Evidence of lumpy skin disease virus over-wintering by transstadial persistence in *Amblyomma hebraeum* and transovarial persistence in *Rhipicephalus decoloratus* ticks

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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. decoloratus, R. appendiculatus* and *A. hebraeum.* The over-wintering of ticks off the host as part of their life cycles is well known: *A. hebraeum* and *R. appendiculatus* over-winter, for example, on the

ground as engorged nymphs / unfed (emergent) adults while *R. 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. 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 decoloratus*, lumpy skin disease virus, transstadial, transovarial transmission

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 and 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 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 et al. 2011, Norval et al. 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 et al. 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 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 et al. 2006). The potential for vertical transmission also makes *R. 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. 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.

Materials and methods

Study area

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.

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.

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. decoloratus* females fed to repletion on LSDV-free cattle were supplied by ARC-OVI.

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 Nuttall (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^{TM} *Plus* Demi, USA), the needle was inserted, beyond the bevelled tip, into the hemocoel, 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. 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.

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. 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.

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.

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. 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).

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.

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 et al. 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 GAG TTG 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.

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.

Briefly, 3 to 4 µm thick tick sections were mounted on positively charged microscope slides and dried overnight in an oven at 38°C. The tick sections were de-waxed in xylene for 10 minutes, serially rehydrated (3 minutes each in 100%, 96% and 70% ethanol), incubated in 3% hydrogen peroxide (in methanol) for 15 minutes at room temperature (22-25°C) and rinsed three times in distilled water. The slides were then incubated in citrate buffer (pH of 6) at 96°C for 14 minutes and cooled for 15 minutes at room temperature before rinsing twice in distilled water and in PBS buffer for 5 minutes. The non-specific immunoglobulin binding was blocked by incubating the slides 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 specific for capripoxviruses (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.

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.

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 (MCCI) 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 MCCI's between the two groups. The presence of an overlap in the MCCI's between two groups would indicate "no true difference" while lack of overlap would indicate a true difference between them.

Results

Tick moulting –A. 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.

Tick oviposition and hatching- R. decoloratus

Rhipicephalus 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. 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%.

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 1).

Table 1 : Summarised real-time PCR and virus isolation results for *Amblyomma hebraeum* (nymphs and adults) and *Rhipicephalus decoloratus* (eggs and larvae)

Details	**Incubation**	**Real time PCR of tick homogenate**		**VI**	**Real time PCR of VI**	
		No. positive (%)	**MCCI range of C_t value**	**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
R. decoloratus eggs (inoculated)	RT	9 (90)	33.41–36.95	Not tested		
R. 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. 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)

The virus was detected in 10 (100%) *R. 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 1). The negative control tick samples tested negative.

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 (Figure 1) 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 1).

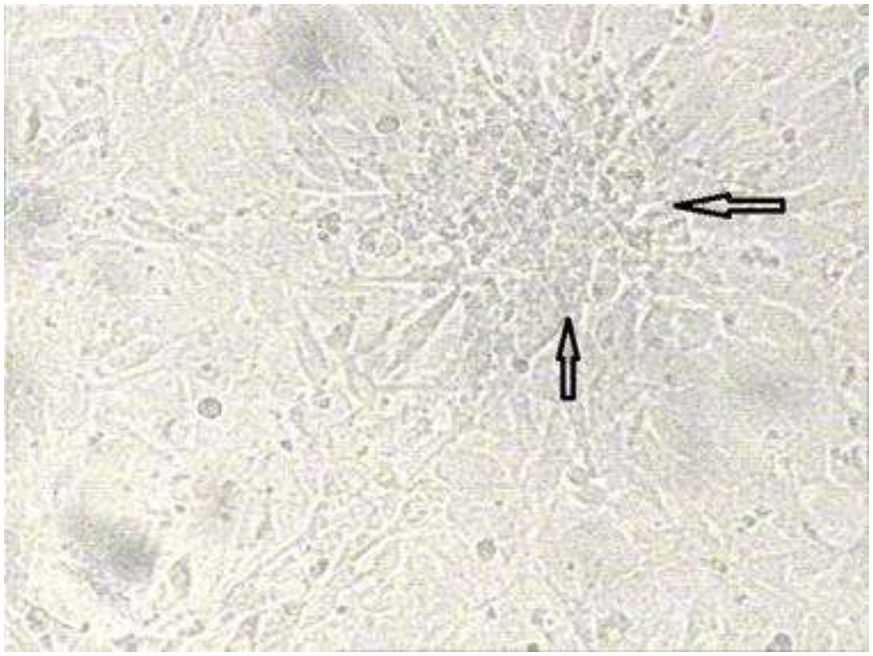

Fig. 1 : Cytopathic effects of lumpy skin disease virus on bovine dermis cell monolayer inoculated with tick homogenate of *Amblyomma hebraeum* moulting ticks seen by clumping of cells (*arrows*)

Cytopathic effect was seen within 4 days of the passage for larval samples of *R. 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 (Table1).

Transmission electron microscopy

The presence of LSDV could not be demonstrated in the *A. hebraeum* tick organs by TEM. However, presence of LSDV particles were demonstrated by TEM from cell cultures that had shown CPE for *A. hebraeum* ticks exposed to cold temperature (Figure 2).

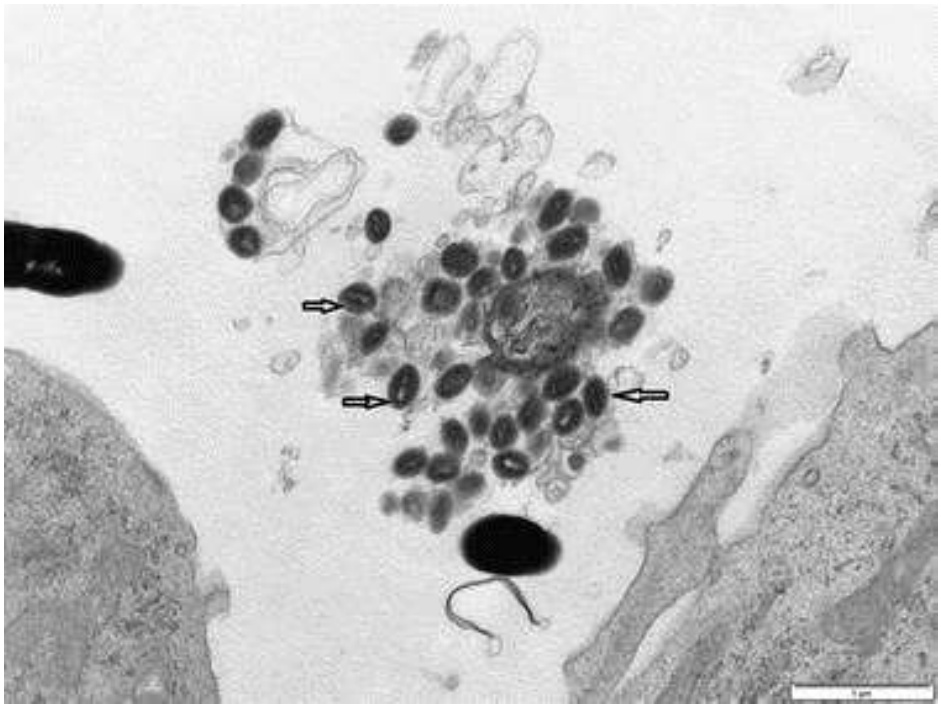

Fig. 2 : Lumpy skin disease virus particles (*arrows*) seen under electron microscope on cell cultures inoculated with tick homogenate of *Amblyomma hebraeum* moulting ticks

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 3). From the room temperature group, four ticks also showed staining in the midgut, fat body, synganglion, testes and lobular accessory glands.

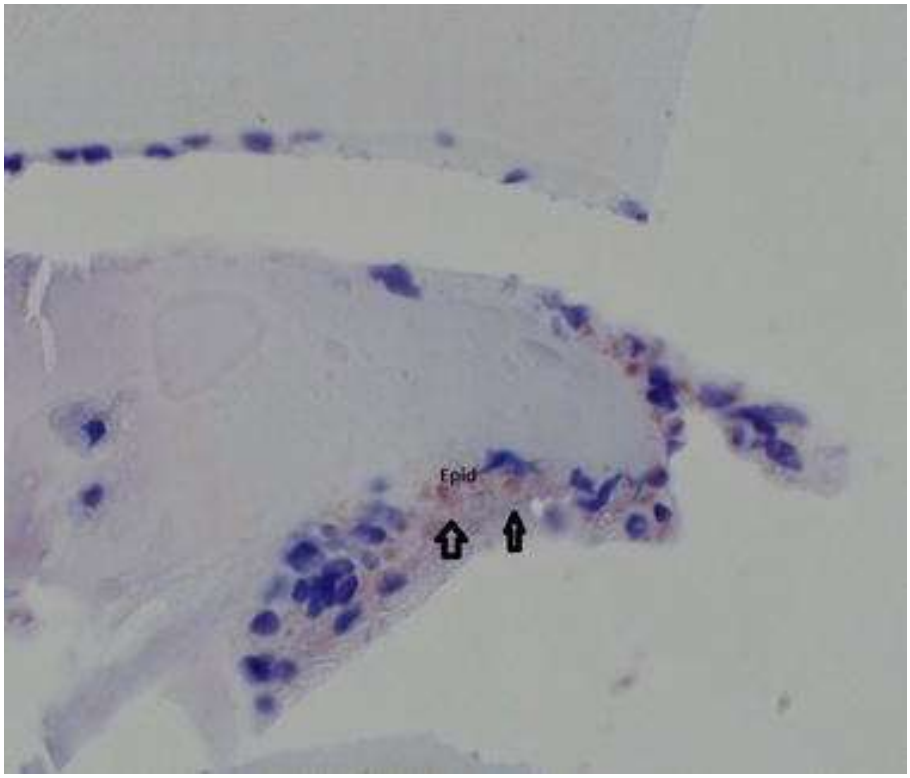

Fig. 3 : Red-brown granular (positive) labelling in the epidermis (Epid) of an *Amblyomma hebraeum* adult

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 et al. 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.

In this study, the survival of *A. hebraeum* adults exposed to cold temperature (5 - 20°C) was 97%, which was comparable to the 98% of those kept at room temperature (25°C). Egg

laying was reduced in *R. 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. decoloratus* (Table 2).

Table 2 : Comparison of Monte Carlo confidence interval ranges at 95 % confidence level

Details	**Comparison of CI range**	**Conclusion**
Adult (RT) versus adult (RT)	Overlap	No difference
Nymph (CT) versus adult (CT/RT)	No overlap	Different
Nymph (CT) versus VI nymph (CT)	No overlap	Different
VI adults (CT) versus VI nymphs (CT)	No overlap	Different
VI adults (CT) versus VI adults (RT)	Overlap	No difference
Adults (CT) versus VI adults (CT)	Overlap	No difference
Eggs (CT) versus eggs (RT)	Overlap	No difference
Eggs (CT) versus larvae (CT)	Overlap	No difference
Eggs (RT) versus larvae (RT)	Overlap	No difference
Larvae(CT) versus larvae (RT)	Overlap	No difference

RT room temperature, *CT* cold temperature

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 detected was possibly the intact viral inoculum. 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 (Table1). 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 *Culicoides bolitinos* and *C. imicola* (Paweska et al. 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) (Table1, Table 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 and 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 and Nuttall 2004). For instance, infection in the epithelial tissues, fatty bodies and synganglia, which survive histolysis (Balashov et al. 1983, Sonenshine 1991) will enable the virus to pass to the next tick developmental 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. 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 and Nuttall 2003). When comparing the MCCI's, no true

difference exists in the viral titres between ticks incubated at room temperature and those exposed to cold temperatures (Table 2). The culturing of virus demonstrates viral infectivity and when comparing the MCCI'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 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 3). 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. 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 *Culicoides 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 et al. 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. 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).

Acknowledgements:

This was a collaborative study between the DVTD, FVS, UP, South Africa and the Pirbright Institute, Pirbright, United Kingdom. The project was funded by the Combating 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). The authors also thank

Dr. Nlingisisi Babayani for guidance on statistical analysis; Ms Karen Eberhson, Rebone Mahlare, Milana Troskie, Rephima Phaswane and Naomi Timmerman for their technical assistance and the ARC-OVI for supplying engorged *A. hebraeum* nymphs and *R. decoloratus* adults.

Conflict of interest: None

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