Amblyomma hebraeum* and *Rhipicephalus appendiculatus* as well as transovarial transmission by *Rhipicephalus (Boophilus) decoloratus* have been reported.

In this study transovarial passage of LSDV to larvae and subsequent transmission to recipient animals were demonstrated. The finding of transovarial passage of LSDV in female ticks shows the potential for *A. hebraeum, R. appendiculatus and R. (B) decoloratus* to be reservoir hosts for LSDV.

Key words: Transovarial transmission, *Amblyomma hebraeum*, *Rhipicephalus appendiculatus, Rhipicephalus (Boophilus) decoloratus*, tick eggs, larvae, lumpy skin disease virus

5.2. Introduction

Lumpy skin disease virus (LSDV) is a member of the genus *Capripoxvirus* in the family *Poxviridae* (King et al. 2011). It causes lumpy skin disease (LSD) which affects all age groups and breeds of cattle. Lumpy skin disease is characterised by high fever, multiple circumscribed firm skin nodules, enlargement of superficial lymph nodes, salivation, lachrimation, rhinitis and keratitis. The disease is of economic importance due to the severe damage to hides, reduced milk and meat production, temporary infertility and trade losses due to movement restrictions placed on cattle from affected areas. (Davies 1981, Kumar 2011, Weiss 1968).

Haematophagous insects have mostly been associated with the transmission of LSDV. In Kenya (1957), the LSD outbreak was associated with a high prevelance of *Aedes natronius* and *Culex mirificens* mosquitoes (Burdin 1959) while the outbreak in Israel (1989) was attributed to *Stomoxys calcitrans* carried by wind from Ismailiya to Egypt (Yeruham et al. 1995). The demonstration of mechanical transmission of LSDV by *Aedes aegypti* in the laboratory suggests the ability of mosquitoes and other biting insects to transmit LSDV under natural conditions (Chihota et al. 2001). Ticks have recently been implicated in the transmission of LSDV (Tuppurainen et al. 2011, Tuppurainen & Oura 2012). Mechanical transmission of the virus by *Rhipicephalus appendiculatus* has been demonstrated (Tuppurainen et al. 2013a), while mechanical and transstadial transmission by *Amblyomma hebraeum* have been demonstrated by Lubinga *et al* (submitted). Lumpy skin disease has also been detected in saliva samples collected from *A. hebraeum* and *R. appendiculatus* adults that fed on infected cattle as nymphs or as adults (Lubinga et al. 2013). Transovarial transmission of LSDV by *R. (B) decoloratus* larvae to cattle was reported by Tuppurainen and co-workers (2013b).

In this study the passage of LSDV from infected female ticks through the eggs to the next generation larvae is demonstrated in *A. hebraeum, R. appendiculatus* and *R. (B) decoloratus*. Transmission to recipient animals by *A. hebraeum* and *R. appendiculatus* larvae was also shown

5.3. Materials and methods

5.3.1. Infection of ticks

Eight LSDV sero-negative Bonsmara cattle were used in the study; six were experimentally infected with LSDV virus and acted as donor animals (Tuppurainen et al. 2013a). The donor animals were paired and each pair hosted adult *A. hebraeum*, *R. appendiculatus* and *R. (B) decoloratus*, respectively. The other two animals were used as recipient animals to demonstrate transmission by *A. hebraeum* and *R. appendiculatus* larvae respectively. The recipient animals were kept in separate rooms in insect-free stables at the University of Pretoria's Bioscience Research Centre (UPBRC). Donor cattle were infected in October 2010 and were removed from the animal facilities by the end of November 2010. The facilities were then cleaned and disinfected according to the standard operating procedures of UPBRC. The recipient animals were brought into the facilities in the beginning of February 2011 and during the time that the recipient cattle were housed at the insect-free isolation unit where there were no animals infected with LSDV or other capripoxviruses. All the experimental procedures for the animals were approved by the Animal Use and Care Committee (AUCC) of the University of Pretoria (UP), South Africa.

A South African LSDV field isolate (248/93), was used in the study to artificially infect donor animals. It was propagated on primary bovine dermis cells for five to six passages at the Virology Section of the Department of Veterinary Tropical Diseases (DVTD), UP. The titre of the virus was 5.95 log $TCID_{50}$/ml.

The donor animals were infected with 3.5 ml of the prepared LSDV inoculum (Tuppurainen et al. 2013a).

Unfed *A. hebraeum* and *R. appendiculatus* adult ticks and *R. (B) decoloratus* larvae were reared at the Agricultural Research Council's Onderstepoort Veterinary Institute (ARC-OVI). These ticks were exposed to LSDV infection by feeding them on two infected donor animals each for *A. hebraeum*, *R. appendiculatus* and *R. (B) decoloratus* during the viraemic stage as follows**:**

Seventy five (F1 adult) *A. hebraeum* males were placed in two cotton cloth bags attached with Genkem contact adhesive glue on the body sides of donor animals on Day 4 post infection (pi). Twenty five females were added to the bags containing the males four days later as described by Lubinga et al (2013). Engorged females were collected after feeding to

repletion for 6 to 8 days. They were incubated in an acaridarium at 25°C and 85% relative humidity (RH) to oviposit and for the eggs to hatch. After hatching, the larvae were further incubated for approximately one month, to allow for their maturation to complete. To test for transovarial passage of LSDV to larvae, four pooled samples from five females containing approximately 300 larvae each were collected and stored at -80°C for testing by real-time polymerse chain reaction (PCR) and virus isolation (VI). To test for transovarial transmission, approximately 500 pooled larvae were placed on the ears of recipient animal RA4 in cotton cloth bags, attached at the base of the ears. This was designated Day 0 post attachment (0 dpa).

To infect *R. appendiculatus*, 100 unfed adult ticks were placed in cotton cloth bags attached to the base of the ears of donor animals. Engorged *R. appendiculatus* females were collected after feeding to repletion on Days 6 to 7 (pi.). They were incubated to lay eggs and for the eggs to hatch as for *A. hebraeum*. Samples of larvae were collected for testing by real-time PCR and VI. Approximately 500 larvae were placed in cotton bags attached to the base of the ears of recipient animal RR2 to test for transovarial transmission by *R. appendiculatus*.

The transovarial transmission of LSDV by *R. (B) decoloratus* was reported by Tuppurainen and co-workers (2013b). In the present study, a sample of the potentially infected larvae were tested for LSDV by real-time PCR and VI to confirm the transovarial passage of LSDV by *R. (B) decoloratus* as described for *A. hebraeum* larvae.

At the time of tick feeding, the presence of LSDV in donor animals was indicated by clinical signs and confirmed by serum neutralisation test (SNT) and real-time PCR (Lubinga et al. 2013, Tuppurainen et al. 2013a, Tuppurainen et al. 2013b).

Successful transovarial transmission to recipient animals was tested by clinical examination, VI, SNT and real-time PCR. Blood was collected from recipient animals (RA4 and RR2) on 0, 5, 7- 9, 12 - 16, 20 - 22, 27 and 28 (dpa). Blood was collected in EDTA vacutainer tubes for real-time PCR, in heparin tubes for VI and serum for the SNT. Blood samples were stored at -20°C until processed.

5.3.2. Clinical monitoring of animals

Donor animals were monitored daily from Day 0 – 30 days pi, for rise in body temperature, development or presence of circumscribed skin lesions and enlargement of peripheral lymph nodes. Similarly, recipient animals were observed from Day 0 to Day 30 post attachment (pa)

of potentially infected ticks. Depending on the severity of these parameters, the disease was classified as severe, i.e. with high fever (body temperature above 40°C) and skin lesions more than 6 mm in diameter; mild disease i.e. mild fever (39.1°C to 40°C) and small or transient skin lesions less than 6 mm; no disease or inapparent where no clinical signs were detected. Animals that developed severe clinical signs including eruptive lesions were treated with antibiotics to prevent secondary bacterial infections.

5.3.3. Homogenisation of larvae

Approximately 1 g of pooled larvae were washed three times in phosphate buffered saline containing Ca^{2+} and Mg^{2+} (PBS^{+}) and 0.2% Gentamycin (Genta 50, Virbac Animal Health, 50 mg/ml) and 0.5% fungizone (Gibco®fungizone, Life technologies) to reduce other microbial contaminants as well as to rinse off any LSDV adhered to surface of the larvae. The cleaned samples were placed in tubes containing beads and 600 µl of Minimum Essential Medium (MEM) with 5% foetal calf serum, 0.2% gentamycin and 0.5% fungizone and cooled at -80°C for 5 minutes. The larvae were then homogenised using a Precelly's 24 Lysis and Homogenizer (Bertin Technologies, France) at 6500 rpm twice for a total of 5 minutes. Of the tick homogenate collected, 200 µl was used for DNA extraction and 200 µl for VI.

5.3.4. DNA extraction

Extraction of DNA from cattle blood and tick homogenates was based on the protocol used by Tuppurainen and co-workers (Tuppurainen, Venter & Coetzer 2005). Proteins were digested by adding 2- 4 IU of proteinase K (Roche products, Mannheim, Germany) (10 mg/ml) to samples followed by incubation at 56°C for 2 hours (blood) or overnight (tick homogenate).

A real-time PCR, Taqman assay, was used to amplify the extracted DNA (Bowden et al. 2008). An 89 bp region within the *Capripoxvirus* ORF 74 region that encodes the intracellular mature protein P32 was amplified. Capripoxvirus-specific primers and probes with the following sequences were used: Forward primer-CaPV074F1 5'-AAA ACG GTA TAT GGA ATA GAGTT GAA-3', Reverse primer- CaPV074R1 5'-AAA TGA AAC CAA TGG ATG GGA TA-3' and Probe CaPV074P1 5'FAM-TGG CTC ATA GAT TTC CT-MGB/NFQ -3' (Bowden et al. 2008).

The reaction was performed using a Light Cycler®, 2.0 (Roche products, Mannheim, Germany). The reaction mixture comprised 4 µl of 10X FastStart DNA Master^{plus} HybProbe kit (Roche Products), 1 µl (0.1 µM) each of the primers, 1 µl (0.2 µM) of hybridization probe, 0.5 µl of UDG, 8.5 µl of water and 4 µl of DNA template (up to 500 ng). The reaction was run according to the procedures described by Bowden *et al.* (2008). DNA extracted from LSDV infected cell cultures was used as positive control. Negative controls included a water sample processed through the DNA extraction procedures and a no-DNA template water control.

5.3.5. Serum neutralisation test

Neutralizing antibodies were measured using a constant-virus/varying-serum neutralising method (Beard et al. 2010) and bovine dermis cells. Negative sera controls were collected from research animals before LSDV inoculation or attachment of ticks. Sera from artificially infected (donor) animals were used as positive sera controls. Titres were determined as the last dilution that gave 50% neutralization.

5.3.6. Virus isolation

Virus isolation from blood samples was performed according to previously published methods (Tuppurainen, Venter & Coetzer 2005). A similar procedure for VI from tick homogenates was followed. To reduce toxicity to the monolayers, 100 µl of tick-homogenate was added to 900 µl of MEM (1/10 dilution) and 300 µl of the diluted homogenate was added to the monolayers. Monolayers were observed daily for cytopathic effect (CPE). In the absence of CPE, 2 to 3 blind passages on cell cultures were performed and supernatants were tested by real-time PCR to confirm the presence of LSDV and as the cause of CPE. The VI materials for recipient animals (RA4 and RR2) after the third passage were pooled and concentrated using the PEG kit (BioVision Research Products, Mountain view, USA) and also tested by real-time PCR

5.4. Results

The clinical signs observed in donor animals were reported by Tuppurainen et al, (2013); Tuppurainen et al (2013b); Lubinga et al. (2013). Homogenates of *A. hebraeum* larvae tested positive for LSDV by real-time PCR with Ct values of 36.7, 37.23 and 39.87. Homogenates of *R. appendiculatus* larvae were all negative by real-time PCR. One homogenate sample for *R. (B) decoloratus* larvae was positive with a Ct value of 39.75.

Cytopathic effects were observed in cell cultures for *A. hebraeum* and *R. (B) decoloratus* larvae while no CPE was observed on cell cultures for *R. appendiculatus* larvae. Testing by real-time PCR of VI supernatants showed the presence of LSDV in cultures of all 3 species with Ct values between 32.54 and 37.43 (Table 1).

Table 5.1: Real time PCR of tick homogenate and virus isolation supernatants for *Amblyomma hebraeum*, *Rhipicephalus appendiculatus* and *R. (B) decoloratus* larvae

Tick species	Real-time PCR (tick homogenate		Real-time PCR (virus isolation supernatant)	
	Result	C_t value	Result	C_t value
A. hebraeum	Positive	39.87	Positive	32.88
	Positive	36.7	Positive	32.54
	Positive	37.23	Positive	35.69
	Negative	>40	Positive	34.24
R. appendiculatus				
	Negative	-	Positive	36.59
	Negative	-	Negative	-
	Negative	-	positive	35.91
R. (B) decoloratus	Positive	39.75	Positive	37.43
	Negative	>40	Negative	>40
		-	Negative	>40
		-	Positive	33.41

Attachment by *A. hebraeum* larvae on the ears of recipient animals RR2 and RA4 was very high, approaching 100%. The recipient animal (RA4) representing transovarial transmission by *A. hebraeum* showed no increase in body temperature throughout the study, except on 6 dpa when the temperature rose to 39.4°C. All other clinical parameters and temperature for RA4 remained normal for the rest of the observation period.

Serum obtained from recipient animal RA4, was negative by SNT throughout the study. The real-time PCR results of blood samples were also negative for LSDV and no CPE was observed from blood samples of RA4. However, the 2nd passage of cell culture media used for VI, tested positive for LSDV real-time PCR on day 14 pi with a Ct value of 37.7.

Recipient animal RR2 used for *R. appendiculatus* larvae showed a slight temperature raise to 39°C on Day 6 pi, 39.1°C on Day 13 pi and 39.3°C on Day 22 pi. No other clinical signs were observed. Viraemia was detected on days 6, 21 and 23 with C_t values of 39.24, 36.7 and 38.1, respectively. Cytopathic effects were observed on cell cultures inoculated with heparinised blood on days 8 to 22 pi. Testing of VI material by real-time PCR confirmed LSDV with C_t values of 39.13 on Day 19; 39.94 on Day 20 and 38.8 on Day 21 pi. The C_t values from the pooled and concentrated VI material were 35.85 for RA5 and 28.75 for RR2.

5.5. Discussion

The detection of LSDV in larvae of *A. hebraeum, R. (B) decoloratus* and *R. appendiculatus* indicates transovarial passage of LSDV by these tick species. Washing of the larvae three times helped to reduce possible surface contamination of the larvae by the virus, thereby increasing the confidence that the virus detected passed through the larval tissues.

The C_t values for real-time PCR of tick homogenates and the VI material were lower for *A. hebraeum* larvae than for *R. (B) decoloratus* and *R. appendiculatus*. It has been shown that during real-time PCR, the lower the C_t values obtained, the higher the target DNA sequences in the test material (Higuchi et al. 1993). The C_t values obtained from testing larval samples suggest that more efficient transovarial passage of LSDV occurred by *A. hebraeum* than *R. (B) decoloratus* and *R. appendiculatus*. However, transmission tests in recipient animals showed more efficient transovarial transmission by *R. (B) decoloratus* than *R. appendiculatus* and *A. hebraeum*. The recipient animals (RB2 and RB3) of R. *(B) decoloratus* larvae showed more evidence of LSDV infection (i.e. skin lesions, oral lesions, salivation), the C_t values for real-time PCR of blood were lower and the virus was demonstrated from the skin lesions (Tuppurainen et al. 2013b). Low efficiency of transovarial transmission of viruses by 3-host ticks was reported for Crimean Congo Haemorrhagic Fever (CCHF) by *Hyalomma truncatum* (Wilson et al. 1991) and tick borne-encephalitis by *Ixodes ricinus* (Danielova & Holubova 1991). This may explain the apparent low efficiency of transovarial transmission of LSDV *A. hebraeum* and *R. appendiculatus* observed in this study.

Clinically, recipient animal RA4 (*A. hebraeum*) did not indicate development of LSD, except for the slight elevation in body temperature on Day 6. Fever is one of the characteristic signs for LSD (Coetzer 2004, Weiss 1968) and a transient rise in body temperature of animals is consistent with findings in the other studies involving *R. appendiculatus* (Tuppurainen et al.

2013a) and *R. (B) decoloratus* (Tuppurainen et al. 2013b), where recipient animals showed a transient raise in temperature between days 4 to 8 of attachment. A similar transient temperature rise was also recorded by Carn and Kitching (1995), when they monitored clinical response to artificial infection with LSDV. However, fever alone is not indicative of LSDV infection, since it is common to most other infectious conditions. The detection of LSDV in blood of RA4 confirms transmission of the virus. Since LSDV was only detected in blood after VI, there is likely to be a very low viraemia. The presence of CPE in cell cultures inoculated with larval homogenates suggests that the virus in the larvae was infectious, thereby showing high potential for transmission to recipient animals.

Transovarial passage of pathogens does not seem to be a common route for *A. hebraeum*. Although it is reported that *A. hebraeum* transmits CCHF (Shepherd et al. 1989, Shepherd et al. 1991), attempts to show transovarial passage of the virus failed and the virus could not be demonstrated in larvae produced by females fed on infected animals (Shepherd et al. 1991). However transovarial transmission by *A. hebraeum* has been demonstrated for *Rickettsia conori* (Kelly & Mason 1991) and *Ehrlichia (Cowdria) ruminantium* (Bezuidenhout & Jacobsz 1986).

Cattle are preferred hosts for both immature and adult *A. hebraeum* ticks and although immature stages (e.g. larvae) attach mostly on the feet, legs and muzzle (Walker 2003), their attachment on the ears in this study was very high. Therefore, the animal host or attachment site does not seem to have adversely affected the transmission of the virus.

Evidence of transovarial passage of LSDV in *R. (B) decoloratus* in this study, strengthens the report of transovarial transmission of LSDV by Tuppurainen and co-workers (2013b). Since *R. (B) decoloratus* is a one host tick (Walker 2003), transmission of pathogens between hosts is expected to be vertical through transovarial passage. Transstadial passage from larvae to nymphs and adults may occur on the same host and this may explain the more prominent development of LSD reported in recipient animals challenged with *R. (B) decoloratus* (Tuppurainen et al. 2013b) compared to the infection developed by animals challenged with larvae of 3-host ticks (*A. hebraeum* and *R. appendiculatus*). *Rhipicephalus (Boophilus) decoloratus* has also been shown to transmit *Babesia bigemina* through vertical transmission (Buscher 1988, Okon et al. 2011). No evidence of transovarial transmission of other viruses by *R. (B) decoloratus* has been reported. An attempt to show transovarial transmission of CCHF by *R. (B) decoloratus* was not successful (Shepherd et al. 1991).

Transovarial passage of LSDV by *R. appendiculatus* in this study was quite poor. The virus was only detected in the larvae after testing the VI material by real-time PCR, with high C_t values of 35 - 36. Similarly, transovarial transmission by *R. appendiculatus* infected larvae to recipient animal RR2 was transient. Infection was indicated clinically by fever (39.3°C) on Day 23 pi. It was confirmed by detection of viraemia by real-time PCR (C_t values of 36.7 and 38.1 on Days 21 and 23, respectively) and detection of LSDV by real-time PCR in VI material on Day 14 dpa as well that of the pooled and concentrated VI supernatants.

This study demonstrates transovarial passage of LSDV by *A. hebraeum, R. appendiculatus* and *R. (B) decoloratus* ticks. The findings of this study and other published LSDV transmission studies (Tuppurainen et al. 2013a, Tuppurainen et al. 2013b) indicate a high possibility for ticks to be reservoir hosts for LSDV in nature. The overwintering period in the life cycle of some tick species such as *R. (B) decoloratus* ticks (after repletion of adults) (Bryson et al. 2002, Walker 2003), may play a significant role in the overwintering of LSDV. The limitations on the number of test animals used did not allow the use of statistical analysis in this study and more comprehensive investigations may be required to fully ascertain transovarial passage of LSDV in ticks in the field settings.

5.6. References

Beard, P.M., Sugar, S., Bazarragchaa, E., Gerelmaa, U., Tserendorj, S., Tuppurainen, E. & Sodnomdarjaa, R., 2010, 'A description of two outbreaks of capripoxvirus disease in Mongolia', *Veterinary microbiology* 142(3), 427-431.

Bezuidenhout, J.D. & Jacobsz, C.J., 1986, 'Proof of transovarial transmission of *Cowdria ruminantium* by *Amblyomma hebraeum*.', *Onderstepoort Journal of Veterinary Research* 53(1), 31-34.

Bowden, T.R., Babiuk, S.L., Parkyn, G.R., Copps, J.S. & Boyle, D.B., 2008, 'Capripoxvirus tissue tropism and shedding: a quantitative study in experimentally infected sheep and goats', *Virology* 371(2), 380-393.

Bryson, N.R., Tice, G.A., Horak, I.G., Stewart, C.G. & Plessis, B.J.A.d., 2002, 'Ixodid ticks on cattle belonging to small-scale farmers at 4 communal grazing areas in South Africa.', *Journal of the South African Veterinary Association* 73(3), 98-103.

Burdin, M.L., 1959, 'The use of histo-pathological examinations of skin material for the diagnosis of lumpy skin disease in Kenya', *Bulletin of epizootic diseases of Africa. Bulletin des epizooties en Afrique* 7 27-36.

Buscher, G., 1988, 'The infection of various tick species with *Babesia bigemina*, its transmission and identification.', *Parasitology research* 74(4), 324-330.

Chihota, C.M., Rennie, L.F., Kitching, R.P. & Mellor, P.S., 2001, 'Mechanical transmission of lumpy skin disease virus by *Aedes aegypti* (Diptera: Culicidae)', *Epidemiology and infection* 126(2), 317-321.

Coetzer, J.A.W., 2004, 'Lumpy skin disease' in pp. 1268-1276, Oxford University Press, Oxford.

Danielova, V. & Holubova, J., 1991, *Transovarial transmission rate of tick-borne encephalitis virus in Ixodes ricinus ticks.* .

Davies, F.G., 1981, *Lumpyskin disease*, , Academic Press, London.

Higuchi, R., Fockler, C., Dollinger, G. & Watson, R., 1993, 'Kinetic PCR analysis: real-time monitoring of DNA amplification reactions.', *Bio/Technology* 11(9), 1026-1030.

Kelly, P.J. & Mason, P.R., 1991, 'Transmission of a spotted fever group rickettsia by *Amblyomma hebraeum* (Acari: Ixodidae).', *Journal of medical entomology* 28(5), 598-600.

King, A., Lefkowitz, E., Adams, M.J. & Carstens, E.B. (eds.), 2011, 'Virus Taxonomy, 1st Edition. Ninth Report of the International Committee on Taxonomy Viruses'.

Kumar, S.M., 2011, 'An outbreak of lumpy skin disease in a Holstein dairy herd in Oman: a clinical report.', *Asian Journal of Animal and Veterinary Advances* 6(8), 851-859.

Lubinga, J.C., Tuppurainen, E.S.M., Stoltsz, W.H., Eberhson, K., Coetzer, J.A.W. & Venter, E.H., 2013, '
Detection of lumpy skin disease virus in saliva of ticks fed on blood of infected cattle', *Exp Appl Acarol* .

Okon, O.E., Opara, K.N., Etim, S.E., Arong, G.A., Oku, E.E. & Iboh, C.I., 2011, 'Experimental transmission o*f Babesia bigemina* by *Boophilus decoloratus* in cattle.', *Research Journal of Parasitology* 6(5), 168-175.

Shepherd, A.J., Swanepoel, R., Cornel, A.J. & Mathee, O., 1989, 'Experimental studies on the replication and transmission of Crimean-Congo hemorrhagic fever virus in some African tick species.', *American Journal of Tropical Medicine and Hygiene* 40(3), 326-331.

Shepherd, A.J., Swanepoel, R., Shepherd, S.P., Leman, P.A. & Mathee, O., 1991, 'Viraemic transmission of Crimean-Congo haemorrhagic fever virus to ticks.', *Epidemiology and infection* 106(2), 373-382.

Tuppurainen, E.S.M., Lubinga, J.C., Stoltsz, W.H., Troskie, M., Carpenter, S.T., Coetzer, J.A.W., Venter, E.H. & Oura, C.A.L., 2013a, 'Mechanical transmission of lumpy skin disease virus by *Rhipicephalus appendiculatus* male ticks', *Epidemiology and infection, Epidemiol.Infect.* 141, 2 425-430.

Tuppurainen, E.S.M., Lubinga, J.C., Stoltzs, W.H., Troskie, M., Carpenter, S.T., Coetzer, J.A.W., Venter, E.H. & Oura, A.L., 2013b, 'Evidence of vertical transmission of lumpy skin disease in Rhipicephalus decoloratus ticks', *Ticks and tick borne diseases* .

Tuppurainen, E.S., Venter, E.H. & Coetzer, J.A., 2005, 'The detection of lumpy skin disease virus in samples of experimentally infected cattle using different diagnostic techniques', *The Onderstepoort journal of veterinary research* 72(2), 153-164.

Tuppurainen, E.S.M. & Oura, C.A.L., 2012, 'Review: lumpy skin disease: an emerging threat to Europe, the Middle East and Asia.', *Transboundary and Emerging Diseases* 59(1), 40-48.

Tuppurainen, E.S.M., Stoltsz, W.H., Troskie, M., Wallace, D.B., Oura, C.A.L., Mellor, P.S., Coetzer, J.A.W. & Venter, E.H., 2011, 'A potential role for ixodid (hard) tick vectors in the transmission of lumpy skin disease virus in cattle', *Transboundary and Emerging Diseases* 58(2), 93-104.

Walker, A., 2003, *Ticks of domestic animals in Africa : a guide to identification of species*, Bioscience Reports, Edinburgh.

Weiss, K.E., 1968, 'Lumpy skin disease virus', *Virology Monographs* 3 111-131.

Wilson, M.L., Gonzalez, J.P., Cornet, J.P. & Camicas, J.L., 1991, 'Transmission of Crimean-Congo haemorrhagic fever virus from experimentally infected sheep to *Hyalomma truncatum* ticks.', *Research in virology* 142(5), 395-404.

Yeruham, I., Nir, O., Braverman, Y., Davidson, M., Grinstein, H., Haymovitch, M. & Zamir, O., 1995, 'Spread of lumpy skin disease in Israeli dairy herds', *The Veterinary record* 137(4), 91-93.

Chapter 6

Demonstration of lumpy skin disease virus infection in *Amblyomma hebraeum* and *Rhipicephalus appendiculatus* ticks using immunohistochemistry

Submitted to Tick and tick-borne diseases Journal

Manuscript number: TTBDIS-D-13-00090

6.1. Abstract

Lumpy skin disease (LSD) is an OIE listed disease of cattle. It is caused by the lumpy skin disease virus (LSDV), a member of the genus *Capripoxvirus*. Epidemiological evidence has associated transmission of the virus with biting insects, with most outbreaks occurring in wet seasons and along water courses. The outbreaks of LSD in Kenya (1957) were associated with *Culex mirificus* and *Aedes natrionus* mosquitoes while the spread to Israel (1986) was associated with the stable fly, *Stomoxysis calcitrans*. Recent studies have shown the capability of ticks to act as vectors for LSDV. Recent reports indicated mechanical transmission of the virus by *Rhipicephalus appendiculatus*, mechanical/intrasstadial and transstadial transmission by *A. hebraeum* and transovarial transmission by *R. (B) decoloratus*.

Although mechanical/intrastadial and transstadial passages of LSDV have been reported following the detection of the virus in saliva of *R. appendiculatus* and *A. hebraeum*, the tick organs where the virus persists have not been reported.

This study reports the immunohistochemical detection of LSDV antigens in tick salivary glands, epithelial tissues, haemocytes, the synganglion, reproductive tissues, and the fatty body following intrastadial and transstadial passage of LSDV in *A. hebraeum* and *R. appendiculatus* adults.

6.2. Introduction

Lumpy skin disease (LSD) is a World Organisation for Animal Health (OIE) listed disease of cattle (World Organisation for Animal Health 2012) It is caused by the lumpy skin disease virus (LSDV), a member of the genus *Capripoxvirus*. Other members of this genus are sheep poxvirus and goat poxvirus (King et al. 2011). Lumpy skin disease was first detected in Northern Rhodesia (Zambia) in 1921 and spread southwards to South Africa (Weiss 1968) and later, northwards up to Egypt (House et al. 1990). The disease has now spread to the Middle East in Israel (1989), Saudi Arabia (1992), Yemen (1995), United Arab Emirates (2000), Oman (2010) and recently also Lebanon (2013) (Tuppurainen & Oura 2012, Woods 1988, Yeruham et al. 1995). Epidemiological evidence implicates biting insects in the transmission of the virus. Most outbreaks have been reported in the wet season and along water courses where there is also an abundance of biting flies (Weiss 1968). The 1957 outbreak in Kenya was associated with high activity of *Culex mirificus* and *Aedes natrionus* mosquitoes (MacOwan 1959). The spread into Israel, in 1986, was associated with the stable fly, *Stomoxysis calcitrans* (Yeruham et al. 1995).

Recent studies have reported mechanical transmission of LSDV by *Rhipicephalus appendiculatus* and transovarial *transmission* of the virus by *Rhipicephalus (Boophilus) decoloratus* (Tuppurainen et al. 2013a, Tuppurainen et al. 2013b). Transstadial and intrastadial persistence of LSDV in saliva of *R. appendiculatus* and *A. hebraeum* adults previously fed on infected animals as either nymphs or adults, has also been demonstrated (Lubinga et al. 2013).

Ixodid ticks normally have single prolonged blood meals per development stage. For instance, *R. appendiculatus* nymphs will feed for 5 to 6 days and *A. hebraeum* nymphs for 7 to 8 days (Cupp 1991, Sonenshine 1991, Walker 2003). Pathogens in the blood meal have to survive and cross the midgut barrier before reaching the haemocoele from where other tick organs including the midgut, haemocytes, ovaries, synganglion (tick brain) and the salivary glands become infected (Booth et al. 1989, Booth et al. 1991b). Biological persistence of pathogens allows passage of the pathogens across tick stages (transstadial), vertically in the ovaries (transovarial) and sometimes within the same tick stage following interrupted feeding (intrastadial) (Labuda & Nuttall 2004, Sonenshine 1991). The specific tick organs affected have not been described.

As part of a broader study to determine the role of ixodid ticks in the transmission of LSDV, this study reports on the specific organ infection of *R. appendiculatus* and *A. hebraeum* adults by LSDV following intrastadial and transstadial persistence.

6.3. Materials and methods

6.3.1. Cattle

Eleven sero-negative Bonsmara cattle (*Bos indicus* and *Bos taurus* cross-bred cattle), were purchased from a farm in the Pretoria area, South Africa from a herd where vaccination against LSDV is not practised. They were approximately 18 months old and weighed between 190 and 250 kg. They were brought to the Faculty of Veterinary Science (FVS), University of Pretoria (UP) 14 days before onset of the trial for them to acclimatise to the environment and kept in insect-free stables at the UP's Bioscience Research Centre (UPBRC). Five of them were used as donor animals to infect ticks while 6 were used as recipient animals to host potentially infected ticks. The experimental procedures for the animals were approved by the Animal Use and Care Committee (AUCC) of the UP.

6.3.2. Preparation of the virus

A virulent South African LSDV field isolate (248/93), propagated on primary bovine dermis cells was used in the study. It was passaged five times in cell cultures at the Virology Section of the Department of Veterinary Tropical Diseases (DVTD), UP. The final titre of the virus was 5.95 log $TCID_{50}$/ml.

6.3.3. Source of the ticks

Laboratory reared unfed nymph and F1 adult *A. hebraeum* and *R. appendiculatus* ticks were supplied by the Agricultural Research Council's Onderstepoort Veterinary Institute (ARC-OVI).

6.3.4. Experimental infection of donor animals

Four donor animals were infected with the prepared LSDV inoculum on Day 0 post infection (pi), by injecting a volume of 2.5 ml of the virus suspension intravenously in the jugular vein and 0.25 ml intradermally at each of 4 sites, front and rear on each side of the body (Lubinga et al. 2013, Tuppurainen et al. 2013a). Two donor animals (DA1 and DA2) hosted *A. hebraeum* clean ticks while the other two (DR1 and DR2) hosted *R. appendiculatus* clean

ticks. The fifth donor animal DA3 hosted the second group of unfed F1 *A. hebraeum* and *R. appendiculatus* adults.

6.3.5. Test for intrastadial persistence in *A. hebraeum*

One of the virus inoculation sites on the lateral aspects of the body of donor animal DA3 was covered by a cotton cloth containment bag adhered to the skin with Genkem contact adhesive glue (Qualichem and Genkem, South Africa). On Day 4 pi, 75 unfed F1 *A. hebraeum* males were placed in the lateral bag. Four days later, i.e. Day 8 pi, 25 F1 *A. hebraeum* females were added to the same bag with males since *A. hebraeum* females only attach in response to aggregation and attachment pheromones secreted by males that had fed for approximately four days (Norval, Andrew & Yunker 1989, Norval et al. 1992). All the ticks (males and females) were collected after females fed for 4 days (Day12 pi) to test for LSDV using immunohistochemistry, specifically immunoperoxidase staining (IHC), electron microscopy (EM) and real-time PCR.

6.3.6. Test for transstadial persistence in *A. hebraeum*

To demonstrate transstadial passage of LSDV in *A. hebraeum* ticks, 500 unfed *A. hebraeum* nymphs were placed on the ears in containment bags attached to the base of the ear of each donor animal (DA1 and DA2) on Day 7 pi as described by Lubinga et al (2013). After feeding to repletion for 7-8 days, the engorged nymphs were collected, washed, dried and incubated for moulting in an acaridarium at 25°C and 85% relative humidity (RH). Two months after emergence, 200 F2 males were placed on each of the recipient animals RA5 and RA6 in containment bags placed on the lateral sides of the body to test for LSDV transmission, on Day 0 post attachment (pa). Four days later (Day 4 pa), 200 females were added to the bags containing males. Four days after adding females (Day 8 pa), 100 ticks (males and females) were collected for the detection of LSDV by IMPS, EM and real-time PCR.

6.3.7. Test for intrastadial persistence in *R. appendiculatus*

To investigate mechanical/intrastadial passage of the virus by *R. appendiculatus,* 100 adult ticks were placed in containment bags attached to the base of one ear of donor animal (DA3). After 4 days of feeding they were collected and tested for the presence of LSDV by IMPS EM and real-time PCR.

6.3.8. Test for transstadial persistence in *R. appendiculatus*

To investigate transstadial passage of LSDV in *R. appendiculatus*, 100 *R. appendiculatus* nymphs (total 200) were placed on one ear of each donor animal (DR1 and DR2) in the same way that adult ticks were placed above. After feeding to repletion for approximately 5 to 6 days, the engorged nymphs were collected and incubated to moult to adults as described above for *A. hebraeum* nymphs. As in *A. hebraeum*, 150 emergent adults were placed on recipient animal RR3 to test for transstadial transmission of LSDV. Fifty partially fed adults were collected after feeding for 4 days for testing by IMPS, EM and real-time PCR.

6.3.9. Animal infection tests

Infection in donor and recipient animals was determined by monitoring clinical signs and testing blood samples for LSDV by real-time PCR, virus isolation (VI) and serum neutralisation test (SNT) (Tuppurainen et al. 2013a).

6.3.10. Virus isolation

Virus isolation was carried out according to a standard operations procedure (SOP) of the Virology Section of the DVTD, UP, based on OIE guidelines (World Organisation for Animal Health 2012).

6.3.11. Real-time PCR

Real-time PCR was done on the tick homogenate samples as well as on cell culture supernatants. Blood samples from donor and recipient animals were also tested by real-time PCR (Lubinga et al. 2013, Tuppurainen et al. 2013a).

6.3.12. Immunohistochemistry

To determine the presence of LSDV in specific tick organs following interrupted feeding (intrastadial passage of the virus), 9 F1 *A. hebraeum* and 5 *R. appendiculatus* adults were collected from recipient animal RR3. They were sectioned in the sagittal plane and fixed overnight in 10% phosphate buffered formalin. Both halves of each tick were then embedded in paraffin and processed according to standard protocols of the Pathology Section in the Department of Paraclinical Sciences, FVS, UP.

To determine the presence of LSDV antigens in specific tick organs following transstadial passage of the virus, 5 unfed F2 and 10 partially fed F2 adults from recipient animals (RA5

and RA6) were processed as above. Similarly, to demonstrate the presence of the virus during transstadial infection in *R. appendiculatus*, 4 unfed F2 adults and 17 partially fed F2 adults on recipient animal RR3 were processed as above. Four un-infected ticks were processed in the same way and used as negative controls.

Pilot studies were conducted to optimise the immunoperoxidase labelling technique. Briefly, three 3 to 4 µm-thick tick sections were mounted on positively charged microscope slides and dried overnight in an oven at 38°C. After de-waxing in xylene for 10 minutes, the specimens were rehydrated through a graded ethanol and distilled water series (3 minutes each in 100%, 96% and 70% ethanol). Endogenous peroxidase activity was quenched by incubating the tick sections in 3% hydrogen peroxide (in methanol) for 15 minutes at room temperature (22-25°C) and rinsed three times in distilled water. For the purpose of antigen retrieval, slides were incubated in citrate buffer (pH of 6) at 96°C for 14 minutes, followed by cooling for 15 minutes at room temperature and then rinsed twice in distilled water and in PBS buffer for 5 minutes. To block non-specific immunoglobulin binding, the slides were incubated with normal horse serum (1:10 dilution) for 20 minutes at room temperature. The blocking serum was decanted and replaced with the primary F80G5 monoclonal antibody (anti–S057) (Babiuk et al. 2008), diluted 1:1000 and incubated overnight with the sections. The slides were then rinsed three times in distilled water and then in PBS buffer for 10 minutes. The secondary antibody, a biotinylated polyclonal rabbit anti-mouse antibody (Catalogue no: EO354, DakoCytomation, Denmark), was incubated with the tick sections for 30 minutes in a humidified chamber at room temperature. The slides were rinsed in distilled water for 3 minutes before rinsing in PBS for 10 minutes. The sections were incubated with the peroxidase conjugated avidin biotinylated complex (Catalogue no: PK6100, Vector laboratories, USA) for 30 minutes at room temperature and rinsed twice as before. A Vector® Nova red substrate (catalogue no: SK-4800, Vector laboratories, USA) was reconstituted according to manufacturer's instructions and incubated with the sections at room temperature. During this time, the positive-tissue control, a section of skin with characteristic lesions from a cow with confirmed LSD (PCR, EM and IHC-positive on a skin sample) was monitored at 100X magnification for positive labelling, using the light microscope in the IHC laboratory. As soon as there was evidence of clear, specific positive labelling in the positive-tissue control section, all of the tick sections were immediately rinsed in a distilled water bath to halt the substrate reaction. The sections were then counterstained with Mayer's haematoxylin for 20 seconds and rinsed under running tap water for 10 minutes. The sections were

routinely dehydrated through 70%, 96% and 100% alcohol, cleared in xylene and mounted in Entellan® (Merck Chemicals, Darmstadt, Germany). Specific positive labelling was confined to cytoplasmic granular labelling in tick cells and tissues, comparable with the labelling in target cells in the positive-tissue control.

To determine the limit of detection for the IMPS, bovine dermal cell monolayers were cultured on 8-well glass-slides (AEC-Amersham, LTD, South Africa). In each well, 400 µl of MEM containing 480,000 bovine dermis cells per ml was aliquoted and incubated for a day to allow the cells to form a confluent monolayer. Ten-fold serial dilutions of the virus (titre 4.5 Log $TCID_{50}$/ml) were performed and 10 µl of the respective virus dilution was inoculated into individual wells. After four days incubation, the wells were examined for cytopathic effects. The supernatant was removed for testing by real-time PCR to confirm presence of LSDV. The monolayers were air-dried and went through the process of immunoperoxidase staining according to protocols of the Section of Pathology. The stained slides were examined for the presence of LSDV. The viral titre for the highest dilution to show LSDV antigen staining was taken as the limit of detection.

6.4. Results

6.4.1. Infection of donor and recipient animals

Donor animal DA3 showed mild LSDV signs with mild prescapular lymph node enlargement from Day 11 pi until Day 27 pi. Lumpy skin disease virus DNA was detected by real-time PCR, from blood samples, between Day 11 and Day 26 pi with C_t values between 34.4 and 39.8.

To demonstrate transstadial transmission by *R. appendiculatus* the recipient animal was assessed for lumpy skin disease. Recipient animal, RR3, developed small circumscribed skin lesions on the neck on Day 12 post attachment (pa) until Day 22 pa. The highest body temperature was 39.1°C on Days 5 and 13 pa. Lumpy skin disease virus DNA was detected by real-time PCR from blood samples on Days 16, 20, 21, and 23 pa with Ct values between 34 and 39 but no sero-conversion was detected by SNT. Cytopathic effect was observed from blood samples on cell cultures after the second passage between Days 9 and 27 pa which was confirmed by real time PCR to be due to LSDV on samples for Day 20 pa (C_t value 38.7).

6.4.2. Infection in ticks

Of the 50 ticks examined by IHC, 41 (82%) showed positive labelling for LSDV. The *A. hebraeum* ticks which partially fed on the infected animal as adults (F1 adults) showed positive labelling in seven (78%) of the samples. The *A. hebraeum* F2 adults, which fed on a infected blood meal as nymphs, were positive in four (80%) of the unfed adults and eight (80%) of the fed F2 adults. *Rhipicephalus appendiculatus* showed positive labelling in four (80%) of the F1 partially fed adults, two (50%) unfed F2 adults and 16 (94%) fed adults. Non-infected negative-tissue control ticks did not show any specific labelling (Figure 6.1). The IHC results are summarised in Table 6.1.

The salivary glands were most affected with the highest infection occurring in F2 fed *R. appendiculatus* adults (82%), followed by F1 *R. appendiculatus* fed adults (80%) and F2 *A. hebraeum* fed adults (60%) (Table 6.1). In salivary glands, granular labelling of the LSDV antigen was seen in the cytoplasm of secretory cells of both type II and III alveoli (Figures 6.2, 6.3 & 6.4). Specific positive labelling was also seen in the cuticular layer of the salivary lobular ducts and in the bicuspid valves of the salivary gland acini.

Infection in the reproductive organs was especially prominent in fed F2 *R. appendiculatus* adults (65%), fed F2 *A. hebraeum* (50%) and unfed F2 *A. hebraeum* (60%) adults but was not seen in unfed F2 *R. appendiculatus* adults. In males, the most obvious infection of the reproductive tract was observed in the spermatogonia and spermatozoa of the testes (Figure 6.3) and in the vas deferens (Figures 6.4 & 6.5). labelling was also seen in the cells of the accessory sex glands. Infection in the female reproductive tract was noted in the cytoplasm of oogonia and

Table 6.1: Summary of immunohistochemistry results

Tick species	S/ gl	epidermis	pharynx	muscles	syng	rep system	midgut	faty body	h/cytes	MT	trachea	Pos	examined
A. hebraeum -IST	3 (33%)	1 (11%)	0 (0%)	2 (22%)	0 (0%)	2 (22%)	2 (22%)	0 (0%)	0 (0%)	0 (0%)	5 (56%)	7 (78%)	***9***
A. hebraeum-TST, unfed	1 (20%)	0 (0%)	0 (0%)	1 (20%)	0 (0%)	3 (60%)	0 (0%)	1 (20%)	0 (0%)	0 (0%)	3 (60%)	4 (80%)	**5**
A. hebraeum-TST, fed	6 (60%)	3 (30%)	0 (0%)	3 (30%)	1 (10%)	5 (50%)	1 (10%)	0 (0%)	5 (50%)	1 (10%)	2 (20%)	8 (80%)	**10**
R. appendiculatus-IST	4 (80%)	2 (40%)	0 (0%)	2 (40%)	0 (0%)	2 (40%)	1 (20%)	0 (0%)	2 (40%)	0 (0%)	3 (60%)	4 (80%)	**5**
R. appendiculatus-TST, fed	14 (82%)	5 (29%)	2 (12%)	10 (59%)	3 (18%)	11 (65%)	5 (29%)	6 (35%)	5 (29%)	1 (6%)	8 (47%)	16 (94%)	**17**
R. appendiculatus-*TST, unfed*	2 (50%)	0 (0%)	0 (0%)	2 (50%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (25%)	2 (50%)	**4**
Overall	**30 (60%)**	**11 (22%)**	**2 (4%)**	**20 (40%)**	**4 (8%)**	**23 (46%)**	**9 (18%)**	**7 (14%)**	**12 (24%)**	**2 (4%)**	**22 (44%)**	**41 (82%)**	***50***

IST= Intrastadial passage, TST= transstadial passage, S/gl= salivary glands, syng= synganglion, h/cytes= haemocytes, MT= malpighian tubules, Pos= total positive samples

in the lumen of the ovary, and in the walls and lumen of oviducts and connecting tubes (Figures 6.6, 6.7 & 6.8).

Cytoplasmic labelling was also seen in the haemocytes of five (50%) F2-fed adult *A. hebraeum*, five (29%) fed F2 adult *R. appendiculatus* and five (40%) F1 adult *R. appendiculatus* ticks where all types of haemocytes, including the prohemocytes and plasmocytes were labelled (Figures 6.4 & 6.9).

Infection of the muscles was seen in all groups of ticks with the highest rates in fed F2 *R. appendiculatus* (59%). labelling of muscles was mostly seen in the cytoplasm of connective sheaths enclosing the muscle bundles (Fig. 8). The midgut was labelled in the epithelial cells of two (22%) F1 *A. hebraeum* adults, one (10%) F2 fed *A. hebraeum* adult, one (20%) F1 *R. appendiculatus* adult and five (29%) F2 fed *R. appendiculatus* adults (Figs. 6 &7). The synganglia showed focal/multifocal granular labelling in one (10%) unfed F2 *A. hebraeum* adult and three (18%) fed F2 *R. appendiculatus* adults. labelling was seen in the cytoplasm of neuro-secretory cells of the cortex (Fig. 6). Labelling of the fat body was seen in one (20%) unfed F2 *A. hebraeum* adult and six (35%) fed F2 *R. appendiculatus* adults. Labelling was seen in the cytoplasm of both cells and in the matrix of fat bodies (Figures 6.6 & 6.7). Labelling was also seen in the malpighian tubules.

The limit of detection on stained monolayers was at a dilution of 10^{-4}, which corresponded to a viral titre of 10^{-1} log $TCID_{50}$/ml. This is also to the highest dilution where cytopathic effects were seen. Real-time PCR was able to detect the virus in monolayers up to a dilution of 10^{-7}, which corresponded to a concentration of log 10^{-3} log $TCID_{50}$/ml.

Real-time PCR confirmed the presence of LSDV (C_t value 36.69) in a sample of F2 *A. hebraeum* adults and in a sample of F1 *R. appendiculatus* (C_t value 36.05). These ticks were from the same batch as those examined by IHC.

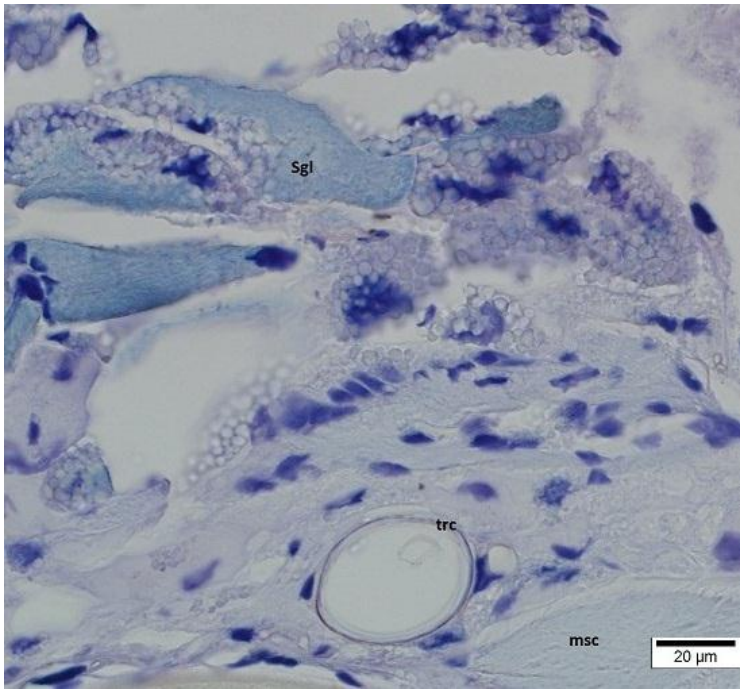

Figure 6.1: Non-infected negative-tissue control tick showing a lack of LSDV-specific immunoreactivity in salivary glands (sgl), muscle fibres (msc), tracheae (trc)

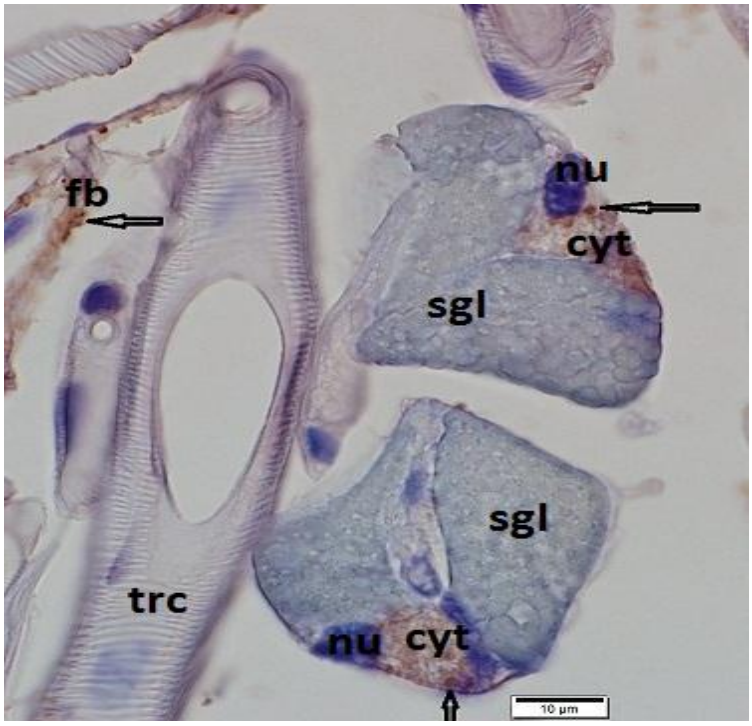

Figure 6.2: Salivary glands. Granular red-brown (LSDV-positive) labelling (arrows) in the cytoplasm (cyt) of alveolar cells of salivary glands (sgl) and fatbody (fb). Nu=nucleus.

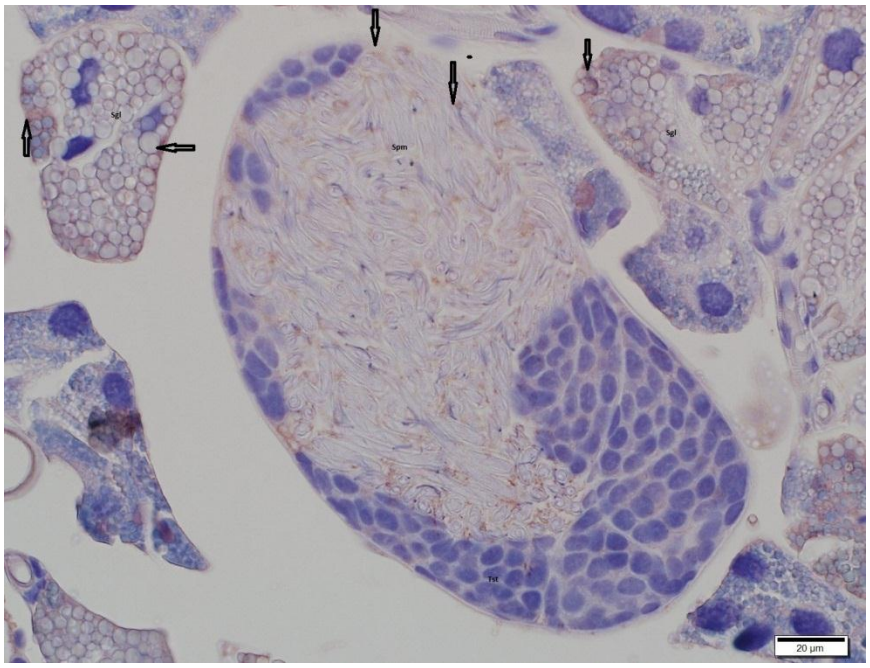

Figure 6.3: Red-brown granular labelling (arrows) in spermatozoa (spms) of testes (tst) and secretory granules of salivary glands (sgl)

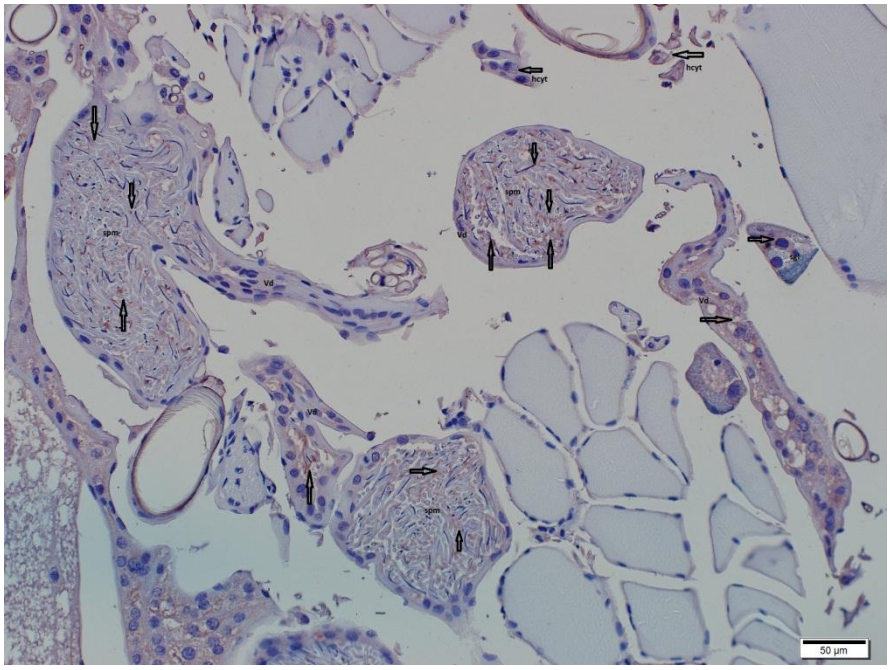

Figure 6.4: Red-brown granular labelling (arrows) in spermatozoa (spm) of the vas deferens (vd), the cytoplasm of haemocytes (hcyt) and secretory cells of the salivary glands (sgl)

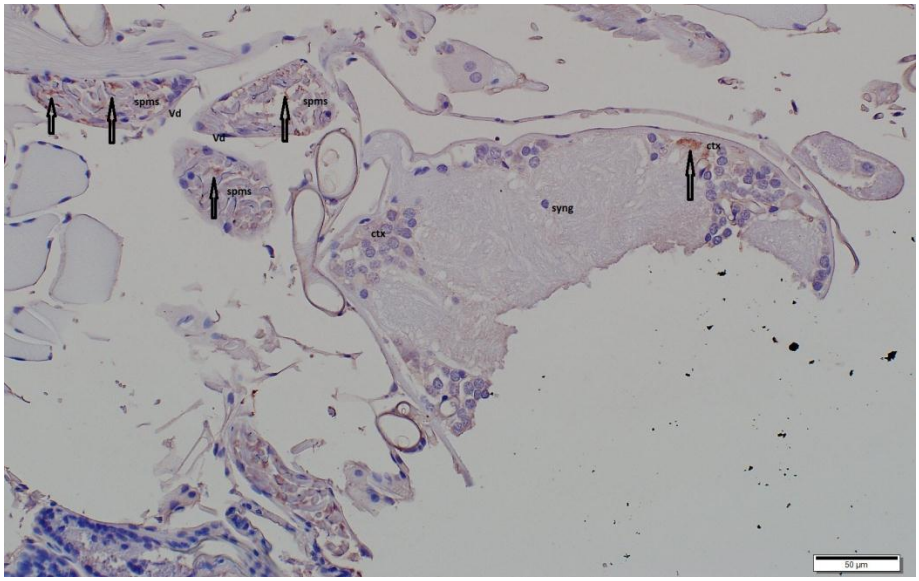

Figure 6.5: Red-brown granular labelling (arrows) in cortex (ctx) of synganglion (syng) and spermatozoa (spms) in vas deferens (Vd)

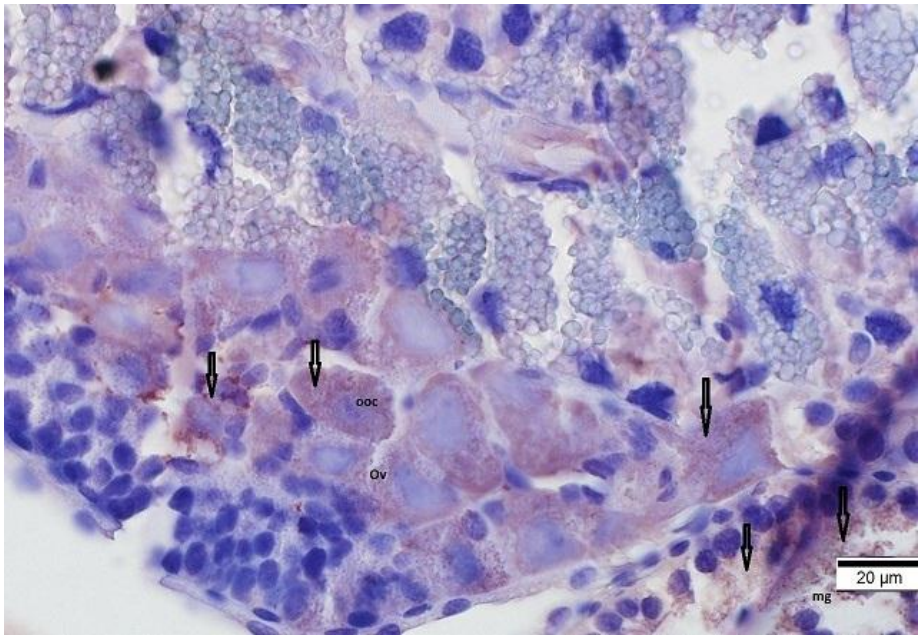

Figure 6.6: Intracytoplasmic Red-brown granular labelling (arrows) in the oocytes (ooc) of the ovary and in the midgut (mg)

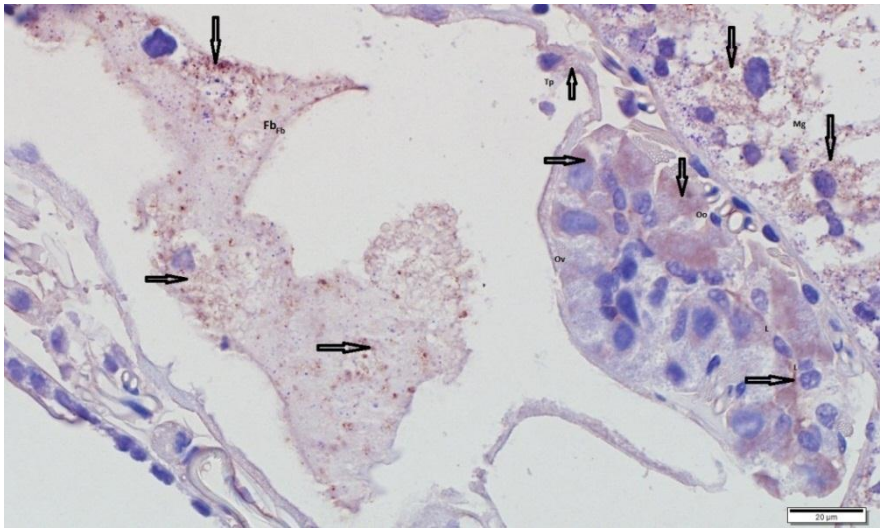

Figure 6.7: Intracytoplasmic red-brown granular labelling (arrows) in the oocytes (ooc) of the ovary and in the midgut (mg) and fatbody (Fb)

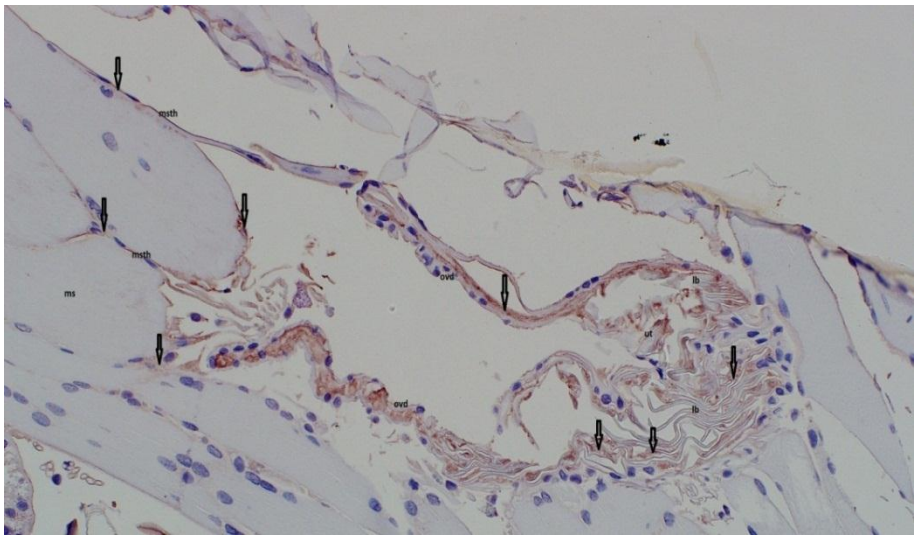

Figure 6.8: Red-brown granular labelling (arrows) in muscle sheaths (msth) around muscle bundles (ms) and in the oviducts (ovd) and labyrinth (lb) of common uterus (ut).

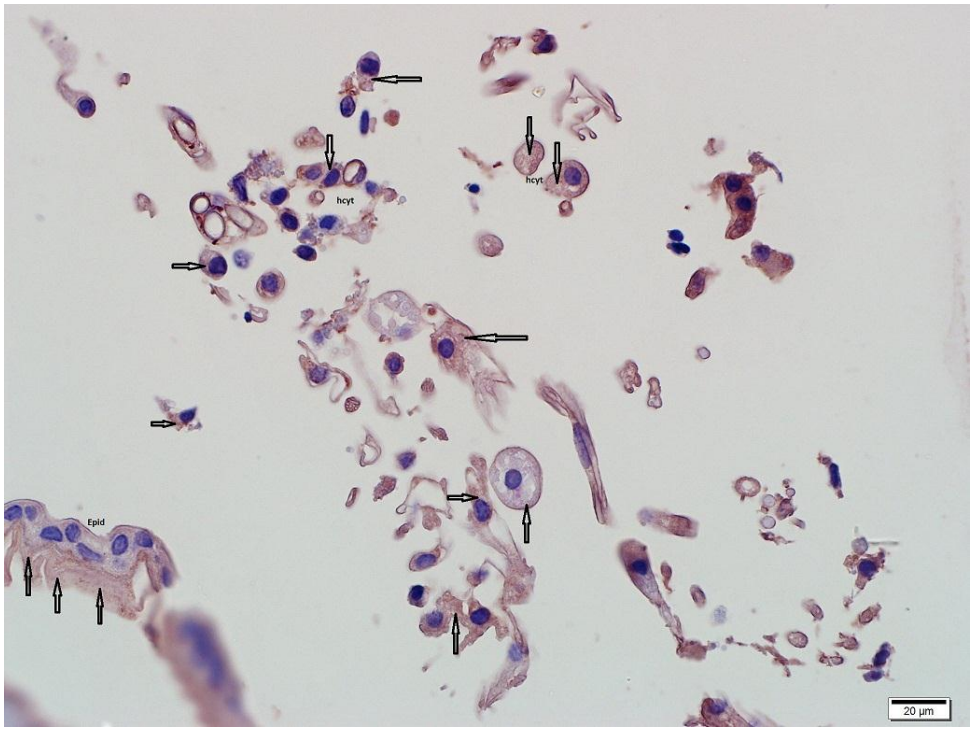

Figure 6.9: Red-brown granular labelling (arrows) in cytoplasm of haemocytes (hcyt) and epidermis (Epid)

6.5. Discussion

The competence of a vector is determined by the ability of pathogens to survive the hostile midgut environment (Jones et al. 1989). In this study, the vector competence of *A. hebraeum* and *R. appendiculatus* for LSDV was demonstrated by the detection of LSDV antigens, beyond the midgut in the organs of the haemocoele and the epidermis using IHC (Table 6.1). Similar organ involvement was demonstrated for thogoto virus in *R. appendiculatus* (Booth et al. 1989, Kaufman&Nuttall 2003) and dugbe virus in *A. hebraeum* (Booth et al. 1991a, Booth et al. 1991b). Similarly, *Ixodes hexagonus* ticks were reported to be competent vectors for bluetongue virus when the virus was demonstrated to cross the gut wall and spread to the testes, ovaries and salivary glands (Bouwknegt et al. 2010). Both transstadial and transovarial persistence of Nairobi sheep disease virus in *R. appendiculatus* was reported although the organs affected were not determined (Davies 1982, Lewis 1946).

The high in salivary glands reported in this study may suggest not only a high affinity of LSDV for the salivary glands but also that the virus is at end point in the tick's infection cycle. Since tick saliva is the major medium of transmission of tick pathogens (Labuda&Nuttall 2004, Randolph 2009), the salivary glands function as the main outlet of pathogens from ticks. These results, therefore, provide further evidence of vector competence by both *A. hebraeum* and *R. appendiculatus* for LSDV and are consistent with the detection of LSDV in the saliva of these ticks reported by Lubinga *et al.* (2013).

Both F1 adult *A. hebraeum* and *R. appendiculatus* subjected to interrupted feeding showed infection in organs other than salivary glands (Table 6.1), thereby demonstrating biological (intrastadial) development of LSDV. In nature, interrupted feeding in ticks has been reported. Males have been shown to detach after their mated female partner has engorged and dropped, in response to attraction pheromones secreted by unmated feeding females (Leahy, Hajkova & Bouchalova 1981). *Rhipicephalus sanguineus* males were seen to move between co-housed dogs (Little, Hostetler & Kocan 2007) while host to host movement of *A. hebraeum* males has also been described (Andrew&Norval 1989). Therefore, *A. hebraeum* and *R. appendiculatus* males are capable of intrastadial transmission of LSDV as they parasitize other hosts in search of unmated female partners. Intrastadial transmission has also

been reported for *Erhlichia canis* by *R. sanguinius* males (Bremer et al. 2005) and *Erhlichia ruminantium* by *A. hebraeum* males (Andrew&Norval 1989).

All tick stages have been shown to resume feeding when they are interrupted before they have taken enough blood to develop to the next stage, for example following grooming or death of the host (Andrew&Norval 1989, Wang&Nuttall 2001). This phenomenon has been attributed to the reprogramming of salivary gland protein expression (Wang, Henbest & Nuttall 1999). Interrupted feeding, therefore, is not restricted to male ticks and leads to increased disease transmission within an epidemic and may have important implications on the dispersal of ticks from dead animals (Andrew&Norval 1989, Wang&Nuttall 2001).

During moulting, some tick organs such as salivary glands undergo histolysis, a process of tissue dissolution (Balashov 1972, Sonenshine 1991). The vector competence for transstadial transmission of a pathogen, therefore, depends on the persistence of the pathogen in tissues that are not affected by histolysis during moulting (Kaufman&Nuttall 2003, Labuda&Nuttall 2004, Nuttall et al. 1994). The presence of LSDV antigen in the synganglia, epidermis, haemocytes and reproductive organs, which do not undergo histolysis (Balashov, Raikhel & Hoogstraal 1983, Sonenshine 1991) reported in this study, suggests these tissues/organs may be sites for LSDV transstadial survival in *A. hebraeum* and *R. appendiculatus*. The involvement of haemocytes suggests systemic distribution to organs and since haemocytes are responsible for synthesis of non-cellular connective tissue sheaths, the infection of tissue sheaths covering the muscle bundles may be a direct consequence of haemocyte infection. During moulting, the epithelial cells of the epidermis secrete cuticle of the exoskeleton just as the epithelial cells of the tracheae secrete the cuticular intima in the tracheae (Sonenshine 1991, Till 1961). Epithelial cells are therefore ideal for persistence of LSDV during transstadial development. Similarly, the fat body plays a storage role during moulting (Balashov, Raikhel & Hoogstraal 1983, Sonenshine 1991) and the apparent susceptibility of the fat body to LSDV infection seen in this study, may enable the virus to persist in the fat bodies between stages.

The demonstration LSDV in female reproductive organs such as the ovaries shows the potential for subsequent transovarial passage of LSDV by both *A. hebraeum* and *R. appendiculatus* females. This confirms the observations of transovarial passage and transmission of LSDV by *A. hebraeum* and *R. appendiculatus* (Lubinga et al, unpublished data). The virus, in this study, was also detected in the male reproductive organs especially

the sperm cells in the testes and vas deferens. Therefore, apart from the possibility that female ticks become infected from either an infected blood meal or from co-feeding with infected ticks (Labuda et al. 1993, Labuda et al. 1993), uninfected females may also be infected by sexual transmission from infected males, which may then be followed by transovarial passage of the virus. Sexual transmission and transovarial passage has been shown for Crimean Congo Haemorrhagic Fever in *Hyalomma trancatum* (Gonzalez et al. 1992).

Transstadial passage of LSDV in *R. appendiculatus* and *A. hebraeum* adults may play a role in the persistence of the virus during inter-epidemic periods. Both tick species over-winter on the ground after the parasitic nymphal stage as they moult to adults (Bryson et al. 2002, Horak, Gallivan & Spickett 2011, Schroder, Uys & Reilly 2006, Short&Norval 1981) and both (*A. hebraeum* and *R. appendiculatus*) adults become active in summer (Horak, Gallivan & Spickett 2011, Short et al. 1989), notably when the presence of LSD also increases (Coetzer 2004, Weiss 1968). This suggests a possible role for ticks in the transmission of the disease. Ticks, especially *A. hebraeum*, are known to have long life spans, i.e. they can survive for several years without a blood meal (Sonenshine 1991, Walker 2003) and viruses have the potential to survive in the ticks during this time (Sonenshine 1991).

The antibody and immunohistochemical technique employed in the present study could detect LSDV in tissues at a viral titre of 10^{-1} log $TCID_{50}$/ml, which is less sensitive than that of real-time PCR which was determined at 10^{-3} log $TCID_{50}$/ml. Immunohistochemistry has the advantage of not being compromised by cross-contamination when compared to real-time PCR. However, it is clear from the study, that only the tissues/organs that were present in the sections were available for immune-detection. The tissues/organs remote from the cut sections were therefore excluded from the IHC test. This limitation could feasibly be reduced by examining serial sections.

This study was able to show for the first time the tick organs and tissues infected by LSDV during both intrastadial and transstadial persistence of the virus in ticks. The study also revealed the potential vector competency of *A. hebraeum* and *R. appendiculatus* for LSDV, which is also supported by the reports of mechanical transmission of LSDV by *R. appendiculatus* (Tuppurainen et al. 2013a), mechanical/intrastadial and transstadial transmission by *A. hebraeum* (Lubinga et al, submitted) and the transstadial transmission by

R. appendiculatus. Studies to detect LSDV in ticks in natural outbreaks will further validate these results.

6.6. References

Andrew, H.R. & Norval, R.A.I., 1989, 'The role of males of the bont tick (*Amblyomma hebraeum*) in the transmission of *Cowdria ruminantium* (heartwater)', *Veterinary parasitology* 34(1-2), 15-23.

Babiuk, S., Bowden, T.R., Parkyn, G., Dalman, B., Manning, L., Neufeld, J., Embury-Hyatt, C., Copps, J. & Boyle, D.B., 2008, 'Quantification of lumpy skin disease virus following experimental infection in cattle', *Transboundary and Emerging Diseases* 55(7), 299-307.

Balashov, Y., S., Raikhel, A.S. & Hoogstraal, H., 1983, *An atlas of ixodid tick ultrastructure.* Entomological Society of America, College Park, MaryLand; USA.

Balashov, Y.S., 1972, *Blood-sucking ticks (Ixodoidea)-vectors of diseases of man and animals.* Entomological Society of America.

Booth, T.F., Davies, C.R., Jones, L.D., Staunton, D. & Nuttall, P.A., 1989, 'Anatomical basis of Thogoto virus infection in BHK cell culture and in the ixodid tick vector, *Rhipicephalus appendiculatus*.', *Journal of General Virology* 70(5), 1093-1104.

Booth, T.F., Marriott, A.C., Steele, G.M. & Nuttall, P.A., 1991a, 'Dugbe virus in ticks: histological localization studies using light and electron microscopy', *Archives of Virology, Supplementum* (1), 207-218.

Booth, T.F., Steele, G.M., Marriott, A.C. & Nuttall, P.A., 1991b, 'Dissemination, replication, and trans-stadial persistence of Dugbe virus (Nairovirus, Bunyaviridae) in the tick vector *Amblyomma variegatum*', *American Journal of Tropical Medicine and Hygiene* 45(1), 146-157.

Bouwknegt, C., Rijn, P.A., Schipper, J.J.M., Hölzel, D., Boonstra, J., Nijhof, A.M., Rooij, E.M.A. & Jongejan, F., 2010, 'Potential role of ticks as vectors of bluetongue virus', *Experimental and Applied Acarology* 52(2), 183-192.

Bremer, W.G., Schaefer, J.J., Wagner, E.R., Ewing, S.A., Rikihisa, Y., Needham, G.R., Jittapalapong, S., Moore, D.L. & Stich, R.W., 2005, 'Transstadial and intrastadial experimental transmission of *Ehrlichia canis* by male *Rhipicephalus sanguineus*.', *Veterinary parasitology* 131(1/2), 95-105.

Bryson, N.R., Tice, G.A., Horak, I.G., Stewart, C.G. & Plessis, B.J.A.d., 2002, 'Ixodid ticks on cattle belonging to small-scale farmers at 4 communal grazing areas in South Africa.', *Journal of the South African Veterinary Association* 73(3), 98-103.

Coetzer, J.A.W., 2004, 'Lumpy skin disease' in pp. 1268-1276, Oxford University Press, Oxford.

Cupp, E.W., 1991, 'Biology of ticks', *Veterinary Clinics of North America, Small Animal Practice* 21(1), 1-26.

Davies, F.G., 1982, 'Observations on the epidemiology of lumpy skin disease in Kenya', *The Journal of hygiene* 88(1), 95-102.

Gonzalez, J.P., Camicas, J.L., Cornet, J.P., Faye, O. & Wilson, M.L., 1992, 'Sexual and transovarian transmission of Crimean-Congo Hemorrhagic-vever virus in *Hyalomma-truncatum* ticks', *Research in virology* 143(1), 23-28.

Horak, I.G., Gallivan, G.J. & Spickett, A.M., 2011, 'The dynamics of questing ticks collected for 164 consecutive months off the vegetation of two landscape zones in the Kruger National Park (1988-2002). Part I. Total ticks, Amblyomma hebraeum and Rhipicephalus decoloratus.', *Onderstepoort Journal of Veterinary Research* 78(1), Arte2.

House, J.A., Wilson, T.M., el Nakashly, S., Karim, I.A., Ismail, I., el Danaf, N., Moussa, A.M. & Ayoub, N.N., 1990, 'The isolation of lumpy skin disease virus and bovine herpesvirus-4 from cattle in Egypt', *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc* 2(2), 111-115.

Jones, L.D., Davies, C.R., Steel, G.M. & Nuttall, P.A., 1989, 'Vector Capacity of Rhipicephalus-Appendiculatus and Amblyomma-Variegatum for Thogoto and Dhori Viruses', *Medical and veterinary entomology* 3(2), 195-202.

Kaufman, W.R. & Nuttall, P.A., 2003, 'Rhipicephalus appendiculatus (Acari: Ixodidae): dynamics of Thogoto virus infection in female ticks during feeding on guinea pigs.', *Experimental parasitology* 104(1/2), 20-25.

King, A., Lefkowitz, E., Adams, M.J. & Carstens, E.B. (eds.), 2011, 'Virus Taxonomy, 1st Edition. Ninth Report of the International Committee on Taxonomy Viruses'.

Labuda, M., Jones, L.D., Williams, T., Danielova, V. & Nuttall, P.A., 1993, 'Efficient transmission of tick-borne encephalitis virus between cofeeding ticks.', *Journal of medical entomology* 30(1), 295-299.

Labuda, M. & Nuttall, P.A., 2004, 'Tick-borne viruses.', *Parasitology* 129(Supplement), S221-S245.

Labuda, M., Nuttall, P.A., Kožuch, O., Elečková, E., Williams, T., Žuffová, E. & Sabó, A., 1993, 'Non-viraemic transmission of tick-borne encephalitis virus: a mechanism for arbovirus survival in nature', *Experientia* 49(9), 802-805.

Leahy, M.G., Hajkova, Z. & Bouchalova, J., 1981, 'Two female pheromones in the metastriate tick Hyalomma dromedarii (Acarina, Ixodidae).', *Acta Entomologica Bohemoslovaca* 78(4), 224-230.

Lewis, E.A., 1946, 'Nairobi sheep disease; the survival of the virus in the tick *Rhipicephalus appendiculatus*.', *Parasitology* 37 55-9.

Little, S.E., Hostetler, J. & Kocan, K.M., 2007, 'Movement of *Rhipicephalus sanguineus* adults between co-housed dogs during active feeding.', *Veterinary parasitology* 150(1/2), 139-145.

Lubinga, J.C., Tuppurainen, E.S.M., Stoltsz, W.H., Eberhson, K., Coetzer, J.A.W. & Venter, E.H., 2013, '
Detection of lumpy skin disease virus in saliva of ticks fed on blood of infected cattle', *Exp Appl Acarol* .

MacOwan, K.D.S., 1959, 'Observations on the epizootiology of lumpy skin disease during the first year of its occurrence in Kenya', *Bulletin of epizootic diseases of Africa. Bulletin des epizooties en Afrique* 7 7-20.

Norval, R.A.I., Andrew, H.R. & Yunker, C.E., 1989, 'Pheromone-mediation of host-selection in bont ticks (*Amblyomma hebraeum* Koch).', *Science (Washington)* 243(4889), 364-365.

Norval, R.A.I., Peter, T., Sonenshine, D.E. & Burridge, M.J., 1992, 'Responses of the ticks *Amblyomma hebraeum* and *A. variegatum* to known or potential components of the aggregation-attachment pheromone. III. Aggregation', *Experimental & applied acarology* 16(3), 237-245.

Nuttall, P.A., Jones, L.D., Labuda, M. & Kaufman, W.R., 1994, 'Adaptation of arboviruses to ticks.', *Journal of medical entomology* 31(1), 1-9.

Randolph, S.E., 2009, 'Tick-borne disease systems emerge from the shadows: the beauty lies in molecular detail, the message in epidemiology.', *Parasitology* 136(12), 1403-1413.

Schroder, B., Uys, A.C. & Reilly, B.K., 2006, 'A survey of free-living ixodid ticks on a commercial game farm in the Thabazimbi District, Limpopo Province, South Africa', *Journal of the South African Veterinary Association* 77(3), 141-144.

Short, N.J., Floyd, R.B., Norval, R.A.I. & Sutherst, R.W., 1989, 'Development rates, fecundity and survival of developmental stages of the ticks *Rhipicephalus appendiculatus, Boophilus decoloratus and B. microplus* under field conditions in Zimbabwe.', *Experimental & applied acarology* 6(2), 123-141.

Short, N.J. & Norval, R.A.I., 1981, 'The seasonal activity of Rhipicephalus appendiculatus Neumann 1901 (Acarina: Ixodidae) in the highveld of Zimbabwe (Rhodesia).', *Journal of Parasitology* 67(1), 77-84.

Sonenshine, D.E., 1991, *Biology of ticks*, Oxford University Press, Oxford.

Till, W.M., 1961, *A contribution to the anatomy and histology of the brown ear tick Rhipicephalus appendiculatus Neumann.* .

Tuppurainen, E.S.M., Lubinga, J.C., Stoltsz, W.H., Troskie, M., Carpenter, S.T., Coetzer, J.A.W., Venter, E.H. & Oura, C.A.L., 2013a, 'Mechanical transmission of lumpy skin disease virus by *Rhipicephalus appendiculatus* male ticks', *Epidemiology and infection, Epidemiol.Infect.* 141, 2 425-430.

Tuppurainen, E.S.M., Lubinga, J.C., Stoltzs, W.H., Troskie, M., Carpenter, S.T., Coetzer, J.A.W., Venter, E.H. & Oura, A.L., 2013b, 'Evidence of vertical transmission of lumpy skin disease in Rhipicephalus decoloratus ticks', *Ticks and tick borne diseases* .

Tuppurainen, E.S.M. & Oura, C.A.L., 2012, 'Review: lumpy skin disease: an emerging threat to Europe, the Middle East and Asia.', *Transboundary and Emerging Diseases* 59(1), 40-48.

Walker, A., 2003, *Ticks of domestic animals in Africa : a guide to identification of species,* Bioscience Reports, Edinburgh.

Wang, H., Henbest, P.J. & Nuttall, P.A., 1999, 'Successful interrupted feeding of adult Rhipicephalus appendiculatus (Ixodidae) is accompanied by reprogramming of salivary gland protein expression.', *Parasitology* 119(2), 143-149.

Wang, H. & Nuttall, P.A., 2001, 'Intra-stadial tick-borne Thogoto virus (Orthomyxoviridae) transmission: accelerated arbovirus transmission triggered by host death.', *Parasitology* 122(4), 439-446.

Weiss, K.E., 1968, 'Lumpy skin disease virus', *Virology Monographs* 3 111-131.

Woods, J.A., 1988, 'Lumpy skin disease--a review', *Tropical animal health and production* 20(1), 11-17.

World Organisation for Animal Health, 2012, *Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees), Volume 1*. .

Yeruham, I., Nir, O., Braverman, Y., Davidson, M., Grinstein, H., Haymovitch, M. & Zamir, O., 1995, 'Spread of lumpy skin disease in Israeli dairy herds', *The Veterinary record* 137(4), 91-93.

Chapter 7

Evidence of lumpy skin disease virus over-wintering by transstadial persistence in *Amblyomma hebraeum* and transovarial persistence in *Rhipicephalus (Boophilus) decoloratus* ticks

Submitted to Experimental and Applied Acarology journal

Manuscript number: APPA-D-13-00065

7.1. Abstract

Lumpy skin disease (LSD) is a debilitating cattle disease caused by the lumpy skin disease virus (LSDV), belonging to the genus *Capripoxvirus*. Epidemics of the disease usually occur in summer, when insect activity is high. Limited information is available on how LSDV persists during inter-epidemic periods. Transmission of LSDV by mosquitoes such as *Aedes aegypti* has been shown to be mechanical, there is no carrier state in cattle and the role of wildlife in the epidemiology of the disease seems to be of minor importance. Recent studies in ticks have shown transstadial persistence of LSDV in *Rhipicephalus appendiculatus* and *Amblyomma hebraeum* as well as transovarial persistence of the virus in *R. (B) decoloratus, R. appendiculatus* and *A. hebraeum.* The over-wintering of ticks on the ground as part of their life cycles is well known: *A. hebraeum* and *R. appendiculatus* over-winter, for example, on the ground after feeding as nymphs/unfed adults while *R. (B) decoloratus* over-winters on the ground as engorged females. In this study, transstadial and transovarial persistence of LSDV from experimentally infected *A. hebraeum* nymphs and *R. (B) decoloratus* females after exposure to cold temperatures of 5°C at night and 20°C during the day for 2 months was reported. This observation suggests possible over-wintering of the virus in these tick species.

Key words: *Amblyomma hebraeum*, *Rhipicephalus (Boophilus) decoloratus*, lumpy skin disease virus, transstadial, transovarial transmission, Over-wintering

7.2. Introduction

Lumpy skin disease (LSD) is a debilitating disease of cattle caused by the lumpy skin disease virus (LSDV), a member of the genus *Capripoxvirus*. Outbreaks occur most commonly in Africa and on occasion, in some countries of the Middle East (Davies 1991, Kumar 2011, Woods 1988, Yeruham et al. 1995). In most instances, epidemics of LSD are associated with conditions that favour high activity of biting flies, especially during very high rainfall. The 'reservoir/s' of the virus during inter-epidemic periods have not been established. Only mechanical transmission in insects has been reported, such as in *Aedes aegypti* mosquitoes (Chihota et al. 2001). There is no carrier state in cattle and, although the role of wildlife is not well established, a carrier state in wild ungulates is unlikely (Coetzer 2004, Hunter & Wallace 2001).

Recent transmission studies with ticks on animals demonstrated mechanical/intrastadial and transstadial transmission by *Amblyomma hebraeum* (Lubinga et al, submitted) and *Rhipicephalus appendiculatus* adult ticks (Tuppurainen et al. 2013a). Transovarial transmission of the virus was demonstrated in *Rhipicephalus (Boophilus) decoloratus* (Tuppurainen et al. 2013b). Both intrastadial and transstadial passage of LSDV has been demonstrated in *R. appendiculatus* and *A. hebraeum* through detection of LSDV in saliva of adult ticks fed as either adults or nymphs respectively (Lubinga et al. 2013).

Amblyomma hebraeum, the bont tick, is a three-host tick common in Southern Africa (Horak, Gallivan & Spickett 2011, Norval, Andrew & Meltzer 1991, Walker 2003). It has a long life span and overwinters after engorgement as nymphs. The adult stages emerge early in summer (Horak 1982, Horak, Gallivan & Spickett 2011, Norval 1977, Walker 2003). Transstadial persistence of LSDV in *A. hebraeum* makes this tick species a likely candidate for over-wintering of the virus.

Rhipicephalus (Boophilus) decoloratus is a one-host tick; wide spread in southern Africa and a common parasite of ungulates. It has several generations per year (Walker 2003), but with peak numbers found on animals in summer. They appear to over-winter on the ground (Bryson et al. 2002a, Schroder, Uys & Reilly 2006). The potential for vertical transmission also makes *R. (B) decoloratus* a possible over-wintering host for LSDV.

In this study, the passage of LSDV from engorged *A. hebraeum* nymphs to adults and from engorged female *R. (B) decoloratus* to larvae were investigated under cold temperatures (5°C

at night and 20°C during the day) in order to determine their possible role in the over-wintering of LSDV.

7.3. Materials and methods

7.3.1. Study area and ethics statement

The study was conducted in between April and May 2012 at the University of Pretoria (UP), Faculty of Veterinary Science (FVS) in the Department of Veterinary Tropical Diseases (DVTD). The experimental procedures for the animals were approved by the Animal Use and Care Committee (AUCC) of the University of Pretoria, South Africa on 26/07/2010 (Project No. V043-10).

7.3.2. The virus

The virus used in the study is a virulent South African LSDV field isolate (248/93), which was propagated on primary bovine dermis cells for five to six passages at the Virology Section of the Department of Veterinary Tropical Diseases (DVTD), University of Pretoria (UP). The final titre of the virus was 5.95 log $TCID_{50}$/ml.

7.3.3. Tick origin

Amblyomma hebraeum engorged nymphs (n=362), fed to repletion on sheep, were supplied by the Agricultural Research Council's Onderstepoort Veterinary Institute (ARC-OVI), Pretoria, South Africa on the day of dropping off the host. Similarly, 50 engorged *R. (B) decoloratus* females fed to repletion on LSDV-free cattle were supplied by ARC-OVI.

7.3.4. Tick inoculation

The engorged ticks were artificially infected on the same day of dropping from the host by intracoelomic injection, based on the method used by Kaufman and Nuttal (1996). Briefly, the engorged ticks were placed on an adhesive tape, dorsal side down. Using a 0.3 ml syringe with a 30 gauge needle (BD Micro-Fine™ *Plus* Demi, USA), the needle was inserted, beyond the bevelled tip, into the hemocoele, at the rear margin of the tick and 5 to 10 µl of virus suspension was inoculated. The needle was withdrawn after a minute to reduce leakage of inoculant. Forty of the fifty *R. (B) decoloratus* female ticks were injected and the remaining 10 were kept as negative controls. Similarly, 312 *A. hebraeum* engorged nymphs were injected and 50 kept as negative controls.

7.3.5. Incubation of ticks

The *A. hebraeum* nymphs were divided into two incubation groups. One group comprising 160 nymphs (140 injected and 20 negative controls) was incubated to moult at room temperature (25°C) and 85% relative humidity (RH). The other group of 202 nymphs (172 inoculated and 30 negative controls) was incubated at temperatures simulating the approximate maximum and minimum temperatures experienced during winter in the northern part of South Africa i.e. 20°C during the day from 08:00 to 17:00 hrs (9 hrs) and at 5°C during the night from 17:00 to 08:00 hrs the following day (15 hrs). The relative humidity remained at 85% throughout. They were observed for the progress in moulting. After 60 days of incubation, ticks kept at fluctuating cold temperatures were incubated at room temperature until moulting was completed.

Similarly, *R. (B) decoloratus* females were divided into two groups of 25 ticks each, with 20 injected ticks and 5 negative controls per group. One group was left at room temperature and the other exposed to cold temperatures as described for *A. hebraeum* above. Similarly, the incubation period at cold temperatures was 60 days before they were incubated at 25°C.

7.3.6. Tick dissection

Ticks were rinsed twice in sterile deionised water, dried on filter paper and then rinsed twice in phosphate buffered saline containing Ca^{2+} and Mg^{2+} (PBS^{+}). Ten *A. hebraeum* emergent adults from each group of incubation were dissected by removing the dorsal part of body or idiosoma (scutum or conscutum). Briefly, using a number 11 surgical blade, an incision was made along the lateral margins of the tick under a stereomicroscope (Nikon SMZ 800, Japan). The scutum or conscutum was lifted and separated from the rest of the body parts using pointed surgical forceps. The salivary glands were identified as white grape-like structures on the antero-lateral aspects of the tick. The midguts were seen as black tubes with branches (diverticulae) extending in various directions. The synganglion was identified as a white mass in the midline just antero-ventral to the midgut and between the anterior parts of the salivary ducts. The salivary glands, midguts and synganglia were collected for testing.

7.3.7. Homogenisation of tick samples

Homogenisation of *A. hebraeum* adult ticks was performed by combining techniques used by Bell-Sakyi and co-workers (2009) and Sang and co-workers (2006). Briefly, ticks were rinsed twice in sterile deionised water and dried on filter paper after which they were rinsed twice in

PBS^+ with double the normal dose (0.2%) of Gentamycin (50 mg/ml, Genta 50, Virbac Animal Health). The ticks were cut into 4 parts and placed into tubes with beads (Roche Diagnostics, Mannheim, Germany) containing 1 ml of MEM with 0.2 mg/ml Gentamycin. The tubes were then cooled at -80°C for 5 minutes and crushed using a Magnalyser (Roche Diagnostics) at 6500 rpm for 1 minute. The supernatant was collected in 1.5 ml Eppendorf tubes (Eppendorf, South Africa) and stored at -80°C for further use in virus isolation and real-time PCR. Homogenised tick samples included 10 moulting nymphs (incubated at cold temperatures), 10 emergent adult ticks of each of the groups incubated at cold and room temperatures and their negative controls and pooled tick organs of the salivary glands, midguts and synganglia from both cold and room temperature incubation groups.

From each pair of inoculated female *R. (B) decoloratus*, a sample of the laid eggs and hatched larvae were collected in 1.5 ml micro tubes and rinsed three times in PBS^+ with 0.2% Gentamycin and 0.5% Amphotericin B. approximately 5 g were homogenised as above. A negative control from each temperature group was also homogenised.

The tick egg homogenate samples were stored for testing by real-time PCR while larval homogenate samples were stored for testing by real-time PCR and virus isolation (VI).

7.3.8. Virus isolation

Virus isolation was carried out according to standard operation procedures of the DVTD, UP based on OIE guidelines (OIE, 2010). Briefly; 100 µl tick homogenate was diluted into 900 µl MEM with 5% foetal calf serum and 2% Gentamycin (Genta 50, Virbac Animal Health) to minimise cytotoxicity and inoculated onto bovine dermis cells in 24 well-plates and incubated at 37°C for an hour. After the incubation, the cells were washed with PBS^+ followed by addition of fresh medium, (i.e. 3 ml MEM containing 5% foetal calf serum) (Highveld Biological, SA) and 0.2% Gentamycin (Genta 50, Virbac Animal Health) and incubation at 37°C. The cells were observed daily for cytopathic effects (CPE). In the absence of CPE a second or third passage was done. The used cell culture media were stored at -80°C and was tested by real-time PCR to confirm that the CPE was caused by LSDV.

7.3.9. Real-time PCR

Real-time PCR was performed on the tick homogenates and on the cell culture media from virus isolation tests. Extraction of DNA from tick homogenates was based on the protocol followed by Tuppurainen and co-workers (Tuppurainen, Venter & Coetzer 2005).

The extracted DNA was tested by a real-time PCR Taqman assay, which amplifies an 89 bp region within the capripoxviruses ORF 74 region that encodes the intracellular mature protein P32 (Bowden et al. 2008, Tulman et al. 2001). Sequences for the *Capripoxvirus*-specific primers and probes used were as follows: Forward primer-CaPV074F1 5'-AAA ACG GTA TAT GGA ATA GAGTT GAA-3', Reverse primer- CaPV074R1 5'-AAA TGA AAC CAA TGG ATG GGA TA-3' and Probe CaPV074P1 5'FAM-TGG CTC ATA GAT TTC CT-MGB/NFQ -3' (Bowden et al. 2008). The assays were run using a Light Cycler®, 2.0 machine (Roche Diagnostics, Mannheim, Germany). The reaction mixture included 4 µl of 10X FastStart DNA Master^{plus} HybProbe kit (Roche products, Mannheim, Germany), 1 µl (0.1 µM) each of the primers, 1 µl (0.2 µM) of hybridization probe, 0.5 µl of UDG, 8.5 µl of water and 4 µl of DNA template (up to 500 ng). The reaction was run following procedures described by Bowden *et al.* (2008). Briefly, the temperature was raised to 40°C for 10 minutes to activate UDG (Roche products, Mannheim, Germany), followed by a step of FastStart *Taq* polymerase enzyme activation for 10 minutes at 95°C. The amplification involved 45 cycles of denaturation at 95°C for 10s, annealing at 60°C for 60s and extension at 72°C for 1s (single acquisition mode). DNA extracted from known infected cell cultures was used as positive control. Negative controls included a water sample processed through the DNA extraction procedures and a no-DNA template water control. For DNA extracted from cell culture supernatant, clean cell culture medium was used as negative control, while un-inoculated ticks were used as negative controls for tick homogenate DNA assays.

7.3.10. Immunoperoxidase staining

Ticks were cut in their sagittal section and fixed in 10% phosphate buffered formalin. They were imbedded in paraffin and processed according to standard protocols of the Pathology Section, Faculty of Veterinary Science, UP. Un-inoculated ticks were used as tick negative controls.

7.3.11. Transmission electron microscopy

Tick organs submitted for transmission electron microscopy (TEM) were tested according to protocols of the Electron Microscopy Unit at the Department of Anatomy, Faculty of Veterinary Science, UP. These included samples of salivary glands, synganglia and midguts of 10 *A. hebraeum* adults from each of the cold and room temperature groups and their negative controls. Cell culture material inoculated with ticks (n=4) from the cold temperature group, showing CPE, were also processed for TEM.

7.3.12. Statistical analysis

The comparative analysis of the effect of exposure to cold temperature during incubation (moulting/oviposition) was done using a bootstrap method (Tarr 2012) with 1000 times Monte Carlo simulation (Buckland 1984, Tan Gang 2012). Briefly, the lower and upper Monte Carlo confidence intervals (MMCI) of C_t values from real-time PCR results of each group were computer generated in excel using Monte Carlo simulations. True differences between any two groups were determined by examining for the presence or absence of an overlap in MMCI's between the two groups. The presence of an overlap in the MMCI's between two groups would indicate "no true difference" while lack of overlap would indicate a true difference between them.

7.4. Results

7.4.1. Tick moulting –*Amblyomma hebraeum* nymphs

On Day 1, *A. hebraeum* nymphs incubated at room temperature showed signs of the outer cuticle turning pale and shrinking except for 4 of the inoculated nymphs which became black, a sign of death. These remained black for the rest of the observation period. The rest kept increasing in pallor and by Day 10 of incubation, the coat colour of the nymphs started turning yellow. The coat continued to thin and increased in transparency and by Day 22, the new inner coat was visible and sex of the emergent adult tick could be seen by Day 26. On Day 29, the shedding of the outer coat was seen in 8 inoculated ticks (6 females and 2 males) and 6 non-inoculated ticks (4 females, 2 males). Moulting in the rest of the ticks incubated at room temperature was complete by Day 36. Only 4 nymphs from this group died.

The *A. hebraeum* nymphs incubated under cold temperatures turned slightly pale on Day 1 and remained in this state until Day 60, when their incubation was changed to room temperature. No mortality of the nymphs was detected up to Day 60. Six days later, the coat of the moulting nymphs turned yellow and became thinner with increasing transparency. By day 80 i.e. 20 days after change of incubation to room temperature, the outer coat was very transparent and the new inner coat was visible. The shedding of the outer coat was seen on Day 84 in 5 ticks (4 females, 1 male) of the inoculated group and 6 ticks (4 females, 2 males) of the non-inoculated group. By Day 90 the moulting was complete. However 5 nymphs from the inoculated group and 1 among the non-inoculated ticks failed to moult. The exact time of death was not determined.

7.4.2. Tick oviposition and hatching- *Rhipicephalus (Boophilus) decoloratus*

Rhipicephalus (Boophilus) decoloratus females incubated at room temperature began laying eggs on Day 4 of incubation. They continued laying eggs up to Day 19 (15 days of ovipositing). By this time, the outer coat of the females turned yellow and their size was considerably. Hatching of these eggs into larvae was noted on Day 32 of incubation and continued until Day 50 (18 days of hatching). All the females in this group survived until completion of ovipositing.

The *R. (B) decoloratus* females that were incubated under cold temperatures did not lay eggs during this time (i.e. up to Day 63). Oviposition commenced on Day 64, i.e. 1 day after raising the incubation temperature to 25°C and continued on up to about Day 80 (16 days of laying). The hatching of larvae was noted on Day 82 up to Day 96 (14 days of hatching). Two ticks in this group seemed to have died before they started laying eggs. Four of the inoculated ticks and 3 control ticks in this group died before they completed laying eggs. The rate of hatching was very close to 100%.

7.4.3. Real-time PCR

All *A. hebraeum* adults (100%) kept at room temperature tested positive with C_t values between 28.94 and 34.33. All the samples of moulting nymphs still under cold temperatures tested positive with C_t values between 23.33 and 27.84. All adults exposed to cold temperatures tested positive with C_t values between 31.03 and 38.41 (Table 7.1).

The virus was detected in 10 (100%) *R. (B) decoloratus* egg samples from females that were exposed to cold temperatures (C_t values between 32.82 and 37.83) and in 9 (90%) egg samples from females in the room temperature group with C_t values between 30.10 and 37.54. The virus was detected in all larval samples with C_t values between 31.21 and 35.57 for the room temperature group and between 30.95 and 34.03 for the cold temperature group (Table 7.1). The negative control tick samples tested negative.

7.4.4. Virus isolation

Cytopathic effect (CPE) was observed in 6 of the 10 (60%) adult *A. hebraeum* samples for the room temperature group after the second passage. CPE was also seen in all samples of moulting ticks in the cold temperature group after the first passage by Day 4 as well as in samples of the adult ticks in the cold temperature group after the second passage. Testing of

virus isolation material by real-time PCR confirmed the presence of LSDV DNA with C_t values between 34.7 and 39.18 for the adults in the room temperature group, between 15.17 and 35.91 for the moulting nymphs and between 31.60 and 38.46 for the adults in the cold temperature group (Table 7.1).

Table 7.1: summarised real time PCR and virus isolation results for A. hebraeum (nymphs and adults) and *R. (B) decoloratus* (eggs and larvae)

Details	Incubation	Real time PCR of tick homogenate		VI	Real time PCR of VI	
		No. positives	MCCI range of C_t values	No. showing CPE	No. positive	MCCI range of C_t value range
A. hebraeum adults (inoculated)	RT	10 (100%)	30.58 – 32.89	6 (60%)	6	33.15 - 34.67
A. hebraeum adults (inoculated)	CT	10 (100%)	32.56 – 35.75	10 (100%)	10	31.48 - 36.05
A. hebraeum Nymphs (inoculated)	CT	10 (100%)	25.04 - 27.18	10 (100%)	10	16.35 – 22.90
A. hebraeum Nymphs (inoculated)	CT	10 (100%)	25.04 - 27.18	10 (100%)	10	16.35 – 22.90
R. (B) decoloratus eggs (inoculated)	RT	9 (90%)	33.41 – 36.95	Not tested		
R. (B) decoloratus eggs (inoculated)	CT	10 (100%)	34.59 – 36.14	Not tested		
R. decoloratus Larvae (inoculated)	RT	10 (100%)	32.51 – 34.17	10 (100%)	5	35.44 - 38.16
R. (B) decoloratus Larvae (inoculated)	CT	10 (100%)	33.41 – 36.97	10 (100%)	6	35.87 - 38.03

RT- room temperature; CT- cold temperature; CPE- cytopathic effect; MCCI- Monte Carlo confidence interval (95% confidence level)

Cytopathic effect was seen within 4 days of the passage for larval samples of *R. (B) decoloratus* of both groups. LSDV was confirmed in both groups with C_t values between 35.44 and 38.16 in 5 samples of the room temperature group and between 35.87 and 38.03 in 6 samples of the cold temperature samples (Table 7.1).

7.4.5. Transmission electron microscopy

The presence of LSDV could not be demonstrated in the *A. hebraeum* tick organs by TEM. However, presence of LSDV was demonstrated in cell cultures showing CPE for *A. hebraeum* ticks exposed to cold temperature.

7.4.6. Immunoperoxidase staining

Amblyomma hebraeum ticks from the group incubated at cold temperatures showed positive staining. The affected organs were the epidermis, fat body and trachea (Figure 7.1). From the room temperature group, four ticks also showed staining in the midgut, fat body, synganglion, testes and lobular accessory glands.

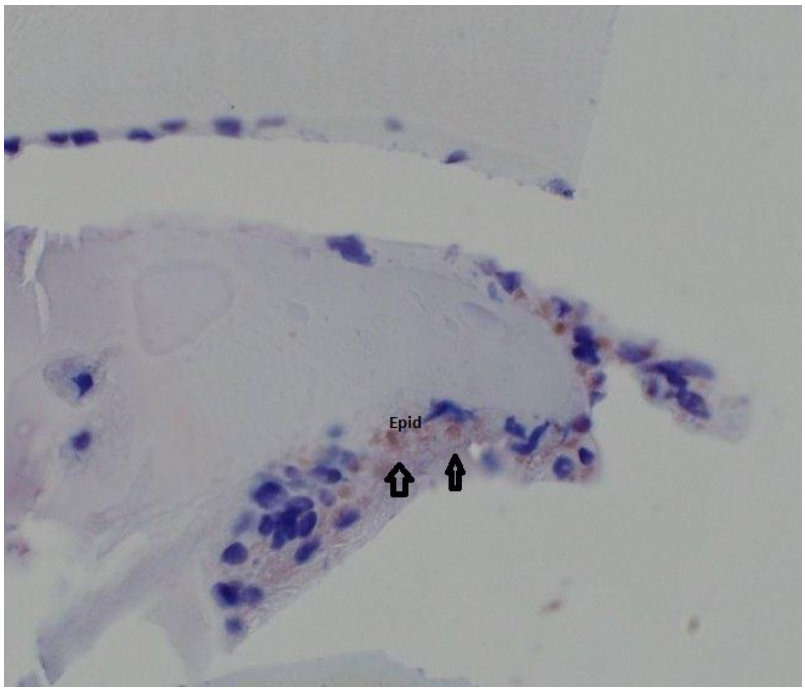

Figure 7.1: Saggital section of Amblyomma hebraeum adult with brown-redish (positive) staining (arrows) in the epidermis (Epid)

7.5. Discussion

During the time of cold temperature incubation (5 to 20°C), the moulting and oviposition were arrested due to ticks entering a state of quiescence, a non-diapause state of dormancy (Belezerov, 2009, Steward et al, 2009). The actual moulting and ovipositing in this study was only observed to occur when the incubation temperatures were increased to above 22˚C. This is in agreement with observations that *A. hebraeum* nymphs that drop in winter only emerge as adults in summer (Horak, Gallivan & Spickett 2011, Walker 2003), although in nature, diapause also plays a role in inducing and terminating the dormancy (Belozerov 2009, Madder et al. 1999). It was observed in this study that once moulting or ovipositing commenced following cold temperature dormancy, the time frame and stages of moulting or ovipositing and hatching seemed to be similar to those stages without interrupted development.

Table 7.2: Comparison of Monte Carl Confidence Interval ranges at 95% confidence level

Details	Comparison of CI range	Conclusion
Adult (RT) Vs Adult (RT)	Overlap	No difference
Nymph (CT) vs Adult (CT/RT)	No overlap	Different
Nymph (CT) vs VI nymph (CT)	No overlap	Different
VI adults (CT) vs VI nymphs (CT)	No overlap	Different
VI adults (CT) vs VI adults (RT)	Overlap	No difference
Adults (CT) vs VI adults (CT)	Overlap	No difference
Eggs (CT) vs Eggs (RT)	Overlap	No difference
Eggs (CT) vs larvae (CT)	Overlap	No difference
Eggs (RT) vs larvae (RT)	Overlap	No difference
Larvae(CT) vs larvae (RT)	Overlap	No difference

RT= room temperature, CT= cold temperature

In this study, the survival of *A. hebraeum* adults exposed to cold temperature (5 - 20°C) was 96.88%, which was comparable to the 98% of those kept at room temperature (25°C). Egg laying was reduced in *R. (B) decoloratus* females exposed to cold temperature. On average, *A. hebraeum* females were seen to shed their cuticle earlier than males. Monte Carlo's

simulation showed exposure to cold temperature did not affect survival of the virus after moulting in *A. hebraeum* and ovipositing and hatching in *R. (B) decoloratus* (Table 2).

It is also apparent that as the moulting process of *A. hebraeum* nymphs was arrested under cold temperature, the virogenesis was also arrested and the virus possibly remained intact as it was inoculated. This assumption is based on the observation that the virus in the nymphs under the cold temperature had high virus titres as shown by the low C_t values of real-time PCR results (i.e. between 23.33 and 27.66) and a high infectivity on cell cultures occurred, while on the other hand, in ticks which commenced moulting, the virus titre was reduced, as indicated by increasing C_t values for both their homogenate and VI medium (Table 7.1). The "arresting of virogenesis" in arthropods due to low temperature was also demonstrated for BTV, where the virus was reported to cease replicating at 10°C in *Cullicoides bolitinos* and *C. imicola* (Paweska, Venter & Mellor 2002) and at 15°C in *C. sonorensis* (Mullens et al. 1995).

Monte Carlo's simulation of the C_t values also confirmed that there was a difference in the viral titres between the nymphs kept at cold temperatures (C_t = 25.06 – 27.18) and the emergent *A. hebraeum* adults (C_t = 32.56-35.74) (Table 7.1, Table 7.2).

The process of moulting appeared to reduce the viral titre. This is illustrated by the increase in C_t values (from 29.54 to 30.54) as moulting approached completion and the higher C_t values from moulted adults (31.03 to 38.41). The reduction in viral titre in ticks after moulting has been reported for Dugbe (Booth et al. 1991) and Thogoto viruses (Kaufman & Nuttall 2003). It supports the opinion that the physiological state of the tick, such as moulting, influences virogenesis (Nosek et al. 1984). The survival of the virus during moulting depends on the susceptibility to infection of the tick organs that do not undergo histolysis (Labuda & Nuttall 2004). For instance, infection in the epithelial tissues, fatty bodies and synganglia, which survive histolysis (Balashov, Raikhel & Hoogstraal 1983, Sonenshine 1991) will pass the virus to the next stage unlike infection in the salivary glands which will degenerate during moulting (Sonenshine 1991). The decrease in titre may also be affected by the duration of the eclipse phase of the virus (Nosek et al. 1984) and, on the other hand, may be associated with variations in genetic determinants important for virus replication between tick and mammalian hosts (Mitzel et al. 2007, Mitzel et al. 2008). In identifying the potential viral determinants responsible for replication of flaviviruses in tick and mammalian host cells, Mitzel et al. (2008) reported two virus variants that were

responsible for either replication in tick or mammalian cells respectively and suggested these genetic changes in the virus represented host specific determinants for replication.

The infection rate was 100% for both groups of *A. hebraeum* adults and *R. (B) decoloratus* larvae, suggesting that exposure to cold temperature did not affect the number of ticks that became infected and also supports observations that parenteral inoculation of ticks achieves high rates of infection (Kaufman & Nuttall 2003). When comparing the MMCI's, no true difference exists in the viral titres between ticks incubated at room temperature and those exposed to cold temperatures (Table 7.2). The culturing of virus demonstrates viral infectivity and when comparing the MMCI's of virus isolation media for *A. hebraeum* adults between those incubated at room temperature and the ones exposed to cold temperature, no true difference was seen between the two (Table 7.2). We postulate that LSDV-infected ticks that over-winter may be as infective as those that do not over-winter.

The IMPS shows that the virus in *A. hebraeum* adults persists in the epidermis, synganglion and reproductive organs (Figure 7.1). These organs do not undergo histolysis during moulting (Booth et al. 1989) and, therefore, may serve as foci for dissemination to other organs, including the salivary glands. The TEM examination of the tick organs did not reveal any virus particles. Negative results may be attributed to low virus titres (i.e. lower than threshold required for detection by TEM) or that the virus morphology may differ in ticks from that in vertebrate cell cultures (Booth et al. 1989).

The outbreaks of LSD have been seasonal, mostly in summer, when activity of biting insects is high (Coetzer 2004, Weiss 1968). It has not been established where the virus persists during periods when the activity of insects is minimal, such as winter in southern Africa. It has been experimentally demonstrated that *A. hebraeum, R. appendiculatus* and *R. (B) decoloratus* transmit LSDV (Tuppurainen et al. 2011). This study shows the potential for LSDV to over-winter in ticks. It was also reported by White et al. (2005) that BTV may over-winter through vertical passage to larvae stages of *Cullicoides soronesis* vectors. Paweska et al (2002) reported persistence of BTV in artificially infected adult *C. imicola* and *C. bolitinos* exposed to cold temperatures. Ticks are also suspected to contribute to the over-wintering of BTV following the report of transstadial persistence of BTV in *Ixodes hexagonus* and transovarial persistence in *Onithodoros savignyi* (Bouwknegt et al. 2010) and West Nile virus was reported to over-winter in a pool of diapausing female *Culex pipens pipiens* (Farajollahi et al. 2005, Nasci et al. 2001).

The report of the transstadial passage of LSDV in *A. hebraeum* following its detection in saliva of adult ticks fed as nymphs (Lubinga et al. 2013) combined with findings of this study, shows high potential for this tick species to maintain the virus during the winter months when other vectors such as biting flies are least active. Adult *A. hebraeum* ticks normally emerge during the summer months (Schroder, Uys & Reilly 2006, Walker 2003) and are then likely to introduce LSDV to cattle from where the virus may be rapidly spread mechanically by biting insects (Weiss, 1968). Similarly, since transstadial passage of LSDV has also been confirmed by *R. appendiculatus* (Lubinga et al. 2013) and this tick species also over-winters as engorged nymphs and unfed adults (Bryson et al. 2002b), there is a possibility, in nature, that *R. appendiculatus* may also play a role in the over-wintering of LSDV.

The demonstration of transovarial passage of LSDV by *R. (B) decoloratus* following exposure to cold conditions also shows the potential for this tick species to play a role in over-wintering of the virus. This observation is in agreement with observations in southern Africa that hatching of over-wintering eggs is synchronised with rising temperatures in spring (Bryson et al. 2002b).

7.6. Reference

Balashov, Y., S., Raikhel, A.S. & Hoogstraal, H., 1983, *An atlas of ixodid tick ultrastructure.* Entomological Society of America, College Park, MaryLand; USA.

Belozerov, V.N., 2009, 'New aspects in investigations of diapause and non-diapausing types of dormancy in insects and other arthropods.', *Entomologicheskoe Obozrenie* 88(1), 3-15.

Booth, T.F., Davies, C.R., Jones, L.D., Staunton, D. & Nuttall, P.A., 1989, 'Anatomical basis of Thogoto virus infection in BHK cell culture and in the ixodid tick vector, *Rhipicephalus appendiculatus*.', *Journal of General Virology* 70(5), 1093-1104.

Booth, T.F., Steele, G.M., Marriott, A.C. & Nuttall, P.A., 1991, 'Dissemination, replication, and trans-stadial persistence of Dugbe virus (Nairovirus, Bunyaviridae) in the tick vector *Amblyomma variegatum*', *American Journal of Tropical Medicine and Hygiene* 45(1), 146-157.

Bouwknegt, C., Rijn, P.A., Schipper, J.J.M., Hölzel, D., Boonstra, J., Nijhof, A.M., Rooij, E.M.A. & Jongejan, F., 2010, 'Potential role of ticks as vectors of bluetongue virus', *Experimental and Applied Acarology* 52(2), 183-192.

Bowden, T.R., Babiuk, S.L., Parkyn, G.R., Copps, J.S. & Boyle, D.B., 2008, 'Capripoxvirus tissue tropism and shedding: a quantitative study in experimentally infected sheep and goats', *Virology* 371(2), 380-393.

Bryson, N.R., Tice, G.A., Horak, I.G., Stewart, C.G. & Plessis, B.J.A., 2002a, 'Ixodid ticks on indigenous goats owned by small-scale farmers in four communal grazing areas in South Africa', *Journal of the South African Veterinary Association* 73(1), 26-30.

Bryson, N.R., Tice, G.A., Horak, I.G., Stewart, C.G. & Plessis, B.J.A.d., 2002b, 'Ixodid ticks on cattle belonging to small-scale farmers at 4 communal grazing areas in South Africa.', *Journal of the South African Veterinary Association* 73(3), 98-103.

Buckland, S.T., 1984, 'Monte-Carlo Confidence-Intervals', *Biometrics* 40(3), 811-817.

Chihota, C.M., Rennie, L.F., Kitching, R.P. & Mellor, P.S., 2001, 'Mechanical transmission of lumpy skin disease virus by *Aedes aegypti* (Diptera: Culicidae)', *Epidemiology and infection* 126(2), 317-321.

Coetzer, J.A.W., 2004, 'Lumpy skin disease' in pp. 1268-1276, Oxford University Press, Oxford.

Davies, F.G., 1991, 'Lumpy skin disease of cattle: a growing problem in Africa and the Near East', *World Animal Review* 68(3), 37-42.

Farajollahi, A., Crans, W.J., Bryant, P., Wolf, B., Burkhalter, K.L., Godsey, M.S., Aspen, S.E. & Nasci, R.S., 2005, 'Detection of West Nile viral RNA from an overwintering pool of *Culex pipens pipiens* (Diptera : Culicidae) in New Jersey, 2003', *Journal of medical entomology* 42(3), 490-494.

Horak, I.G., 1982, 'Parasites of domestic and wild animals in South Africa. XV. The seasonal prevalence of ectoparasites on impala and cattle in the Northern Transvaal.', *Onderstepoort Journal of Veterinary Research* 49(2), 85-93.

Horak, I.G., Gallivan, G.J. & Spickett, A.M., 2011, 'The dynamics of questing ticks collected for 164 consecutive months off the vegetation of two landscape zones in the Kruger National Park (1988-2002). Part I. Total ticks, *Amblyomma hebraeum* and *Rhipicephalus decoloratus*.', *Onderstepoort Journal of Veterinary Research* 78(1), Arte2.

Hunter, P. & Wallace, D., 2001, 'Lumpy skin disease in southern Africa: a review of the disease and aspects of control', *Journal of the South African Veterinary Association* 72(2), 68-71.

Kaufman, W.R. & Nuttall, P.A., 2003, '*Rhipicephalus appendiculatus* (Acari: Ixodidae): dynamics of Thogoto virus infection in female ticks during feeding on guinea pigs.', *Experimental parasitology* 104(1/2), 20-25.

Kumar, S.M., 2011, 'An outbreak of Lumpy Skin Disease in a Holstein dairy herd in Oman: a clinical report.', *Asian Journal of Animal and Veterinary Advances* 6(8), 851-859.

Labuda, M. & Nuttall, P.A., 2004, 'Tick-borne viruses.', *Parasitology* 129(Supplement), S221-S245.

Lubinga, J.C., Tuppurainen, E.S.M., Stoltsz, W.H., Eberhson, K., Coetzer, J.A.W. & Venter, E.H., 2013, '
Detection of lumpy skin disease virus in saliva of ticks fed on blood of infected cattle', *Experimental and Applied Acarology* .

Madder, M., Speybroeck, N., Brandt, J. & Berkvens, D., 1999, 'Diapause induction in adults of three *Rhipicephalus appendiculatus* stocks.', *Experimental & applied acarology* 23(12), 961-968.

Mitzel, D.N., Best, S.M., Masnick, M.F., Porcella, S.F., Wolfinbarger, J.B. & Bloom, M.E., 2008, 'Identification of genetic determinants of a tick-borne flavivirus associated with host-specific adaptation and pathogenicity', *Virology* 381(2), 268-276.

Mitzel, D.N., Wolfinbarger, J.B., Long, R.D., Masnick, M., Best, S.M. & Bloom, M.E., 2007, 'Tick-borne flavivirus infection in Ixodes scapularis larvae: development of a novel method for synchronous viral infection of ticks.', *Virology* 365(2), 410-418.

Mullens, B.A., Tabachnick, W.J., Holbrook, F.R. & Thompson, L.H., 1995, 'Effects of Temperature on Virogenesis of Bluetongue Virus Serotype-11 in *Culicoides variipenni sonorensis*', *Medical and veterinary entomology* 9(1), 71-76.

Nasci, R., Savage, H., White, D., Miller, J., Cropp, B., Godsey, M., Kerst, A., Bennett, P., Gottfried, K. & Lanciotti, R., 2001, 'West Nile virus in overwintering Culex mosquitoes, New York City, 2000', *Emerging Infectious Diseases* 7(4), 742-744.

Norval, R.A.I., 1977, 'Ecology of the tick *Amblyomma hebraeum* Koch in the Eastern Cape Province of South Africa. I. Distribution and seasonal activity', *Journal of Parasitology* 63(4), 734-739.

Norval, R.A.I., Andrew, H.R. & Meltzer, M.I., 1991, 'Seasonal occurrence of the bont tick (*Amblyomma hebraeum*) in the southern lowveld of Zimbabwe', *Experimental & applied acarology* 13(2), 81-96.

Nosek, J., Korolev, M.B., Chunikhin, S.P., Kozuch, O. & Ciampor, F., 1984, 'The replication and eclipse-phase of the tick-borne encephalitis virus in *Dermacentor reticulatus*.', *Folia parasitologica* 31(2), 187-189.

Paweska, J., Venter, G. & Mellor, P., 2002, 'Vector competence of South African Culicoides species for bluetongue virus serotype 1 (BTV-1) with special reference to the effect of temperature on the rate of virus replication in *C. imicola* and *C. bolitinos*', *Medical and veterinary entomology* 16(1), 10-21.

Schroder, B., Uys, A.C. & Reilly, B.K., 2006, 'A survey of free-living ixodid ticks on a commercial game farm in the Thabazimbi District, Limpopo Province, South Africa', *Journal of the South African Veterinary Association* 77(3), 141-144.

Sonenshine, D.E., 1991, *Biology of ticks*, Oxford University Press, Oxford.

Tan Gang, 2012, 'Natural ventilation performance of single room building with fluctuating wind speed and thermal mass', *Journal of Central South University of Technology* 19(3), 733-739.

Tarr, G., 2012, 'Small sample performance of quantile regression confidence intervals', *Journal of Statistical Computation and Simulation* 82(1), 81-94.

Tulman, E.R., Afonso, C.L., Lu, Z., Zsak, L., Kutish, G.F. & Rock, D.L., 2001, 'Genome of lumpy skin disease virus', *Journal of virology* 75(15), 7122-7130.

Tuppurainen, E.S.M., Lubinga, J.C., Stoltsz, W.H., Troskie, M., Carpenter, S.T., Coetzer, J.A.W., Venter, E.H. & Oura, C.A.L., 2013a, 'Mechanical transmission of lumpy skin disease virus by *Rhipicephalus appendiculatus* male ticks', *Epidemiology and infection, Epidemiol.Infect.* 141, 2 425-430.

Tuppurainen, E.S.M., Lubinga, J.C., Stoltzs, W.H., Troskie, M., Carpenter, S.T., Coetzer, J.A.W., Venter, E.H. & Oura, A.L., 2013b, 'Evidence of vertical transmission of lumpy skin disease in *Rhipicephalus decoloratus* ticks', *Ticks and tick borne diseases* .

Tuppurainen, E.S., Venter, E.H. & Coetzer, J.A., 2005, 'The detection of lumpy skin disease virus in samples of experimentally infected cattle using different diagnostic techniques', *The Onderstepoort journal of veterinary research* 72(2), 153-164.

Tuppurainen, E.S.M., Stoltsz, W.H., Troskie, M., Wallace, D.B., Oura, C.A.L., Mellor, P.S., Coetzer, J.A.W. & Venter, E.H., 2011, 'A potential role for ixodid (hard) tick vectors in the transmission of lumpy skin disease virus in cattle', *Transboundary and Emerging Diseases* 58(2), 93-104.

Walker, A., 2003, *Ticks of domestic animals in Africa : a guide to identification of species*, Bioscience Reports, Edinburgh.

Weiss, K.E., 1968, 'Lumpy skin disease virus', *Virology Monographs* 3 111-131.

Woods, J.A., 1988, 'Lumpy skin disease--a review', *Tropical animal health and production* 20(1), 11-17.

Yeruham, I., Nir, O., Braverman, Y., Davidson, M., Grinstein, H., Haymovitch, M. & Zamir, O., 1995, 'Spread of lumpy skin disease in Israeli dairy herds', *The Veterinary record* 137(4), 91-93.

Chapter 8

General discussion

When the first cases of LSD were reported in Northern Rhodesia (now Zambia) in 1929, the cause was thought to be poison. After it was reported that the disease is caused by an infectious agent, transmission was associated with biting flies (Weiss 1968). This was supported experimentally through mechanical transmission by *Aedes aegypti* mosquitoes (Chihota et al. 2001).

The present study has demonstrated the role of ticks in the transmission of LSDV by showing their ability to transmit the virus to animals and demonstrating persistence of the virus in ticks following interrupted feeding (mechanically / intrastadially), developing into a new stage (transstadially) and vertically, through infected females to next generation larvae (transovarially). The survival of LSDV in ticks under cold temperatures in order to verify the possible over-wintering of the virus in ticks contributed important scientific knowledge to the epidemiology of the disease.

Most recipient animals began to show viraemia by the sixth day of tick attachment, which was about the same time that clinical signs began to manifest. This synchronisation links the clinical signs observed to LSDV infection. The positive real-time PCR results were associated with high C_t values indicating low viral titres. The low viral titres could be the reason why it was difficult to detect neutralising antibodies in serum, except for the recipient animal used for testing of mechanical transmission by *R. appendiculatus,* reported in another study (Tuppurainen et al. 2013) and could also explain the lack of overt disease symptoms in recipient animals.

Vector competence of ticks was demonstrated by the finding of virus crossing the "midgut barrier" of most adult ticks following both intrastadial and transstadial passage. The presence of LSDV was shown in haemocytes and other organs of the haemocoele as well as the detection of LSDV in tick saliva. The presence of the virus in saliva and tick organs following interrupted feeding also shows that infection of tick organs occurs within four days of ingesting an infected blood meal.

The study on over-wintering of the virus in ticks showed the progressive lowering of the virus titres in moulting ticks as moulting advanced towards completion. Under normal

circumstances, biological development is associated with amplification of the pathogen and would have been expected that the virus titres would be increasing as the moulting progressed. This suggests that viral replication is being suppressed in these ticks during moulting. One explanation could be a variation in the genetic determinants for virus replication between tick and mammalian cells. If the determinants for viral replication in tick cells are different to those in the mammalian hosts, there could be suppression in viral replication when transferred from mammalian to tick cells. Virus replication will increase after selection of a virus population with determinants favouring replication in tick cells. The same is expected of the virus population inoculated from the ticks to the animals. A variation in genetic determinants is not expected to affect mechanical transmission since there is no biological change in the virus. This could explain why higher virus titres enough to induce the development of neutralising antibodies were introduced during mechanical transmission by *R. appendiculatus* (Tuppurainen et al. 2013) and supports observations of major outbreaks being associated with a very high prevalence of flying insects, which are associated with mechanical transmission (Weiss 1968). Therefore, under natural conditions, ticks may help to introduce the virus to a susceptible herd, through biological transmission, while high populations of flying insects may amplify the outbreak by mechanical transmission.

The clinical signs of the disease are often either inapparent or transient and viraemia in infected animals are very low. Consequently, the neutralising antibody levels are low and using the existing SNTs may even be undetectable. Results obtained by real-time PCR are associated with high C_t values, which in many cases are border-line between true positives and false positives due to non-specific reactions of primers. However, the determination of the limit of detection using multiple sets of samples increases the confidence in selecting the cut-off C_t value. The use of virus isolation to amplify the virus has in most cases improved the detection by real-time PCR in this study. In some cases, it was difficult to culture the virus especially in cases where the virus source was the ticks. The variations in genetic determinants for replication of the virus between tick and mammalian cells may also have played a role here but the samples may also have contained contaminants which killed the cells before the virus had an opportunity to multiply.

In conclusion, *Rhipicephalus (Boophilus) decoloratus* passes LSDV vertically from infected females to the larvae and the emergent larvae are infective for susceptible animals. *Rhipicephalus (Boophilus) decoloratus* may assist in the over-wintering of LSDV. This is supported by the survival of the virus during the delayed egg laying and hatching induced by

cold temperatures of the winter. When the feeding of *R. (B) decoloratus* males was interrupted, there was a failure in re-attachment. Therefore it is difficult to state whether *R. (B) decoloratus* males play a role in mechanical/intrastadial transmission in the field.

Rhipicephalus appendiculatus and *A. hebraeum* can transmit LSDV both mechanically and biologically following interrupted feeding. They can also pass and transmit LSDV between tick stages and are capable of maintaining the virus over long periods, including over-wintering of the virus. Lumpy skin disease virus can persist through transovarial passage in *A. hebraeum* and *R. appendiculatus*, hence both *A. hebraeum* and *R. appendiculatus* are possible maintenance and reservoir hosts for the virus.

To support these findings future studies should include investigating LSDV infection in free-living ticks following a natural outbreak. The replication and quantification of LSDV in tick organs should be determined. Similarly, during moulting, there is a need to determine the actual organs where the virus survives before spreading to other organs. The minimum viral load for transmission and the minimum number of ticks required to cause LSDV infection per animal should be studied.

Apart from the above roles of transmission and maintenance of LSDV, ticks are also expected to contribute to spread LSDV to other areas of the world. Ticks may be spread over long distances by movement of their animal host, including while feeding on migrating birds. The change of climate due to global warming is making it possible for ticks to successfully survive and quest in areas where previously they could not survive due to very cold conditions. It is recommended that tick control should form an important component of LSD control.

References

Chihota, C.M., Rennie, L.F., Kitching, R.P. & Mellor, P.S., 2001, 'Mechanical transmission of lumpy skin disease virus by *Aedes aegypti* (Diptera: Culicidae)', *Epidemiology and infection* 126(2), 317-321.

Tuppurainen, E.S.M., Lubinga, J.C., Stoltsz, W.H., Troskie, M., Carpenter, S.T., Coetzer, J.A.W., Venter, E.H. & Oura, C.A.L., 2013, 'Mechanical transmission of lumpy skin disease virus by *Rhipicephalus appendiculatus* male ticks', *Epidemiology and infection, Epidemiol.Infect.* 141, 2 425-430.

Weiss, K.E., 1968, 'Lumpy skin disease virus', *Virology Monographs* 3 111-131.