

A potential role for ixodid (hard) tick vectors in the transmission of lumpy skin disease virus in cattle

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Summary

Lumpy skin disease (LSD) is an economically important cattle disease. The disease is endemic in many African countries but outbreaks have also been reported in Madagascar, and the Middle East. The aim of this study was to investigate the potential role of ixodid (hard) ticks in the transmission of the disease. Cattle were infected with a virulent, South African field isolate of lumpy skin disease virus (LSDV). Three common African tick species (genera *Rhipicephalus*, *Amblyomma* and *Rhipicephalus (Boophilus)*) in different life cycle stages were fed on the infected animals during the viraemic stage and on skin lesions. Post-feeding, the partially fed male ticks were transferred to the skin of non-infected “recipient” animals, while females were allowed to lay eggs which were then tested using the polymerase chain reaction (PCR) method and virus isolation. Nymphs were allowed to develop for 2 to 3 weeks after which time they were tested. The non-infected “recipient” cattle were closely monitored and both skin and blood samples were tested using PCR and virus isolation, and serum samples were tested by the serum neutralization test.

This is the first report showing molecular evidence of potential transmission of LSDV by ixodid ticks. The study showed evidence of transstadial and transovarial transmission of LSDV by *R. (B.) decoloratus* ticks, and mechanical or intrastadial transmission by *R. appendiculatus* and *A. hebraeum* ticks.

Keywords

Capripoxvirus; lumpy skin disease virus; transmission; ixodid; tick; vector; *Rhipicephalus appendiculatus*; *Amblyomma hebraeum*; *Rhipicephalus (Boophilus) decoloratus*

Introduction

Lumpy skin disease virus (LSDV) which belongs to genus *Capripoxvirus* within the family *Poxviridae* and subfamily *Chordopoxvirinae* (Buller et al, 2005) causes a disease in cattle that is characterized by fever, enlarged superficial lymph nodes, pox lesions in the skin and mucous membranes of the digestive and respiratory tracts, excessive salivation, lachrymation and nasal discharge. All cattle breeds and age groups are susceptible (Weiss, 1968), however the disease is more severe in young animals and cows in the peak of lactation. The length of the incubation period under field conditions is unknown but under experimental conditions it varies from one to four weeks (Coetzer, 2004; Tuppurainen et al, 2005).

The disease is included in the list of Notifiable Diseases of the World Organization of Animal Health (OIE). Abortions, infertility problems in males and females, a loss in body weight and a sharp drop in milk yield may cause considerable economic losses to farmers during outbreaks. Ulceration of nodular pox lesions leaves permanent scars which decrease the value of the hides for use in the leather industry (Green, 1959). The costs of control and eradication programmes for LSD and indirect costs caused by restrictions or a total ban on the international trade of live animals or animal products following an outbreak can be substantial.

In general, poxviruses have been shown to enter the host through the skin or respiratory tract (Fenner et al, 1987), but direct contact between infected and susceptible animals is considered to be an inefficient route of infection of LSDV. In experimentally infected animals only 50 % are likely to develop clinical signs (Weiss, 1968). Virus is present in the saliva, ocular and nasal discharges (Weiss, 1968), and semen (Irons et al, 2005) of infected animals. Therefore, food or water contaminated by any of these and artificial insemination may serve as sources of infection.

It has long been suspected that one or more insect species may play a role in the transmission of LSDV (Weiss, 1968). The occurrence of the disease is closely related to warm and wet weather conditions, and an abundance of insects (Haig, 1957; Wood, 1988). The virus was isolated from *Stomoxys calcitrans* and *Biomyia fasciata* by Du Toit and Weiss in 1960 (Weiss, 1968) and mechanical transmission by *Stomoxys* flies and *Aedes aegypti* mosquitoes was demonstrated by Kitching and Mellor (1986) and Chihota et al (2001), respectively. However, no in depth studies on the potential role of ixodid ticks in the transmission of LSDV have been carried out.

Several diseases caused by poxviruses are transmitted by insect vectors; these include myxomatosis (mosquitoes, fleas and *Cheyletiella* fur mites), Shope fibroma (mosquitoes and fleas), swine pox (lice) and fowl pox (mosquitoes) (Fenner et al, 1952; Kilham and Dalmat,

1955; Brody, 1936). Camelpox virus belongs to the genus *Orthopoxvirus* within the family *Poxviridae* (Buller et al, 2006). The clinical signs and epidemiology of camelpox are very similar to those caused by capripoxviruses. Wernery et al (1997) were able to isolate camelpox virus on cell culture from camel ticks (*Hyalomma dromedarii*), the virus initially being detected by electron microscope in ticks collected from infected camels.

Ixodid ticks have been shown to transmit several viruses such as those belonging to the families *Flaviviridae* causing encephalitis-like diseases (e.g. tick borne encephalitis, Kumlinge disease, and louping ill), *Bunyaviridae* causing haemorrhagic fevers (e.g. Nairobi sheep disease and Crimean Congo haemorrhagic fever) and *Reoviridae* causing Colorado tick fever (Nuttal, 1994). In addition to the oro-nasal route of transmission, African swine fever virus, which belongs to the genus *Asfivirus* of the *Asfarviridae* family, is also transmitted by argasid (soft) ticks of the *Ornithodoros* genus (Dixon et al, 2005). The virus replicates in these ticks and transstadial, transovarial and sexual routes of transmission have been demonstrated (Plowright et al, 1970; Plowright et al, 1974).

Little is known about how and where LSDV survives between outbreaks of the disease in cattle, nor whether or not there are reservoir hosts for the virus. A possibility which warrants investigation is that virus survives in tick populations, which might explain the sudden re-appearance of the disease after an absence of several years. The life cycle of many species of ticks (such as *Amblyomma* spp) is long and individual life cycle stages may survive for years without a blood meal (Sonenshine, 1991). Ticks may feed on several different mammal, bird and reptile species. Those which feed on birds or mammals could effectively transmit a virus such as LSDV in the field. Adult *R. appendiculatus* ticks prefer to feed on large and medium-sized ruminants and their larvae and nymphs feed on most domestic and wild ruminant species and other mammal species. Adult *A. hebraeum* ticks feed on larger wild and domestic ruminants, whereas their larvae and nymphs parasitize several species of large and small mammals, birds and tortoises (Norval and Horak, 2004). After completing a blood meal larvae and nymphs drop off their host and moult into the next life cycle stage. *A. hebraeum* nymphs actively seek hosts, but those of *R. appendiculatus* wait until a suitable host appears. Moulting and questing may take several months, especially in the case of *Amblyomma* spp. (Norval and Horak, 2004). Hardly any data exist on the susceptibility of wild ruminants and other large or small mammals for capripoxviruses, whereas some information does exist for other members of the family *Poxviridae*; cowpoxvirus within the genus *Orthopoxvirus*, for example, has been shown to survive in small rodents, particularly in ground squirrels, gerbils, voles and woodmice between outbreaks of cowpox in cattle (Chantrey et al, 1999).

The aim of this study was to investigate the transmission of LSDV by three species of laboratory-reared ticks: *R. appendiculatus* (brown ear tick), *A. hebraeum* (bont tick) and *R. (B.) decoloratus* (African blue tick) all of which are common in sub-Saharan Africa. The ticks

were fed on LSDV-infected cattle and the presence of the virus in different life cycle stages of the ticks and their tissues, such as mouthparts, salivary glands and gut tissue was investigated.

Materials and methods

Animals: Eight seronegative, Pinzgauer heifers, approximately 13 months of age and 250 kg in body weight, from a herd in which vaccination against LSD was not practised, were used as experimental animals. They were housed in the insect-proof, high-containment bio-security animal facility of the University of Pretoria's Biological Research Unit (UPBRC), Faculty of Veterinary Science, Onderstepoort. All the experimental procedures were approved by the Animal Use and Care Committee of the University of Pretoria.

Ticks: Laboratory-reared larvae of *R. (B.) decoloratus* species and both nymphs and adults of *R. appendiculatus* and *A. hebraeum* ticks were obtained from ClinVet International (Pty) Ltd laboratories, Bloemfontein, South Africa. Additional *A. hebraeum* adults were provided by Onderstepoort Veterinary Institute (ARC-OVI), South Africa.

Experimental infection of animals: The donor animals were infected by both the intravenous (IV) and intradermal (ID) routes. A virulent South African LSD field isolate (248/93) was passaged 5 to 6 times on primary bovine dermis cell culture. The final titre of the virus was 3.5 log TCID₅₀ / ml for the ID and 4.5 log TCID₅₀ / ml for the IV routes of inoculation. A volume of 2.5 ml was inoculated into the jugular vein and 0.25 ml per site intradermally at 4 sites on the back of the animals.

The experimental animals were divided into two separately housed groups: five donor animals (numbers 604, 605, 609, 610 and 611) and three recipient animals (numbers 613, 701 and 702). The donors were infected with LSDV and laboratory-reared ticks were fed on them during the viraemic stage. Some of the ticks were placed on LSD skin lesions. After feeding on the infected animals the partially fed male ticks and some partially fed female ticks were transferred to non-infected recipient animals on which they were allowed to complete their blood meal.

Attachment, feeding and collection of ticks: The ticks were placed inside cotton cloth bags which were glued using Genkem Contact Adhesive Glue to shaved skin on the back or around the base of the ears of the cattle. The experimental design is illustrated in Figure 1.

R. (B.) decoloratus

R. (B.) decoloratus is a one-host tick with larvae, nymphs and adults feeding on the same host. Approximately 500 *R. (B.) decoloratus* larvae were placed to feed on animal 604 at 3 days post-infection (dpi). Similarly, a batch of 500 larvae and a batch of 1000 larvae were

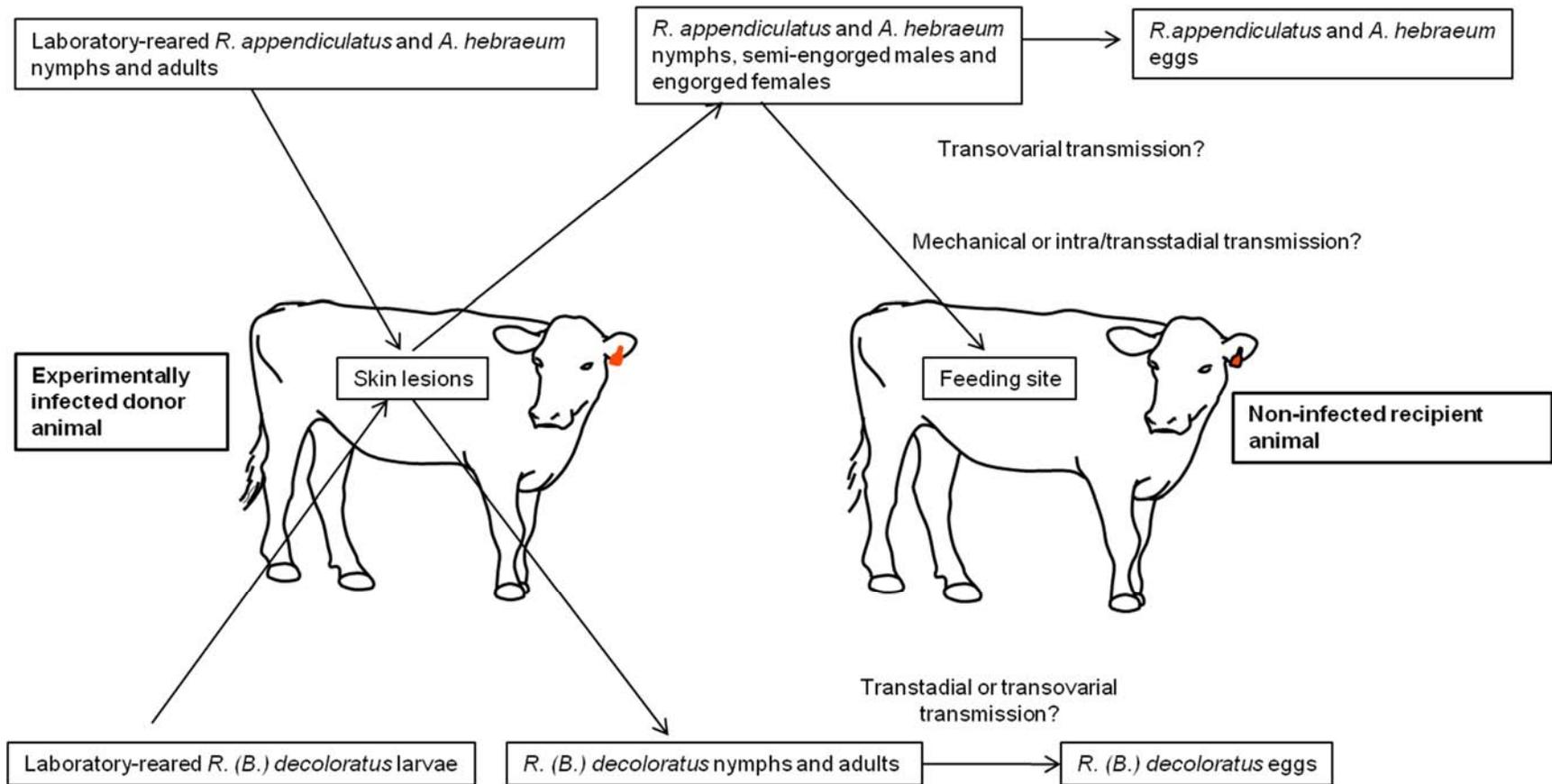


Fig. 1. Schematic representation of the experimental design

introduced into separate skin bags on animal 605. *R. (B.) decoloratus* females, that were placed to feed on animal 605, began engorging 20 days after attachment of larvae. Most of the engorged nymphs, unengorged and engorged adults were collected 21 to 27 days post-attachment. *R. (B.) decoloratus* males were collected between 21 and 35 dpi. All *R. (B.) decoloratus* ticks were collected by hand. Less than 10 fully engorged females were obtained for oviposition.

R. appendiculatus

R. appendiculatus larvae, nymphs and adults feed on different hosts (3-host ticks). These ticks were allowed to feed inside the skin bags of the donor animals for 6 to 7 days after which loose ticks were collected from the skin bags. Nymphs and females detached when they were fully engorged. Males had several small blood meals and were partly engorged when harvested. Therefore the actual feeding time of individual nymphs and adults may differ from the number of days they were kept inside the skin bags.

To ensure the feeding of the ticks during the viraemic stage *R. appendiculatus* nymphs and adults were placed on donor animals at 4 dpi. Approximately 150 nymphs were fed on both ears of animal 609 (which was used only for nymphs) and 100 adults, comprising a mixture of males and females, were placed into both ear bags of animal 610 (which was used only for adults). Approximately 400 nymphs and 100 adults were placed in the bags covering the skin lesions of viraemic animals 604 and 605 at 10 dpi. Because animal 604 was euthanized at 17 dpi the feeding time of ticks on this animal was shorter than planned (4 days). After harvesting *R. appendiculatus* nymphs were incubated at 28 °C and 85 % relative humidity (RH) for 17 to 24 days and were then stored at -80°C until tested for LSD viral antigen. The adult ticks were separated into females and males. The females were washed in phosphate buffered saline (PBS) and incubated at 28 °C and 85 % RH until oviposition and then the females and eggs were tested for the presence of LSDV. The partially fed males were transferred to recipient animal 613.

Approximately 40 partially fed *R. appendiculatus* males (previously fed on animal 610 during the viraemic stage of LSD but not on skin lesions) as well as partially fed males previously fed on skin lesions of viraemic animal 605 were added into the left ear bag of the recipient animal 613. Approximately 50 of these ticks re-attached to feed in clusters. Males and partially fed females that had been feeding on the skin lesions of the viraemic animals 604 and 605 were introduced into the right ear bag of animal 613.

Full-thickness skin biopsies as well as biopsy punches of the skin from the feeding sites of *R. appendiculatus* males on the left ear of animal 613 were collected after 4 to 14 days of attachment of the infected ticks. Partially engorged *R. appendiculatus* males and females, and full-thickness skin biopsies from the right ear were collected 9 to 12 days post-attachment of the infected ticks.

Amblyomma hebraeum

A. hebraeum ticks are also 3-host ticks. Donor animal 611 was used to investigate the possible transmission of LSDV by *A. hebraeum* nymphs and adults. Fifteen males were placed on the back of this animal at 4 dpi in order to encourage the attachment of the females. Five females were introduced into the same bag 5 days later. The males were allowed to feed for 9 days during the viraemic stage, at the intradermal inoculation site of the LSDV prior to them being transferred to recipient animal 702. Approximately 25 *A. hebraeum* males were placed on the skin lesions of viraemic donor animal 604 at 10 dpi and were allowed to feed for 7 days before being transferred to continue their feeding on recipient animal 702. The females were allowed to feed on donor animal 611 for 6 days until fully engorged and, after harvesting they were kept at 28 °C and 85 % RH, and allowed to lay eggs. The females and their eggs were subjected to testing by PCR and virus isolation.

Amblyomma hebraeum nymphs were placed into the ear bags (approximately 100 per ear) of donor animal 611 at 7 dpi. They were allowed to feed for 4 to 8 days after which they were harvested and maintained at 28 °C and 85 % RH for approximately 30 days. Then they were tested as above. In order to avoid damage to the mouthparts all the *Amblyomma* ticks were collected after they had detached by themselves from the donor animals and therefore the actual feeding time may differ from the number of days ticks were kept in the skin bags.

Some “wild” male *A. hebraeum* ticks were found attached to the skin under the belly and perineal area of animals 609, 610 and 611 upon their initial arrival at the animal facility. These ticks were not removed but were allowed to feed during the viraemic stage after which they were transferred to animal number 702 at 16 dpi.

The number of adult *Amblyomma* ticks available in this study was low and recipient animal 702 had only one skin bag into which the different batches of ticks were added. The skin samples and ticks were collected on the same day and the ticks were removed by hand. The number of days the different batches of ticks were allowed to feed in the skin bag varied from 10 to 14 days. Laboratory-reared *A. hebraeum* males that had previously fed on animal 611 during the viraemic stage of LSD were allowed to feed on recipient animal 702 for 13 days, *A. hebraeum* males, which had previously fed on the skin lesions of the viraemic animal 604, were allowed to feed for 10 days and “wild” *A. hebraeum* males were allowed to feed for 14 days on recipient animal 702 before ticks were removed and skin biopsies collected.

Collection of blood, serum and skin samples: The cattle were closely monitored for the presence of clinical signs of LSD and their body temperatures were recorded daily. Blood in EDTA and serum samples were first collected immediately prior to the inoculation of the donor animals (day 0) or the attachment of the ticks to the recipient animals and then EDTA blood was collected at 4, 7, 9, 10, 11, 14, 16, 18 and 21 dpi and serum was collected at 4, 7,

9, 14, 18 and 21 dpi. The last serum samples from donor animals were collected at 35 dpi. The last serum samples from recipient animals were collected 25 days after the attachment of the infected ticks.

Using appropriate aseptic surgical techniques biopsies were collected from the skin lesion sites and from normal appearing skin from donor animals 604 and 605, both of which showed severe clinical signs typical of LSDV infection, and from recipient animals 613 and 702 from the feeding sites of infected *R. appendiculatus* and *A. hebraeum* ticks, respectively. Biopsies were collected using either a 0.6 cm diameter Biopsy Punch (Kruuse) or a full-thickness skin biopsy was collected from a site where a cluster of ticks were feeding. Any ticks attached to the skin at the biopsy site were removed from the skin specimen and both the skin sample and the ticks were stored at -80 °C until tested.

Processing of ticks for virus isolation: Ticks collected from donors or recipients were individually dissected in Dulbecco's Modified Eagle's medium (DMEM), their mouthparts being collected before the idiosoma was cut open. Salivary glands and the gut of each tick were separated. The dissected organs of five ticks of the same species, gender and life cycle stage were pooled in 500 µl of DMEM containing gentamycin 0.1 mg/ml (Gentamicin®; Gibco, Invitrogen, Paisley, UK), ampicillin 0.05 mg/ml (Ampicillin Sodium Salt®; Invitrogen, Paisley, UK) and amphotericin B 5 µg/ml (Amphotericin B Solution; Sigma-Aldrich, Gillingham, UK) and then tested as one sample. The remainder of the viscera of each tick were also collected, pooled and tested using PCR and virus isolation.

The samples were ground in 1.5 ml Eppendorf tubes using a polypropylene pestle (Kimble, Kontes) and stored at -80 °C until tested. In cases in which virus isolation was attempted without storage of the samples they were incubated overnight at 4 °C prior to inoculation onto bovine dermis cell monolayers. A sample volume of 200 µl was used for PCR testing and 300 µl for virus isolation. Briefly, samples were sonicated using a Thistle Scientific Branson Sonifier 150 at a power setting of 2. They were then centrifuged at 600 g for 5 minutes and the supernatant was collected and decanted onto bovine dermis cell monolayers in 25 cm² tissue culture flasks. The flasks were incubated for 2 hours at 37 °C, and then washed with PBS containing Ca²⁺ and Mg²⁺. Fresh medium comprising DMEM supplemented with 2 % foetal calf serum (FCS) and gentamycin 0.05 mg/ml was added to the flasks which were incubated at 37 °C for 12 days. Cell cultures showing no cytopathic effect (CPE) were blind passaged once.

Polymerase chain reaction: The nucleic acid was extracted using a modified extraction method described by Tuppurainen et al (2005). Proteins were digested by adding 2 to 4 IU of proteinase K (Invitrogen, 20 mg/ml) and the samples were incubated at 56 °C overnight. The

DNA was precipitated in 2 volumes of 100 % ethanol and 1/10 of 3 M sodium acetate (pH 5.3). DNA amplification was performed using primers developed from the viral attachment gene. The primers have the following sequences: forward primer: 5'-TCCGAGCTCTTTCCTGATTTTTCTTACTAT-3' and reverse primer: 5'-TATGGTACCTAAATTATATACGTAAATAAC-3' (Ireland and Binopal, 1998). The PCR reaction was carried out in an Eppendorf Mastercycler PCR system (Eppendorf UK Ltd., Cambridge, UK). Amplified products were analysed by agarose gel electrophoresis. The gel was viewed using the Bio-Rad Molecular Imager® Gel Doc™XR System 170 (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). The positive samples gave products of the expected size of 192 bp (Ireland and Binopal, 1998).

Extracted DNA from non-infected *Rhipicephalus*, *Rhipicephalus (Boophilus)* and *Amblyomma* ticks or DNA from non-infected lamb testis cells were used as negative controls. Extracted DNA from scabs or skin lesions collected from experimentally infected donor animals were used as positive controls.

Serum neutralization test: Neutralizing antibodies were measured using a constant-virus/varying-serum neutralization test (Beard et al, 2009). The positive control serum was collected from cattle experimentally infected with LSDV and collected 37 dpi. Serum collected from cattle in the United Kingdom was used as the negative control. Titres were determined as the last dilution in the serum virus mixtures that gave a 50 % end point.

Results

Clinical signs of donor and recipient animals: Only two (604 and 605) of the five donor cattle developed generalized infections with multiple skin lesions characteristic of LSD. Donors 609, 610 and 611 showed transient fever but only a few, if any, skin lesions developed apart from the local reaction at the intradermal injection sites. The subscapular and precrucial lymph nodes were visibly enlarged in all donors. Animals 604 and 605 showed multiple skin lesions, excessive salivation, both nasal and ocular discharges and while several pox lesions in the skin of the muzzle and in the mucous membranes of the mouth and conjunctivae were evident in animal 604. The post-mortem examination of animal 604 revealed pox lesions in the mucous membranes of the respiratory and alimentary tracts.

Animal 604 was euthanized for humane reasons at 17 dpi. The area of skin to which the ticks were attached was excised. On the following day detachment of the nymphs from this excised portion of skin was attempted, however, they were still so firmly attached that it was difficult to remove them without damaging their mouthparts. Many of the nymphs were in the process of moulting to adults and therefore their re-attachment to recipient animal 701 on which they were placed, was poor.

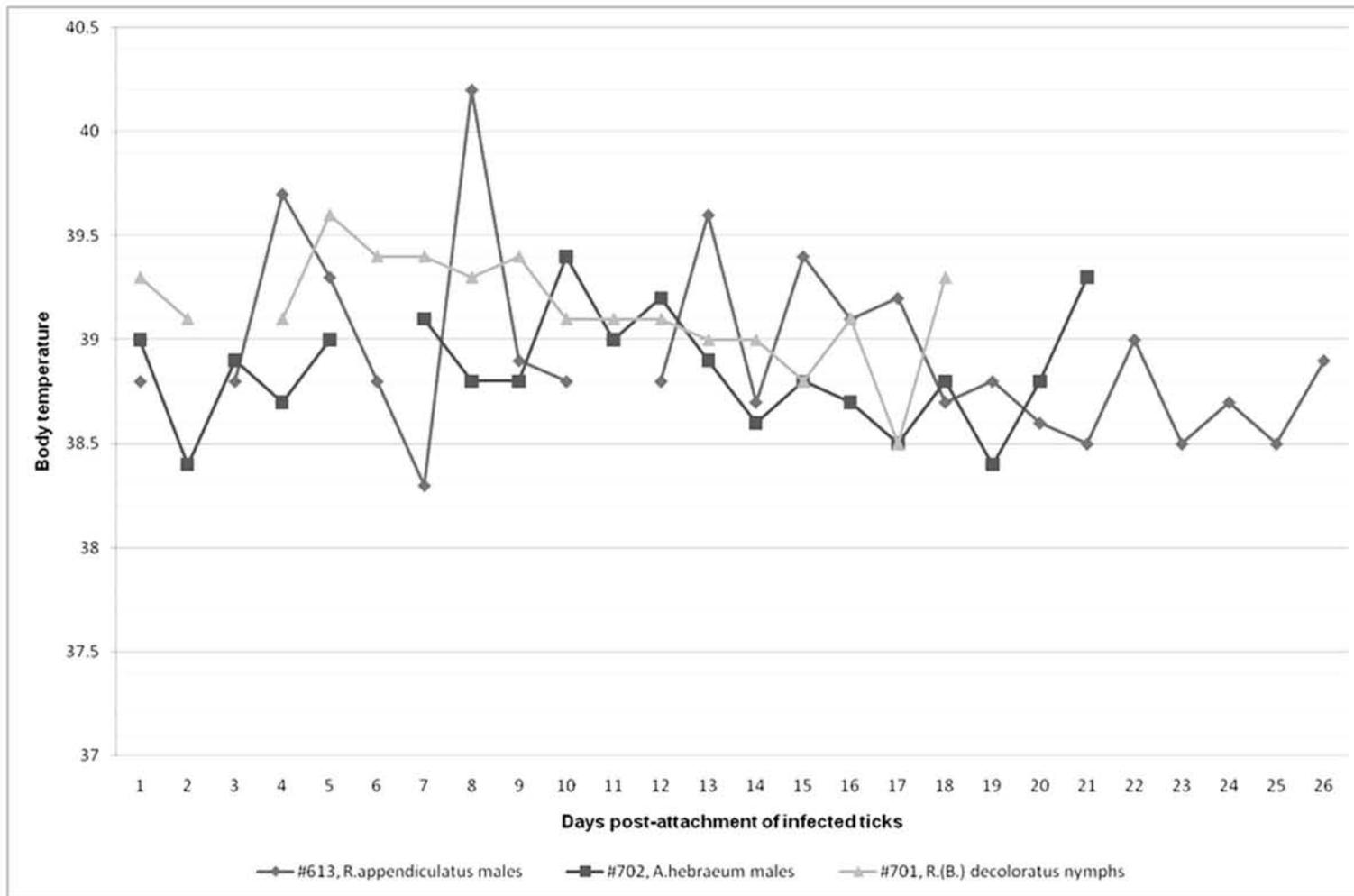


Fig. 2. Body temperatures of the recipient animals, post-attachment of infected ticks

Recipient animal 613 showed a slight transient elevation in body temperature (Fig. 2) approximately 7 days post attachment of infected ticks, and superficial lymph nodes regional to the tick attachment sites, were slightly enlarged. No skin lesions or any other clinical signs characteristic of LSD were observed.

Viraemia: All donor animals developed a viraemia (measured by PCR) between 4 and 18 dpi. Animal 604 became viraemic at 4 dpi and it remained viraemic until it was euthanized at 17 dpi. In animal 605 the viremic stage lasted for 7 days (7 to 14 dpi). Animal 609 was viraemic for 11 days (7 to 18 dpi), and animal 610 for 14 days (4 to 18 dpi). In animal 611 the viraemic stage lasted for 17 days (4 to 21 dpi). No viraemia was detected in any of the recipient animals.

Seroconversion: All donor animals (except animal 604) had seroconverted by 35 dpi on which day the last serum samples were collected. The neutralization assay started to detect a rise in antibody titres 21 dpi. Only one animal (605) showed low antibody levels when tested at 14 dpi. No increased serum antibody levels were detected in animal 604 before it was euthanized at 17 dpi.

No seroconversion was detected in any of the recipient animals. The final serum sample from the recipient animal 613 was collected 25 days post attachment of the *R. appendiculatus* ticks and the last serum sample from animal 702 was collected 21 days post attachment of the *A. hebraeum* ticks.

PCR and virus isolation of the skin samples

Donor animals: Skin biopsies collected from donor animals 604 and 605 while manifesting severe clinical disease tested PCR positive at 9 dpi. A sample of normal appearing skin was also positive in animal 604 which was the more severely affected of the two animals. Scabs collected from animal 610 at 36 dpi and animal 611 at 31 dpi tested PCR positive. As animal 609 did not develop any skin lesions no skin samples were collected from it.

Skin samples of the R. appendiculatus recipient animal 613:

Skin samples collected from the feeding sites of *R. appendiculatus* males from the base of the left ear were negative when tested by PCR and virus isolation. Most of these *R. appendiculatus* males originated from donor animal 610 which was viraemic but did not have skin lesions. However, another smaller group of *R. appendiculatus* males which had previously fed also on the skin lesions of the viraemic animal 605 were added to the same skin bag. The feeding time of these males was only 4 to 7 days on recipient animal 613, whereas the main group *R. appendiculatus* males were allowed to feed longer (12-14 days).

R. appendiculatus males which had previously fed on the skin lesions of viraemic donor animals 604 and 605 were allowed to feed on the skin of the base of the right ear of recipient animal 613 for 9 to 12 days prior to taking of the skin biopsies. Full-thickness skin biopsies were then collected from sites where several ticks were co-feeding in clusters. Lumpy skin disease viral DNA was detected at the feeding site of the ticks by PCR but no live virus was isolated from these skin samples.

Skin samples of the A. hebraeum recipient animal 702:

A. hebraeum males collected from several donor animals were fed on recipient animal 702. Some of these ticks had previously been allowed to feed on viraemic donor animal 611. Another group of *A. hebraeum* males had been feeding on the skin lesions of viraemic animal 604 before being transferred to recipient animal 702. Also "Wild" *A. hebraeum* ticks that were attached to donor animals 609, 610 and 611 on their arrival at the research unit and were thus feeding during the viraemic stage (but not on the skin lesions) were also transferred to the skin of recipient animal 702.

Altogether, *A. hebraeum* males from different origins fed on the skin of the non-infected recipient animal 702 for 10 to 14 days before collection of a full thickness skin biopsy. The section that was excised contained 12 *A. hebraeum* males that were feeding in clusters. Viral DNA was detected by PCR but no live virus was isolated from the skin biopsy.

Virus isolation and PCR results from ticks

Rhipicephalus appendiculatus males, females, nymphs and eggs:

Viral DNA was detected by PCR in the gut of the partially fed *R. appendiculatus* males feeding on the skin of the donor animal 605.

After feeding on the skin lesions of the severely infected and viraemic donor animals 604 and 605, partially fed *R. appendiculatus* males were transferred to feed around the base of the ears of non-infected recipient animal 613 and harvested 9 to 12 days later. The mouthparts and gut of these ticks were PCR positive (Fig. 3).

Some of the fully engorged females that were collected on the skin lesions of the severely infected animal 604 were tested prior to oviposition. The salivary glands and viscera of each female were strongly PCR positive while the pooled mouthparts of these ticks were weakly positive.

The nymphs that had fed on the skin lesions of viraemic donor animal 604 and which were then incubated in a chamber at 85 % RH and 28 °C for 5 days were negative when tested by PCR.

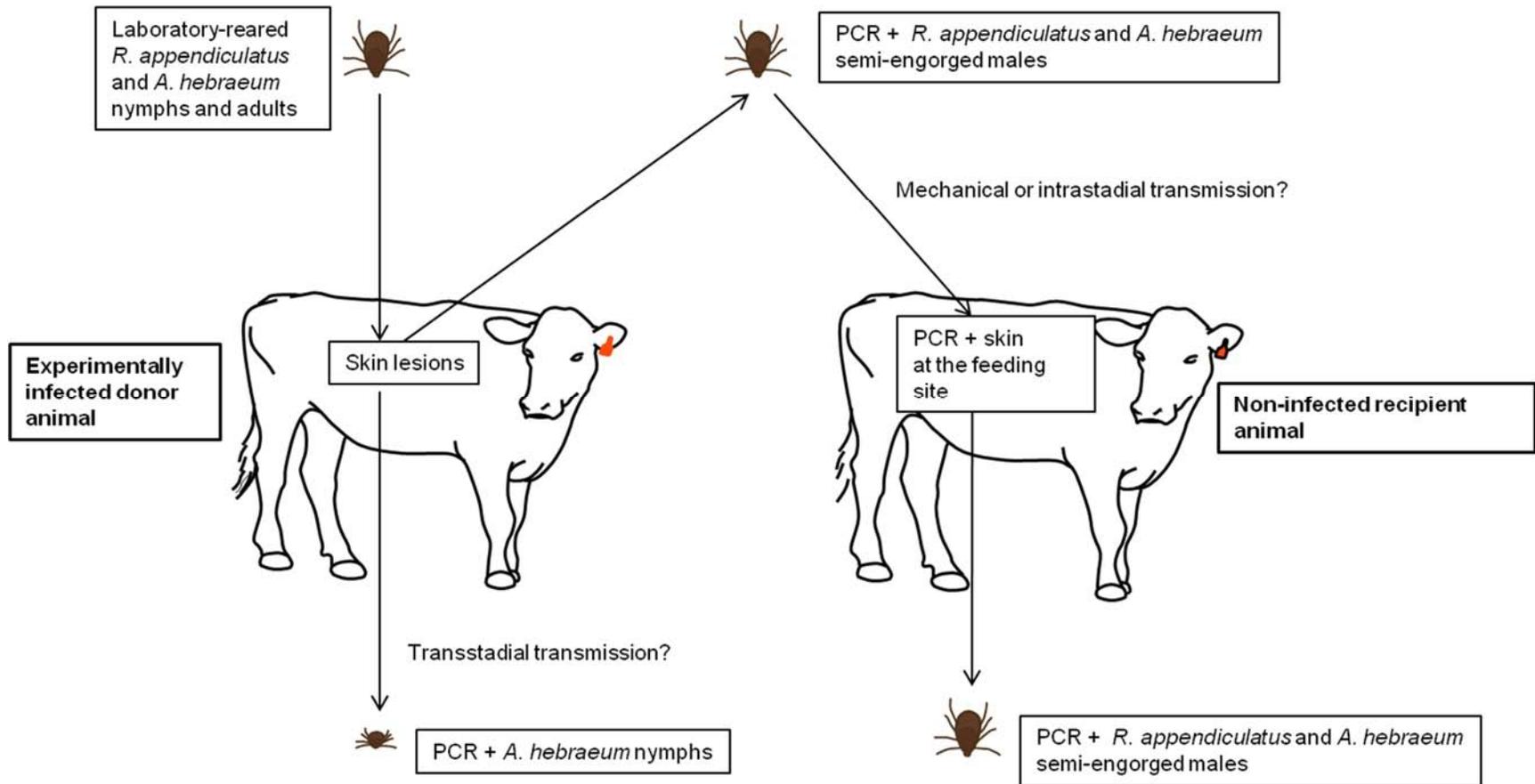


Fig. 3. Schematic representation of the polymerase chain reaction results of *Rhipicephalus appendiculatus* and *Amblyomma hebraeum* ticks after feeding on LSDV infected bovine

Eggs laid by 9 *R. appendiculatus* females that had previously fed around the base of the ear of donor animal 610 were all negative when tested by PCR. This animal had remained viraemic during the whole feeding period of the ticks but it did not develop any skin lesions.

Amblyomma hebraeum males, females, nymphs and eggs:

Partially fed *A. hebraeum* males were collected from the skin of the recipient animal 702 at 10 to 14 days post-attachment. These laboratory-reared or "wild" ticks originated from different donor animals as previously described.

The mouthparts, salivary glands, gut and rest of the viscera of partially fed male *A. hebraeum* ticks were dissected. The mouthparts were separated before the dissection of the idiosoma to avoid possible contamination. Each organ type (3) from 5 ticks was pooled and all tested positive by PCR. In some of the mouthpart specimens a flake of skin or the cement-like substance which secures the attachment of the tick to the host during feeding, was included. When the mouthparts and skin flake or cement from single ticks were separately tested, viral DNA was detected on several occasions. The area of skin of recipient animal 702 to which these ticks had been attached also tested positive by PCR (Fig. 3).

Fully engorged nymphs that had fed on the apparently normal skin of the viraemic donor animal 611 were harvested and kept at 85 % RH at 28 °C for 8 days after which they were stored at -80 °C. The outer surface, cuticle, of each tick was removed and its viscera were tested. The mouthparts of 5 of the ticks were pooled and tested as one sample. Viral DNA was detected in the viscera of the *A. hebraeum* nymphs (Fig. 3).

Fully engorged *A. hebraeum* females and their eggs tested PCR negative. These females had been feeding during the viraemic stage in a skin bag placed directly over the inoculation site of animal 611 that was showing only very mild clinical signs of LSD at the time.

"Wild" *A. hebraeum* males were collected from donor animal 605 at 22 and 23 dpi. Their pooled salivary glands, gut and rest of the viscera tested negative by PCR, and no viral DNA was detected in their mouthparts. "Wild" adult *A. hebraeum* males were also collected from recipient animal 702. These ticks were dissected and tested as above but no viral DNA was detected on their mouthparts, or in salivary glands and gut tissue.

Rhipicephalus (Boophilus) decoloratus males, females and eggs:

Unengorged *R. (B.) decoloratus* females were collected 21 days after attachment as larvae on skin lesions of animal 605. All the internal organs of each tick were tested as a single sample, the organs were not separated or pooled. LSD viral DNA was detected in these samples.

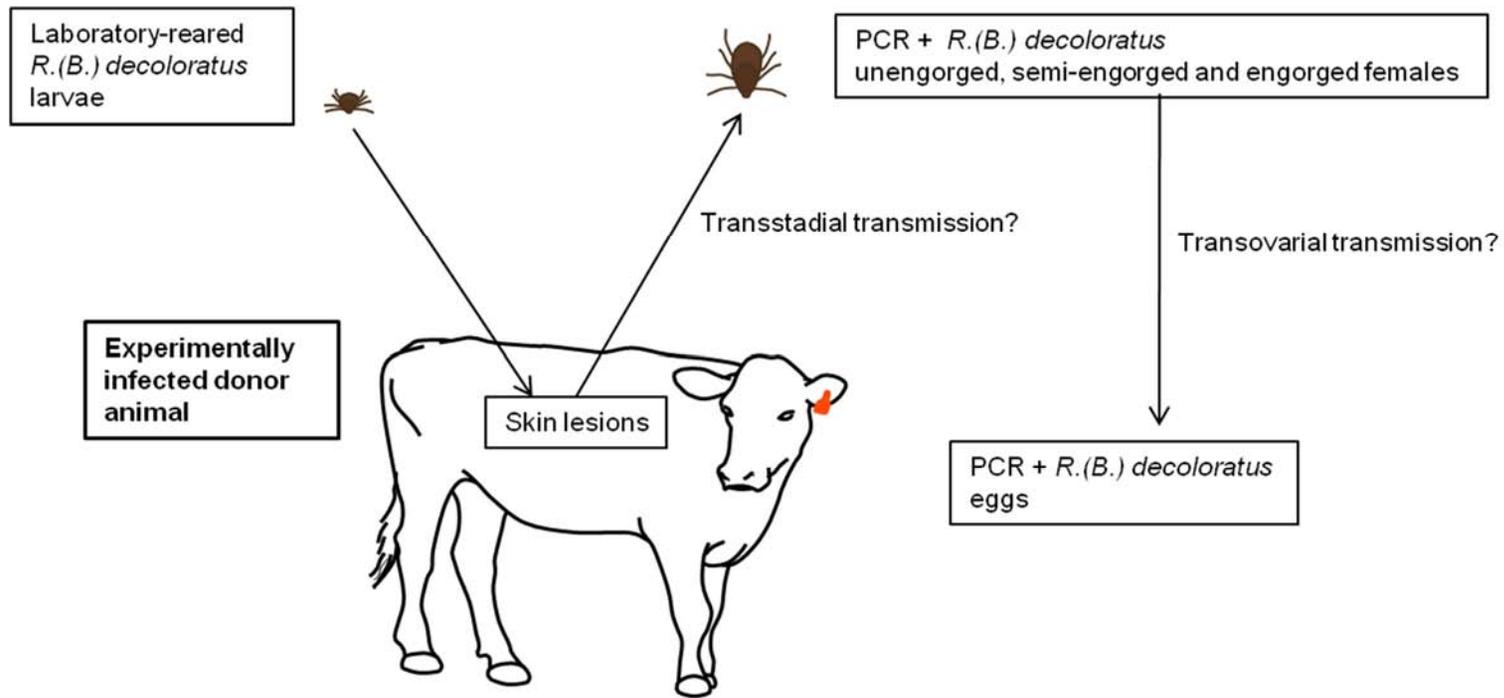


Fig. 4. Schematic representation of the polymerase chain reaction results of *Rhipicephalus (Boophilus) decoloratus* ticks after feeding on LSDV infected bovine



Fig. 5. Polymerase chain reaction results of eggs laid by 2 *R. (B.) decoloratus* females. Lanes 1-3: Eggs laid by female no.1, Lanes 4-6: Eggs laid by female no. 2, Lane 7: extracted DNA from non-infected *R. (B.) decoloratus* tick, Lane 8: LSD skin lesion of donor animal 605, Lane 9: water control, Lane M: 100 bp ladder (Promega).

Partially fed females detached by hand when collected from animal 605 at 21 days after attachment as larvae showed PCR positive salivary glands and gut. A fully engorged gravid female was also PCR positive when all its abdominal organs were tested as one sample by PCR (Fig. 4).

The viscera of partially fed males, however, tested negative for LSD viral DNA. One sample comprising the pooled mouthparts of 3 males which had been collected after feeding on skin of animal number 605 while manifesting multiple skin lesions gave a faintly positive reaction by PCR.

Two *R. (B.) decoloratus* females were allowed to feed on skin lesions of animal 605 for 23 and 24 days, respectively. When fully engorged they were collected and allowed to lay eggs. Viral DNA was detected by PCR in the eggs of both of them (Fig. 5).

Discussion

Interrupted feeding is a natural behavioural pattern of adult *Rhipicephalus* and *Amblyomma* males that detach and re-attach several times throughout the period they spend on the host (Wang et al, 1998). When the host animals are in close contact males may easily swap their hosts. Consequently, transmission of LSDV may occur either mechanically via their mouthparts or intrastadially if the virus survives in cells of the salivary glands. Previous studies have shown that in general the main route of virus transmission by infected ticks is via saliva secreted during feeding (Nuttall et al, 1994). Most of the blood meal of a tick is obtained during the last 24 hours before its detachment. Digestion of a blood meal occurs inside the cells of the midgut (Sonenshine, 1991) which allows an easy entrance for the virus into the tick cells. After ingestion blood is concentrated in the digestive tract of the tick and excessive water and Na⁺ and Cl⁻ ions are rejected back to the host (Kuhnert et al, 1995).

The findings of this preliminary study indicate that mechanical transmission of LSDV may occur by *R. appendiculatus* and *A. hebraeum* males. Viral DNA was detected in the mouthparts of *R. appendiculatus* and *A. hebraeum* males after feeding on experimentally infected cattle. This finding concurred with the observation that the skin at the feeding site of these ticks in recipient animal 613 and 702 tested PCR positive for LSD viral DNA. *Amblyomma hebraeum* ticks have long mouthparts and, in some specimens a small piece of "cement" or skin flake remained attached to the hypostome and tested PCR positive. The salivary glands and gut tissue of *A. hebraeum* males were also PCR positive after feeding on the experimentally infected donor animals.

After feeding on recipient animal 613 for 9 to 12 days some *R. appendiculatus* males still showed PCR positive gut tissue and after feeding for 10 to 14 days on recipient animal 702 viral DNA was still detected in the mouthparts, salivary glands and gut tissue of *A. hebraeum* males. Although in this study we were unable to isolate live virus from the ticks and thus did not obtain conclusive evidence for the biological transmission of LSDV by ixodid ticks it certainly indicates that further more detailed experiments are required in order to rule out the possibility of intrastadial transmission.

The mouthparts and gut of the fully engorged *R. appendiculatus* females which had fed on the skin of the severely affected viraemic animal 604, unsurprisingly, tested PCR positive for LSD viral DNA shortly after their detachment from the host. Their salivary glands also tested PCR positive but because these females were fully engorged and therefore difficult to dissect, this may have been due to the leakage of infected blood from an inadvertently ruptured gut. Their mouthparts, however, were separated prior to the dissection of the body, and therefore the contamination of the mouthparts by viraemic blood is unlikely. In any event, these findings have no bearing on transmission between the vertebrate hosts as fully engorged *R. appendiculatus* females do not re-attach to the hosts. However, partially fed *R. appendiculatus* females are able to detach and re-attach to a new host in the event of interrupted feeding, although the frequency depends on the length of time the ticks have been attached to the first host (Wang et al, 1999). Therefore theoretically, such females may be able to transmit LSDV mechanically or intrastadially between cattle in the event of death of the host or as a result of vigorous grooming by the host.

The duration of attachment of adult ticks and nymphs to the skin of the host may affect the efficiency of transfer of the virus from the infected skin of the host to the non-infected tick. *R. appendiculatus* and *A. hebraeum* are three-host ticks. *R. appendiculatus* is a fast feeder and the total time required to complete a blood meal is approximately 5 to 7 days for both nymphs and adults. *A. hebraeum* adults feed for 6 to 14 days, and nymphs for 5 to 8 days. *R. (B.) decoloratus* is a one-host tick and development from larvae to nymphs and then to adults occurs on a single host and lasts for approximately 23 days (Norval and Horak, 2004). The findings of this study suggest that this long attachment time may indeed enhance the adjustment of the virus to tick cells. The PCR positive engorged nymphs and unengorged adult *R. (B.) decoloratus* females indicate that LSDV may survive in tick cells during the moulting process from nymphs to adults. Therefore, transstadial transmission of the virus in *R. (B.) decoloratus* is a distinct possibility which requires further investigation.

No PCR positive *R. appendiculatus* nymphs were detected despite the fact that the volume of the blood meal consumed by each individual *R. appendiculatus* nymph is larger than that of a *R. (B.) decoloratus* nymph. Interestingly *A. hebraeum* nymphs that were fed on viraemic animals (not skin lesions) tested PCR positive. Further studies are therefore needed to

investigate not only if LSDV is capable of surviving the moulting process of *A. hebraeum* nymphs to adults but also if infected animals that do not show any clinical signs of LSD may serve as a source of transmission of LSDV within a herd by ticks or any other blood-feeding arthropod vectors. In this study donor animal 611 was asymptomatic but it remained viraemic for 17 days.

Previous studies have shown that the levels of vertical transmission of viruses from female ticks to their eggs seem to be generally low (Nuttal et al, 1994). No evidence for transovarial transmission of the LSDV in *A. hebraeum* was found in the present investigation. However, due to the very low number of *A. hebraeum* adults available for the study, no females were fed directly on the skin lesions, only on a viraemic host. *R. appendiculatus* females did feed on the skin lesions of a viraemic animal but no LSD viral DNA was detected in their eggs. In this study only the eggs laid by 8 *A. hebraeum* females and 11 *R. appendiculatus* females were tested and these numbers are considered too low on which to base any definite conclusions. A significant finding of this study was the detection of the viral DNA in *R. (B.) decoloratus* engorged females that had previously fed on the skin lesions of viraemic cattle and in the eggs laid by these females. In addition, PCR positive unlaidd eggs were detected within engorged females.

When vectors feed on a viraemic host, it has been observed that the higher the titre of virus in a blood meal, the higher the number of ticks that become infected (Singh and Anderson, 1968; Davies et al, 1990). However, Carn and Kitching (1995) observed that the feeding of insects on a capripoxvirus-infected animal during the viraemic stage was not sufficient to transmit the infection. It has been demonstrated using a quantitative PCR method that, in animals infected with LSDV, the highest virus load is in the skin lesions (Babiuk et al, 2008) and therefore infected skin is likely to be a better source of infection for ticks or other arthropod vectors than viraemic blood. The findings of this study indicate that ticks collected from donor animals (604 and 605) that showed severe clinical disease with multiple skin lesions were found more often PCR positive than those ticks feeding on only viraemic hosts. Mechanical transmission of LSDV between cattle by ticks or other blood-feeding insect vectors is theoretically possible as the virus is stable and may survive for a long period on the skin and hair of cattle. LSDV has been isolated up to 39 dpi from the skin of the experimentally infected animals, and viral DNA has been detected by PCR from the skin up to 92 dpi (Tuppurainen, et al 2005).

It is possible that, during the process of tick dissection, contamination of the salivary glands with LSDV originating either from the outer surface of the tick or from inadvertently damaged gut tissue could occur. Also contamination of *R. (B.) decoloratus* eggs from the surface of the female tick could occur during the incubation period for oviposition. However, *R. appendiculatus* and *A. hebraeum* eggs tested PCR negative, although the engorged

females were incubated for oviposition in a similar way to female *R. (B.) decoloratus* ticks. Ideally confirmation of the presence of LSDV in salivary glands or eggs should be carried out using parallel immunohistochemistry or immunoperoxidase staining with LSDV specific antibodies.

The number of recipient cattle used in this study was too low to be able to demonstrate clinical signs of LSD with any degree of certainty, although the ticks may well have been able to transmit the virus. However, the final serum samples were collected from the recipient animals only 25 days post attachment of infected ticks, which may have been too early to detect a rise in antibody levels. It is also possible that virus transmitted from the donor animals to the skin of the recipient animals by *R. appendiculatus* and *A. hebraeum* males may not have been infectious, viral DNA originated from dead virus particles or LSDV, although live, may not be able to multiply in tick cells. Due to the limited number of samples obtained in this study it was not possible to carry out multiple attempts to isolate live virus from the tick tissues and feeding sites of infected ticks.

The incidence of clinically apparent disease caused by a virus and transmitted by ticks is dependent on several factors, such as the number of exposures to infected ticks, infection prevalence in ticks, the concentration of infectious virus in ticks, the virulence of the virus isolate and the susceptibility of the host (Gritsun et al, 2003). For example, louping ill infected ticks have been collected from wildlife that did not develop detectable viraemia (Jones et al, 1997). The lack of clinical disease in recipient animals may be due to an insufficient number of infected ticks being attached to them. In this study fewer than 50 potentially infected partially fed *A. hebraeum* males were fed on animal 712. The infection rate in these ticks is not known and therefore the true number of infected ticks was probably lower than 50. In addition, the development of the clinical signs of LSD may be considerably slower or may not occur at all when the recipient animal is exposed to only a few infected ticks. It is also possible that the number of virus particles transmitted by ticks were so low that the host's defence mechanisms in the skin were able to eliminate the infection before the spread of the virus.

Although the numbers of ticks and experimental animals were limited in this study, the data obtained, strongly indicate that transmission of LSDV between hosts may occur via ixodid tick vectors. In future experiments it would be advisable to use a greater number of recipient animals and a significantly larger number of ticks of different life cycle stages to be able to produce statistically meaningful data on the likelihood of transmission of LSDV by hard tick vectors. The significance of this finding would then need to be tested under field conditions.

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