Quantifying the acquisition and retention of lumpy skin disease virus by haematophagus insects and the implications for transmission and control

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Abstract

Robust transmission parameters are required in order to control infectious diseases. With a novel approach, we combine *in vivo* experimental studies with mathematical modelling to estimate transmission parameters of lumpy skin disease virus (LSDV), a vector-transmitted poxvirus of cattle, including the relevance of four potential vector species (*Aedes aegypti*, *Culex quinquefasciatus*, *Stomoxys calcitrans* and *Culicoides nubeculosus*). The probability of LSDV transmission from clinical cattle to the vector correlated with disease severity, and was very low when subclinical animals were considered. All four vector species tested had a similar rate of acquisition of LSDV after feeding on the host, retaining the virus for up to 8 days. We combined our experimental results with other published data on LSDV transmission and vector life history to determine the basic reproduction number of LSDV in cattle mediated by each of the model species, which can be used to inform LSD control programmes.

Key words: poxvirus, lumpy skin disease, transmission, mosquitoes, flies, midges, basic reproduction number, vector, control, *Aedes aegypti*, *Culex quinquefasciatus*, *Stomoxys calcitrans*, *Culicoides nubeculosus*

Introduction

Lumpy skin disease virus (LSDV) is a large DNA virus of the family *Poxviridae* and the etiological agent for lumpy skin disease (LSD) in cattle. LSDV is a rapidly emerging pathogen. First described in Zambia in cattle in 1929, LSDV subsequently spread throughout Africa and into the Middle East [1]. The virus has increased its geographical coverage substantially in the past decade, entering and spreading within Europe and Asia, including Russia, India, Bangladesh and China [2-8]. The virus currently threatens cattle populations in Africa, Europe and Asia. It is important to improve knowledge of the factors that affect the transmission of LSDV in order to control the spread of the virus.

LSD is characterised by fever, weight loss, and prominent multifocal necrotising cutaneous lesions [9], and affect cattle of all ages [10]. Morbidity in disease outbreaks range from 5-26%, and mortality 0.03-2% [2-4,11-13]. Control measures include vaccination, quarantine and partial or complete culling of infected herds. LSD outbreaks and the subsequent control measures cause significant negative economic and welfare impacts in endemic [14-16] and epidemic [17] situations, resulting in food insecurity for affected communities.

Haematophagus dipterans (referred to in this work as "blood-feeding insects"), particularly *Stomoxys calcitrans,* have been associated with outbreaks of LSDV [7,18-20]. In addition, experimental transmission of LSDV from affected to naïve animals (defined by the presence of clinical disease and/or detection of systemic LSDV antigen and/or capripoxvirus-specific antibodies) has being demonstrated via the mosquito *Aedes aegypti* [21], the ticks *Rhipicephalus appendiculatus* [22-24], *Rhipicephalus decoloratus* [25], *Amblyomma hebraeum* [26], the stable fly *Stomoxys calcitrans,* horseflies *Haematopota* spp. and other *Stomoxys* species [27,28]. LSDV DNA has also been detected in other species after feeding on infected cattle or on an infectious blood meal (*Culex quinquefasciatus*, *Anopheles stephensi*, *Culicoides nubeculosus*) [29], or in field-caught pools (*Culicoides punctatus*) [4]. However transmission of LSDV to susceptible

animals has not been confirmed for these species. To date the mode of LSDV arthropod transmission has been assumed to be mechanical as no evidence of active virus replication in insects or ticks has been found [30].

LSDV can be detected in skin lesions, blood (primarily in peripheral blood mononuclear cells), and in nasal, oral and ocular excretions of infected cattle [27,31,32]. Viraemia is considered of short duration and relatively low level, though the virus can survive for longer periods of time in skin lesions [31]. LSDV has also been detected in seminal fluid of diseased bulls [33], making venereal transmission a possibility [34-36]. Subclinical infections (detection of LSDV in animals without cutaneous lesions) [3,27,32] and resistance to LSDV (absence of LSDV and cutaneous lesions following experimental challenge) have been reported, but both are poorly documented. The contribution of subclinical LSD to the transmission of the virus is unclear and a topic of controversy when implementing control measures such as whole-herd culling, particularly when morbidity is low [37,38].

In this study we calculated biologically relevant parameters for the mechanical transmission of LSDV using four blood-feeding insects species previously reported to acquire LSDV (*S. calcitrans*, *Ae. aegypti*, *Cx. quinquefasciatus* and *C. nubeculosus*). These transmission parameters were used to understand the risk of transmission of the virus from experimentally infected cattle to each model species, and then were combined with data from previous studies to determine the basic reproduction number for each species.

Results

Experimental infection of calves with LSDV

Experimental inoculation of calves with LSDV results in clinical and subclinical disease. Eight calves were challenged by intravenous and intradermal inoculation of LSDV in order to

act as donors on which blood-feeding insects could feed. The clinical and pathological findings (summarised in Text S1) have been described previously [9], and resemble those of naturally infected cattle [2,4,8,11,12,37]. Three calves (calves 3, 5, and 9) developed lumpy skin disease, characterised by severe multifocal dermatitis with necrotising fibrinoid vasculitis consistent with field reports of LSD (Figure S1). The cutaneous lesions initially appeared in close proximity to the inoculation site at 5 days post challenge (dpc) for calves 5 and 9, and at distant sites in all three clinical calves at 7 dpc. The five remaining calves (calves 2, 4, 7, 8 and 10) did not develop lesions other than at the inoculation sites (Figure S2A). All eight inoculated calves developed a fever which was more prolonged in calves with clinical signs (Figure S2B). Two non-inoculated in-contact calves (calves 1 and 6) were included in the study and did not develop any clinical signs or lesions consistent with LSD.

LSDV DNA can be detected in blood and skin of clinical and subclinical calves. In the three clinically-affected calves viral DNA was first detected in the blood by qPCR at 5 dpc and remained detectable in all subsequent blood samples (up to 19 dpc). Peak viral DNA levels in the blood (6.9, 5.3 and 5.3 $\log_{10}$ copies/ml in calves 3, 5 and 9, respectively) were reached at 11 dpc (Figure 1). By contrast, viral DNA was detected only intermittently in the blood of four (out of five) subclinically infected calves between 5 dpc and 19 dpc. In addition, genome copy numbers were lower (median: 2.1 $\log_{10}$ copies/ml; range: 1.2 to 2.4 $\log_{10}$ copies/ml) than those in clinically-affected calves (Figure 1). Although negative for LSDV in whole blood, the peripheral blood mononuclear cell (PBMC) fraction of calf 7 was positive for viral DNA on days 7, 9 and 19 post challenge (Data S1). These results indicate that clinical calves had had more viral DNA present in the blood, and for longer compared to subclinical calves. However, LSDV DNA could be detected at least once in all eight challenged animals between 5 and 19 dpc.

Skin biopsies of cutaneous lesions taken at 7 dpc (calf 9) or 9 dpc (calves 3 and 5) contained abundant viral genomes as measured by qPCR (Figure 1). Viral DNA was detected in all subsequent biopsy samples, with the quantities detected remaining at an approximately constant level for the duration of the experiment (Figure 1). The amount of viral DNA present in the skin lesions varied between the three clinical calves in an analogous fashion to the viral DNA in blood, with the highest concentration of viral DNA detected in skin lesions of calf 3 and least in calf 9 (Figure 1). The peak level of viral DNA in skin was reached after the peak level of viral DNA in blood in all three calves (Figure 1). Viral DNA was detected at three time points in biopsies of normal skin from one subclinical calf (calf 4) at a lower copy number than in the clinically-affected animals; skin biopsies from the other subclinical animals (calves 2, 7, 8 and 10) were all negative for LSDV DNA (Figure 1).

Infectious LSDV is present in larger quantities in the skin compared to blood. Both skin homogenate and PBMC suspension collected between 5 and 19 dpc from clinical calves were titrated to determine the quantity of live virus in these tissues. Although units of measurement are not directly comparable between sample types (i.e. skin vs PBMC), they are representative of the magnitude of exposure that haematophagus insects may encounter during feeding (i.e. mg of skin tissue and µl of blood). In all calves the viral titre from skin homogenate was higher and more constant than from PBMC suspension (Figure 2). Live virus was detected for six consecutive days from 5 dpc in the PBMC fraction of calf 3, whereas in calves 5 and 9 the virus was isolated only in three and two days (respectively) starting at day 7 post challenge. In contrast, all skin samples except one taken from dermal lesions contained live LSDV with a maximum titre of $\log_{10}$ 4.3 PFU/mg skin, which is over 3 $\log_{10}$ greater than the maximum level of virus detected in PBMCs, emphasising the strong cutaneous tropism of LSDV. Biopsies collected from normal skin of clinical calves were negative for live virus (i.e. below 10^{-2} PFU/mg, Data S1) suggesting the virus is highly concentrated in the skin lesions of clinical

animals. Live virus was not detected in blood or skin from subclinical animals (including samples which were qPCR positive).

Humoral response to LSDV inoculation. Serum from the three clinically-affected calves contained antibodies to LSDV at 15-17 dpc as determined by a commercial ELISA test. By the end of the study period all subclinical animals had also developed detectable LSDV antibodies at levels lower than those observed in the clinical animals, but above those of the non-challenged controls (Figure S3). The presence of detectable levels of antibodies confirmed exposure to the virus in all eight challenged animals, although the clinical outcome of challenge varied widely between the eight calves.

Acquisition and retention of LSDV by blood-feeding insects after feeding on donor cattle

We next studied the influence of this disease spectrum on the acquisition and retention of LSDV in blood-feeding insects. To assess the acquisition and retention of LSDV by blood-feeding insects, all eight challenged animals were exposed to two mosquito species, *Ae. aegypti* and *Cx. quinquefasciatus*, one species of biting midge, *C. nubeculosus*, and the stable fly, *S. calcitrans* on days 5, 7, 9, 11, 15, 17 and 19 post challenge. The selected species are potential mechanical vectors with different feeding mechanisms [39], covering those which will feed readily on cattle (*i.e. S. calcitrans*), as well as species models for Culex and Aedes mosquitoes [40,41] and also biting midges [42,43] which would feed on cattle. At each time point, a pot of insects of each species (i.e. four pots in total) was placed on a separate cutaneous nodule on a clinical animal and, a corresponding area of normal skin on a subclinical animal. Blood engorgement, as a measure for detection of insect biting activity, was assessed visually. A subset of the insects from each pot was tested for the presence of LSDV DNA by qPCR at 0, 1, 2, 4 and 8 days post

feeding (dpf) (Table S1). The smaller numbers of insects tested at the later time points reflect the lower numbers surviving for long enough to be tested.

Different models for the proportion of positive insects were compared to assess differences in: (i) the probability of transmission from bovine to insect (i.e. of acquiring LSDV) amongst insect species and between clinical and subclinical donors; and (ii) the duration of viral retention amongst insect species (Tables S2 and S3). Models were compared using the deviance information criterion (DIC), with a model having a lower DIC preferred to one with a higher DIC. Positive insects were those with LSDV DNA amplification by qPCR.

Probability of transmission from bovine to insect. A total of 3178 insects were fed on the eight donor calves (over 7 feeding sessions), of which 180 were positive for viral DNA when tested. A higher proportion of insects were positive after feeding on a clinical donor (173 out of 1159) compared to feeding on a subclinical donor (7 out of 2019) (Figure S4). Comparing the proportion of positive insects for each species after feeding in clinical and subclinical calves (Figure 3) revealed that the probability of transmission from bovine to insect (i.e. of acquiring LSDV) does not differ amongst the four insect species, but that this probability does differ between clinical and subclinical donors (Table S2). For a clinical donor, the probability of transmission from bovine to insect was estimated (posterior median) to be 0.22, while for a subclinical donor it was estimated to be 0.006 (Table 1). This means that an insect feeding on a subclinical animal is 97% less likely to acquire LSDV than an insect feeding on a clinical one (Table 1; Figure 3).

Infectiousness correlates with the level of viral DNA in blood and skin. The relationship between the level of viral DNA in the skin or blood of a calf and the proportion of virus-positive insects resulting from a feeding session was examined. For each feeding session that took place on the three clinical calves, the proportion of insects containing viral DNA post-feeding was

calculated and compared to the viral DNA copy number present in both the blood sample and the skin biopsy taken from the calf on that day (Figure 4). This revealed a dose-response relationship between the levels of viral DNA in skin and blood and the probability of transmission from bovine to insect (or "donor infectiousness"). Furthermore, this relationship was the same for all four insect species (Table S3), irrespective of their different feeding mechanisms. The relationship differed between levels of viral DNA in blood and skin (Table 2; Figure 4), with the probability of transmission being higher when the level of viral DNA in blood was used compared to skin (Figure 4). The fits of the models using levels of viral DNA in blood or skin are similar, suggesting that both are acceptable proxy measures for infectiousness of the donor (Figure S5).

Combining the dose-response relationship (Figure 4) with the time course for levels of viral DNA in blood or skin for each calf (Figure 1) shows how the infectiousness of an animal changes over time and how it varies amongst animals (Figure 1, right-hand column). This highlights the very low probability of transmission from bovine to insect (<0.01 at all time points; cf. estimate in Table 1) for calves which were only subclinically infected. In addition, for those calves which did develop clinical signs, there were differences in both the timing and level of infectiousness amongst the calves, which is a consequence of the underlying differences in viral dynamics in each animal. This is reflected in both the changes over time in the proportion of insects acquiring virus after feeding and differences in this proportion amongst clinical calves (Figure S6).

***Duration of LSDV retention*.** Viral DNA was detected in *Ae. aegypti* and *S. calcitrans* up to 8 dpf, in *C. nubeculosus* up to 4 dpf and in *Cx. quinquefasciatus* up to 2 dpf (Figure 3). However, few *Cx. quinquefasciatus* mosquitoes survived to 4 or 8 dpf (Table S1), resulting in uncertainty about the duration of retention in this species (Figures 3 & 4). The mean duration of viral

retention differed amongst the four insect species in the present study (Figure 3; Table S2), being the longest for *Ae. aegypti* (5.9 days) and *S. calcitrans* (5.5 days), followed by *Cx. quinquefasciatus* (4.5 days), and *C. nubeculosus* (2.4 days) (Figure 3; Table 1). The corresponding virus inactivation rate (i.e. the reciprocal of the mean duration of retention) was 0.17/day for *Ae. aegypti* and 0.18/day for *S. calcitrans*, 0.22/day for *Cx. quinquefasciatus* and 0.42/day for *C. nubeculosus* (Table 1).

Levels of retained LSDV. The median amount of viral DNA in homogenized whole insects was the same when tested at different days post feeding for three (out of the four) species: *Ae. aegypti* (Kruskal-Wallis test: χ^2=0.98, df=4, *P*=0.91), *Cx. quinquefasciatus* (Kruskal-Wallis test: χ^2=3.62, df=2, *P*=0.16) or *S. calcitrans* (Kruskal-Wallis test: χ^2=2.74, df=4, *P*=0.60) (Figure S6). However, the median level of viral DNA was lower for individual *C. nubeculosus* tested at later times post feeding (Kruskal-Wallis test: χ^2=10.8, df=3, *P*=0.01) (Figure S7). These results are consistent with a mechanical rather than a biological form of vector-transmission.

Probability of transmission from insect to bovine

Three previous studies have investigated the transmission of LSDV from insects to cattle, where insects of species included in the present study were allowed to feed on an infected donor and were subsequently allowed to refeed on a naïve recipient [21,27,29]. The number of positive insects refeeding was not determined in these studies. By combining LSDV acquisition and retention results of the present study with challenge outcomes of the aforementioned studies (i.e. whether or not transmission occurred), it is possible to estimate the probability of transmission from insect to bovine. This probability was highest for *Ae. aegypti* (0.56), intermediate for *C. nubeculosus* (0.19) and *Cx. quinquefasciatus* (0.11) and lowest for *S. calcitrans* (0.05) (Table 1). However, there is considerable uncertainty in the estimates for all

species, but especially for *Ae. aegypti*, *C. nubeculosus* and *Cx. quinquefasciatus* (Table 1), which makes it difficult to compare estimates across species.

Basic reproduction number for LSDV

The basic reproduction number (R_0) is defined as "the average number of secondary cases caused by an average primary case in an entirely susceptible population" [44]. For LSDV, R_0 combines the parameters related to transmission (Table 1) with those related to vector life history (i.e. biting rate, vector to host ratio and vector mortality rate; Table S4) to provide an overall picture of the risk of transmission by the four insect species [45]. The basic reproduction number was estimated to be highest for *S. calcitrans* (median R_0=19.1) (Table 1; Figure 5), indicating that this species is likely to be the most efficient vector of LSDV and would be able to cause substantial outbreaks if it were the sole vector in a region. Both *C. nubeculosus* (median R_0=7.1) and *Ae. aegypti* (median R_0=2.4) are also potentially efficient vectors of LSDV (i.e. R_0>1 for these species) and would be able to sustain transmission if either were the sole vector in a region. Finally, *Cx. quinquefasciatus* (median R_0=0.6) is likely to be inefficient at transmitting LSDV (Table 1; Figure 5). It would not be able to sustain transmission on its own, but it could contribute to transmission if other vector species were also present.

Exploring the contribution of clinical and subclinical animals to the basic reproduction number for each species further emphasises the more limited role played by subclinical animals in the transmission of LSDV (Figure 5). For all species, the R_0 for clinical animals alone is very close to that for both clinical and subclinical animals combined (Figure 5). Moreover, the median R_0 for subclinical animals alone is below one for all species, except *S. calcitrans* (Figure 5).

The R_0 values calculated from our data and previous studies provide a summary of the risk of LSDV transmission. A range of blood-feeding insects are likely to support a disease outbreak by transmitting LSDV from a clinical to a naïve animal, particularly biting flies such as *S.*

calcitrans. The R_0 calculations also highlight that, although there may be a significant subset of subclinical animals in an affected herd, they are likely to play at most a minor role in the transmission of the virus.

Discussion

This study describes a controlled experimental model of LSD that mimics disease features described in field outbreaks [2,4,8,11,12,37] and other experimental models [27,32]. Inoculated calves (both clinical and subclinical) were used to measure the acquisition (transmission from bovine to insect) and retention of LSDV by four potential vector species. These data were then used to estimate the risk of transmission by these species with the aim of providing evidence with which to inform decisions during the implementation of measures to control LSDV.

In our experimental model we observed that 37.5% of calves developed generalised LSD with the remaining 62.5% of calves classified as subclinical (no cutaneous nodules, positive qPCR in blood [27]). This attack rate of 0.37 is comparable to other experimental models with field strains of LSDV (0.57 [27] and 0.50 [32]). Reports of animals with subclinical LSD in the field is sparse, with an incidence of up to 31.3% reported [3]. The high detection of subclinical infection in our study may be a result of an intense sampling protocol (compared to the limited sampling of individuals during an outbreak investigation). Further investigation of the true incidence of subclinical LSD in field studies is warranted.

Cattle experimentally infected with LSDV, including in our study, have higher concentrations of LSDV in skin lesions than blood (Figures 1 & 2). In clinically infected animals we identified a relationship between the viral load in skin and blood and the proportion of insects positive for the virus, indicating both tissues are good predictors of the transmissibility of LSDV from donors to vector. Our study did not extend beyond 21 days post challenge however, and this observation may only be true during the initial stage of the disease when the viraemia is

detectable. Donors with different disease severity and therefore different levels of infectiousness would strongly influence the proportion of vectors which acquired virus. This finding may explain the discrepancies between experimental studies which have assessed the transmission of LSDV by vectors [21,29] when the infectiousness of the donors may have been different.

As reported in this study and others [27,31] LSDV can be detected in the blood of cattle prior to the appearance of skin lesions, 5-8 dpc. However, during this time, viraemia is relatively low and in our study few insects were positive for LSDV after feeding (Figure S6). Viraemia rises and peaks after the multifocal skin lesions appear (at around 7 dpc), and this is when the probability of transmission from bovine to insect starts to increase (Figure 1). The probability remains high while viraemia is high and when skin lesions are present. The appearance of skin lesions therefore marks the start of the risk period for virus transmission, and this means that rapid diagnosis and consequent implementation of control measures should be possible and effective at limiting onwards transmission [46,47]. In this study we were only able to follow the animals for 21 days post-challenge with the last exposure of blood-feeding insects to infected calves on day 19, and thus the period for transmission risk could not be established beyond this time point. Nevertheless, under controlled conditions [31], LSDV has been isolated up to 28 (blood) and 39 (skin) days post-challenge, and detected by PCR up to 91 days post-challenge (in skin biopsies). Therefore, LSDV uptake by vectors may occur beyond the reported period in our study.

We found that subclinical donors were much less likely than clinical animals to transmit virus to vectors (Table 1; Figure 3), indicating a substantially reduced role of subclinically infected animals in the transmission of LSDV. For some vector-borne diseases such as dengue fever, malaria, asymptomatic and preclinical individuals may be an important source of the pathogen for vectors and help maintain the transmission cycle [48,49]. The situation with LSDV appears to

be different. The viraemia in subclinical animals is low and skin lesions (representing the major viral load) are absent in these animals. Few vectors therefore acquire LSDV from subclinical cattle, and this reduces the chances of onward transmission to a susceptible host. This is the first time the relative contribution of subclinical infected cattle to onward transmission of LSDV has been quantified.

Lumpy skin disease virus can be mechanically transmitted by stable and horse flies [27,28] and mosquitoes [21]. Mechanical transmission of viruses by blood-feeding vectors can be influenced by their feeding mechanism, ecology and biting behaviour. Stable flies are aggressive feeders with a painful bite which leads to interrupted feeding and to more than one feeding events per day [50,51]. They are also known to regurgitate previous blood intakes while feeding. To penetrate the skin, stable flies rotate sharp teeth on their proboscis (5-8mm long) and form a pool of blood from which they feed [39]. *Culicoides* midges also disrupt the skin barrier using their proboscis (0.1-0.2 mm long). Midges serrate the skin using saw-like blades on their proboscis that cross over each other to produce a pool of blood [39]. Biting midges feed generally less frequently than stable flies as feeding is associating to their gonotrophic cycle (7-10 days, but as a temperature-dependent event it can be as short as 2-3 days) [52]. Mosquitoes do not produce pools of blood, instead they penetrate the skin "surgically" searching for a capillary with their proboscis of (1.5-2.0 mm long), accompanied by a pushing and withdrawing movement until it hits a capillary from which to withdraw blood [53]. Mosquitoes feeding on blood is also associated to their gonotrophic cycle, but multiple feedings have been reported in some species [54,55] Despite these variations in feeding behaviour, all four insect species acquire LSDV at the same rate, indicating that virus acquisition is not influenced by feeding behaviour.

All four insect species in the present study were able to acquire LSDV through feeding on clinical animals and to retain it for several days (Figure 3). In a small proportion of *Ae. aegypti* and *S. calcitrans* LSDV DNA was still present at 8 days post feeding, which was the longest

we investigated, thus longer retention cannot be ruled out. Similar to us, Chihota and co-authors [21], identified that *Ae. aegypti* mosquitoes feeding on animals with clinical LSD were able to acquire and retained the virus for up to six days, though the proportion of virus-positive insects decreased with days post feeding. They observed similar dynamics in *Cx. quinquefasciatus* and *Anopheles stephensi* mosquitoes when using a membrane-feeding system with a LSDV-infected bloodmeal, but when they fed *C. nubeculosus* and *S. calcitrans* on LSDV-infected calves they did not detect the virus beyond the day of feeding (*C. nubeculosus*) or the following day (*S. calcitrans*) [29]. However, our work has identified that LSDV can be retained longer than previously reported in *S. calcitrans* and *C. nubeculosus*, and that a virus decline post-feeding is only detectable for *C. nubeculosus*.

For LSDV, as for other chordopoxviruses including sheeppox virus, fowlpox virus and myxoma virus, the mode of vector-mediated transmission is assumed to be mechanical [21,56-59]. Our data and that of Chihota and co-authors support the theory that LSDV does not replicate in the insect (at least at detectable levels), but the retention of viral DNA in *Ae. aegypti* and *S. calcitrans* at similar levels to those acquired during feeding deserves further investigation [60].

Assessment of acquisition and retention of LSDV genome was performed in whole insect homogenates in our study, and further investigations into the location of virus within the insects were not possible. However an earlier study with *Ae. aegypti* [61] indicated that LSDV DNA persist longer in the head than in the thorax/abdomen. This is consistent with research that found myxoma virus was retained on the mouthparts of *Ae. Aegypti* mosquitoes up to 28 days post feeding [62]. The mechanism by which poxviruses persist for days on the mouthparts of vectors warrants further study.

The detection of LSDV in insect vectors in our study was based on the presence of viral DNA rather than infectious virus particles. Virus titration from homogenates of individual insects

was attempted however we were able to detect live virus only from pooled homogenates of *S. calcitrans* and of *Ae. aegypti* (data not shown), suggesting low numbers of infectious virions are present on each insect. In previous work live LSDV was detected in individual *Ae. aegypti* for up to 6 days following exposure to an infectious calf [21], and live goatpox virus up to 4 days in *S. calcitrans* [59].

The aim of the present study was to use the results of feeding four model vector species on LSDV-infected cattle to estimate parameters related to transmission. Given the large number of insects fed and tested (>3000), the resulting estimates for the probability of transmission from bovine to insect (including the relative risk of transmission from a subclinical animal and the dose-response) are robust, as indicated by the narrow credible intervals for these parameters (Tables 1 and 2). The estimates for the duration of virus retention (or, equivalently, the virus inactivation rate) are more uncertain (Table 1), which reflects difficulties in keeping insects alive to later days post feeding, especially *Cx. quinquefasciatus* (Table S1).

Although not assessed in the present study, we used data from previous transmission experiments [21,27] to estimate the probability of transmission of LSDV from insect to bovine. The small number of studies (and animals in each study) mean that the estimates for this parameter are uncertain, extremely so for *Ae. aegypti*, *Cx. quinquefasciatus* and *C. nubeculosus* (Table 1). This uncertainty is less important for *Cx. quinquefasciatus*, which is unlikely to be an important vector even if were able to transmit LSDV efficiently, but it makes it difficult to determine whether or not *C. nubeculosus* is likely be an important vector.

One previous study assessed the importance of different vector species by calculating R_0 for LSDV from its component parameters [45], based on data from two studies by Chihota and co-authors [21,29]. Despite different values for the underlying parameters, both this and the present study obtained similar estimates of R_0 for *S. calcitrans* (median: 15.5 vs 19.1) and for *Cx.*

quinquefasciatus (median: 0.8 vs 0.6), suggesting the former is likely to be an important vector and the latter is likely to be an inefficient vector of LSDV. The estimates of R_0 for *Ae. aegypti* differed between the studies (median: 7.4 vs 2.4), due to differences in estimates for the mean duration of virus retention (11.2 vs 5.9) and probability of transmission from bovine to insect (0.90 vs 0.22). This suggests that *Ae. aegypti* (or other *Aedes* spp. for which it could be considered a model) may be less efficient vector than previously assumed. Finally, there was a major difference between the studies in their estimates of R_0 for *C. nubeculosus*. The earlier study suggested this species is likely to be a less efficient vector (median R_0=1.8), but the present one suggests it could be an efficient one (median R_0=7.4). This discrepancy is principally related to markedly different estimates for the mean duration of virus retention (0.01 days vs 2.4 days). Moreover, the estimate of R_0 for this species is highly uncertain, largely as a consequence of uncertainty in the estimate of the probability of transmission from insect to bovine (Table 1). *Culicoides* spp. are ubiquitous on cattle farms [63,64] and, consequently, would represent a major transmission risk if they proved to be efficient vectors of LSDV. Hence, it is important that their ability to transmit virus to cattle is assessed.

Linking transmission experiments with mathematical modelling is an uncommon and powerful approach to create robust evidence which can inform policy makers involved in controlling the spread of infectious diseases. Here we have used this approach to investigate the transmission of LSDV, which has recently emerged as a significant threat to cattle in Africa, Asia and Europe. Our evidence indicates that *S. calcitrans* is likely to be an important vector species. It also suggests that *Culicoides* biting midges may be a more efficient vector species, than previously considered. Furthermore, we have demonstrated for the first time that subclinical infected cattle pose only very limited risk of onward transmission of LSDV to potential vectors. This evidence supports LSD control programmes which target clinically-affected cattle for rapid removal, rather than complete stamping-out of all cattle in an affected herd.

Methods

Experimental design

Ethical statement, housing and husbandry. The experimental study was conducted under the project license P2137C5BC from the UK Home Office according to the Animals (Scientific Procedures) Act 1986. The study was approved by the Pirbright Institute Animal Welfare and Ethical Review Board. Cattle were housed in the primary high containment animal facilities (Biosafety Level 3 Agriculture) at The Pirbright Institute. The husbandry of the animals during the study was described previously [9].

Challenge study and experimental procedures. Ten Holstein-Friesian male cattle (referred to as calves) were used for the study, which was done in two experimental replicates of five animals each. Details of the age and weight are provided in Table S5. Eight calves were challenged by intravenous and intradermal inoculation with a suspension of LSDV containing 10^6 PFU/ml [9]. More specifically, 2 ml were inoculated intravenously (jugular vein) and 1 ml was inoculated intradermally in four sites (0.25 ml in each site), two on each side of the neck. The remaining two calves were not challenged and were kept as non-inoculated in-contact controls. Calves were randomly assigned to either the control or challenge groups using a random number generator (excluding control calf 1, which was assigned as a control on welfare grounds following diagnosis with shipping fever pneumonia). The calves were kept for 21 days following the challenge; clinical scores were taken daily and serum, whole blood and skin biopsies [9] collected over the study period. The non-steroidal anti-inflammatory drug meloxicam (0.5 mg/kg body weight) (Metacam 20 mg/ml solution, Boehringer Ingelheim) was used when required on welfare grounds.

Insect exposure. Blood-feeding insects used in the study were: *Aedes aegypti* 'Liverpool' strain, *Culex quinquefasciatus* TPRI line (Tropical Pesticides Research Institute, obtained from

the London School of Hygiene and Tropical Medicine, London, UK), *Stomoxys calcitrans* (colony established in 2011 from individuals kindly provided by the Mosquito and Fly Research Unit, USDA Florida) and *Culicoides nubeculosus* [65]. All insects were reared at The Pirbright Institute under the following insectary conditions. *Ae. aegypti* were reared in pans of 300 larvae per pan, containing approximately 1 litre of water supplemented with fish food and housed at 28 °C, 70% relative humidity (RH) and 12:12 light/dark cycle. *Cx. quinquefasciatus* were reared in pans of 500-800 larvae per larval bowl, containing approximately 1.5 litre of water supplemented with ground guinea pig food and maintained at 26 °C, 50% RH and 16:8 light/dark cycle. *S. calcitrans* were reared in approximately 200 eggs per pot, incubated for 12-13 days in larval pots containing a ratio of 3:2:1 (powdered grass meal, water and corn flour) and a table spoon of yeast. *C. nubeculosus* were reared in approximately 10,000 larvae per pan containing 2 litres of dechlorinated water supplemented with oxoid broth and dried grass/wheat germ mix. Pots of 800 *Culicoides* pupae were made with males and females and allowed to emerge. Both *S. calcitrans* and *C. nubeculosus* were maintained in insectaries at 27 ± 2 °C, 50% RH, with a 16:8 light/dark cycle.

The age and sex composition of the insects at exposure was: female and male *C. nubeculosus* between 0-2 days post-eclosion, female *Cx. quinquefasciatus* and *Ae. aegypti* at 5-7 days post-eclosion and male and female *S. calcitrans* at an average of 4 days post-eclosion (range: 2-7 days). All adult insects were maintained on 10% sucrose and starved 18-24 hours before exposure to the calves.

All eight challenged calves, independent of clinical status, were exposed (for between 5 and 20 minutes) to each of the four insect species on days 5, 7, 9, 11, 15, 17 and 19 post challenge. At each time point each pot of insects was placed on a cutaneous nodule on a clinical animal and a corresponding area of normal skin on a subclinical animal. The hair of the calf at each feeding

site was clipped and/or shaved, and the insects were held in close contact with the skin of the calves in a container covered by a mesh. Around two hours after exposure, insects were anaesthetised under CO_2 and unfed individuals discarded and blood-engorged individuals collected.

For *Ae. aegypti*, *Cx. quinquefasciatus* and *C. nubeculosus* blood engorgement was assessed visually by the presence of blood in the abdominal cavity. However, *S. calcitrans* were all collected "blind" and blood engorgement was confirmed by the detection of the bovine cytochrome *b* gene using qPCR. Those individuals negative for cytochrome *b* at collection were removed from the analysis.

Samples from each insect group taken immediately following blood-feeding assessment (dpf 0) were stored at -80C, and the rest of the insects were maintained for 1, 2, 4 or 8 dpf. After this incubation period, surviving individuals were collected and stored at -80 ºC after the incubation period. Throughout incubation all insects were maintained on 10% sucrose solution, except *S. calcitrans* which were maintained with defibrinated horse blood (TCS Biosciences Ltd) after 2 dpf. All insects were kept in a temperature-controlled room at biocontainment level 3, with a 10:14 light/dark cycle. For the incubation, cardboard/waxed pots containing the insects were placed inside plastic boxes covered by a mesh which were kept under a plastic shelter to minimise temperature and humidity fluctuations. Temperature (mean: 24.8˚C; range: 22.4˚C – 26.4˚C) and RH (mean: 35.9%; range: 18.5% – 48.9%) of the room and of the incubation area were recorded approximately every 15 minutes (RF513, Comark Instruments and HOBO UX100-003, Onset).

Samples. Skin biopsies were weighed on a calibrated scale (EP613C Explorer Pro, OHAUS®) and homogenised in 500 µl high-glucose Dulbecco's modified Eagle's medium (41965, Life Technologies) supplemented with 5% foetal bovine serum (Antibody Production Services Ltd,

Bedford, UK), 100 U/ml penicillin and 100 µg/ml streptomycin (15140122, Life Technologies) and 2.5 µg/ml amphotericin B (15290026, Life Technologies) in a Lysing Matrix A tube (SKU 116910050-CF, MP Biomedicals) using a portable homogeniser (BeadBug Microtube Homogenizer, D1030, Benchmark Scientific Inc.). Whole insects were homogenised using a TissueLyser® (Qiagen, UK) with one or two steel beads of 3 mm (Dejay Distribution, UK) [66] in 200 µl Dulbecco's phosphate buffered saline (PBS, 14190094, Life Technologies), supplemented with penicillin-streptomycin and amphotericin B, as above. Bovine peripheral blood mononuclear cells (PBMC) were isolated from 7 ml of whole blood in EDTA diluted in PBS 1:1. The diluted blood was added to a SepMate™-50 centrifugation tube (Stemcell Technologies) under-layered with Histopaque®-1083 (Sigma-Aldrich). Tubes were centrifuged at 1500×g for 30 minutes, 20 °C with no brake. PBMCs were aspirated from the interface into PBS, washed three times with PBS at 1000×g for 10 minutes at 20 °C. After the final wash, cells were resuspended in 2 ml of RPMI medium (21875091, Life Technologies) supplemented with 10% foetal bovine serum, and penicillin-streptomycin as above. Blood collected without anticoagulants was allowed to clot, spun at 1000×g to 2000×g for 10 minutes in a refrigerated centrifuge and the serum collected. All samples were stored at -80 °C until analysed.

Laboratory assays. Nucleic acid from 200 µl of whole blood, PBMC suspension, skin homogenate or 100 µl of insect homogenate was extracted in a 96-well plate with the MagMAX™ CORE Nucleic Acid Purification Kit (Applied Biosystems, A32700) using protocol A in a KingFisher™ Flex Magnetic Particle Processor (Applied Biosystems) and eluted in 50 µl of buffer. qPCR for LSDV ORF074 detection was performed using a modification of the TaqMan assay described by Bowden *et al.* [67] with the Path-ID™ qPCR Master Mix (Life Technologies #4388644). Briefly, a 20 µl reaction was prepared using 5 µl of template, 400 nM of each primer, 250 nM of the probe and nuclease-free water to the final

volume. Samples were prepared in a 96-well plate and assayed using the Applied Biosystems™ 7500 Fast Real-Time PCR System with the program: 95 °C for 10 min, and 45 cycles of 95 °C for 15 s and 60 °C for 60 s. Tissue culture derived LSDV positive controls were included in the extraction plates, and the copy number of LSDV genome were quantified using gBlocks® Gene Fragments (Integrated DNA Technologies) to generate the standard curve (Text S2). Bovine blood intake by insects was determined using a SYBR green assay (PowerUp™ SYBR™ Green Master Mix, A25779, Life Technologies) for the detection of bovine mitochondrial cytochrome *b* as described by Van Der Saag *et al*. [68] with modifications (Text S3). The assay was performed in 10 µl reaction using 2 µl of template. This assay was specific for bovine cytochrome *b* and melt curve analysis was performed to confirm that only specific amplification occurred. For all qPCR assays a constant fluorescence threshold was set which produced a reproducible Cq values for the positive control samples between runs. A double antigen ELISA (ID Screen® Capripox, IDvet) was used to detect circulating antibodies for LSDV in serum samples following the manufacturer's protocol and analysed with the Multiskan FC Microplate photometer (Thermo Scientific™). Infectious virus titrations of PBMC suspension, insect and skin homogenate was performed by viral plaque quantification in MDBK cells.

Parameter estimation

Full details of parameter estimation are provided in Text S4 (those related to transmission of LSDV) and Text S5 (those related to latent and infectious periods of LSDV in cattle). Briefly:

***Probability of transmission from bovine to insect and virus inactivation rate*.** The numbers of insects positive for viral DNA after feeding on cattle infected with LSDV were used to estimate the probability of transmission from bovine to insect and the virus inactivation rate. The probability that an insect would be positive when tested is

$$p = \beta \exp(-\gamma t). \quad (1)$$

where β is the probability of transmission from bovine to insect, γ is the virus inactivation rate (i.e. the reciprocal of the mean duration of virus retention) and t is the time post feeding at which the insect was tested. Equation (1) combines the probability that an insect acquired virus (β; i.e. the probability of transmission from bovine to insect) and the probability that the insect retained the virus until it was tested at t days post feeding ($\exp(-\gamma t)$).

Differences amongst insect species in the virus inactivation rate and probability of transmission from bovine to insect and in the probability of transmission between subclinical and clinical animals were explored by comparing the fit of models in which these parameters did or did not vary with species or clinical status of the donor cattle. In addition, the dose-response relationship was investigated by allowing the probability of transmission from bovine to insect to depend on the level of viral DNA (in either blood or skin) in the donor animal, so that,

$$\log\left(\frac{\beta}{1-\beta}\right) = d_0 + d_1 V, \quad (2)$$

where d_0 and d_1 are the dose-response parameters and V is the level of viral DNA ($\log_{10}$ copies/ml in blood or $\log_{10}$ copies/mg in skin) in the donor when the insect fed. The different models were compared using the deviance information criterion [69]. The two proxy measures for infectiousness (i.e. level of viral DNA in blood or skin) were compared by computing posterior predictive *P*-values for each insect.

Probability of transmission from insect to bovine. Data on transmission of LSDV from insect to bovine were extracted from the published literature [21,27,29]. In these experiments, batches of insects (of the same species as used in the present study) were allowed to feed on an infected

bovine and then to refeed at later time points on a naïve recipient. The probability of the recipient becoming infected is

$$q = 1-(1-b\beta\exp(-\gamma T))^n, \quad (3)$$

where b is the probability of transmission from insect to bovine, β is the probability of transmission from bovine to insect, γ is the virus inactivation rate, T is the time interval between feeding on the donor and refeeding on the recipient and n is the number of insects which refed. The probability, (3), is the probability that at least one insect (out of the n refeeding) transmitted LSDV, where the probability that an individual insect will transmit is the product of the probabilities that it acquired the virus during the initial feed (β), retained it until refeeding ($\exp(-\gamma T)$) and that it subsequently transmitted LSDV at refeeding (b).

***Latent and infectious periods in cattle*.** Previous estimates for the latent and infectious periods of LSDV [45] were updated using the data on detection of LSDV in blood and skin collected during the present and other recently published studies [27,32]. In addition, the proportion of cattle that develop clinical disease following challenge was estimated using data extracted from the published literature [27,31,32,70,71] and the present study.

Bayesian methods. Parameters were estimated using Bayesian methods. For all analyses, samples from the joint posterior distribution were generated using an adaptive Metropolis scheme [72], modified so that the scaling factor was tuned during burn-in to ensure an acceptance rate of between 20% and 40% for more efficient sampling of the target distribution [73]. The adaptive Metropolis schemes were implemented in Matlab (version 2019b; The Mathworks Inc.) and the code is available online at https://github.com/SimonGubbins/LSDVAcquisitionAndRetentionByInsects. Two chains were allowed to burn-in and then run to generate an effective sample size of around 5,000

samples (assessed using the mcmcse package [74] in R (version 3.6.1 [75]). Convergence of the chains was assessed visually and using the Gelman-Rubin statistic provided in the coda package [76] in R [75]. Different models for the variation amongst species in virus inactivation and probability of transmission from bovine to insect (Tables S2 and S3) were compared using the deviance information criterion [69].

Basic reproduction number for LSDV

The basic reproduction number, denoted by R_0, is the "average number of secondary cases arising from the introduction of a single infected individual into an otherwise susceptible population" [44]. The basic reproduction number for LSDV is,

$$R_0 = \sqrt{\frac{b\beta m a^2}{(\mu+\gamma)}\left(p_C\frac{1}{r_C}+(1-p_C)\rho\frac{1}{r_S}\right)}, \quad (4)$$

where b is the probability of transmission from insect to bovine, β is the probability of transmission from bovine to insect, ρ is the relative risk of transmission from a subclinical compared to a clinical bovine, γ is the virus inactivation rate, p_C is the proportion of cattle that develop clinical disease and $1/r_C$ and $1/r_S$ are the mean durations of infectiousness for clinical and subclinical animals, respectively, all of which were estimated in the present study, and a, m and μ are the biting rate, vector to host ratio and vector mortality rate, respectively. The formal derivation of this expression, (4), is given in Text S6.

Replicated Latin hypercube sampling was used to compute the median and 95% prediction interval for R_0 for each insect species [45]. Parameters were sampled either from their marginal posterior distributions derived in the present study (b, β, ρ, γ, p_C, $1/r_C$ and $1/r_S$; see Tables 1 and S6) or uniformly from plausible ranges (a, m and μ; see Table S4). The mean duration of

infection for clinical animals ($1/r_C$) is based on detection of virus or viral DNA in skin, while that for subclinical animals ($1/r_S$) is based on detection of viral DNA in blood (Table S6).

Data availability

The authors declare that the main data supporting the findings of this study are available within the article and its Supplementary Information files.

Code availability

The code and the data used are available online for readers to access with no restriction at https://github.com/SimonGubbins/LSDVAcquisitionAndRetentionByInsects.

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Conflict of interest

None to report.

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Author contribution

P.M.B., S.G., K.E.D., P.C.H., J.A. and A.J.W. conceptualised the study; B.S.B., L.A., S.B., C.S. and C.B. contributed to the design of the experiments. S.B., W.L., A.V.D., Z.L., E.D., J.S. and M.W. prepared the insects; B.S.B., P.M.B., I.R.H., N.W. and K.E.D. carried out the cattle experiments including collection and preparation of samples. B.S.B. performed the laboratory assays and data acquisition. S.G. performed the mathematical modelling. B.S.B., S.G. and P.M.B. drafted the paper. All authors discussed the results and commented on the manuscript.

Figures

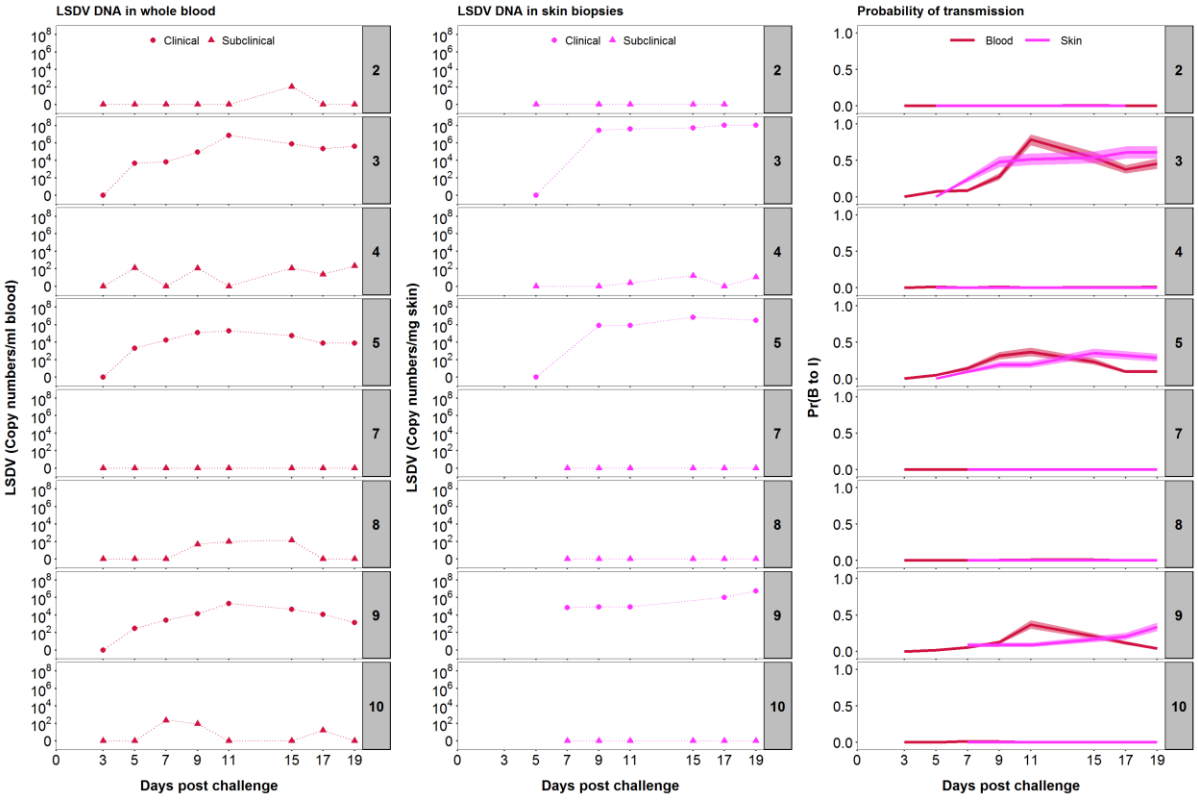

Figure 1. LSDV inoculation of eight calves results in a spectrum of infectiousness. Levels of viral DNA in blood (log_{10} copies/ml; first column) and skin (log_{10} copies/mg; second column) of the inoculated calves at different days post-challenge were quantified by qPCR. Based on the viral DNA levels in blood (red) or skin (magenta) the corresponding probability of transmission from bovine to insect ("infectiousness") was calculated using a dose-response relationship (third column). Lines and shading show the posterior median and 95% credible intervals for the probability, respectively.

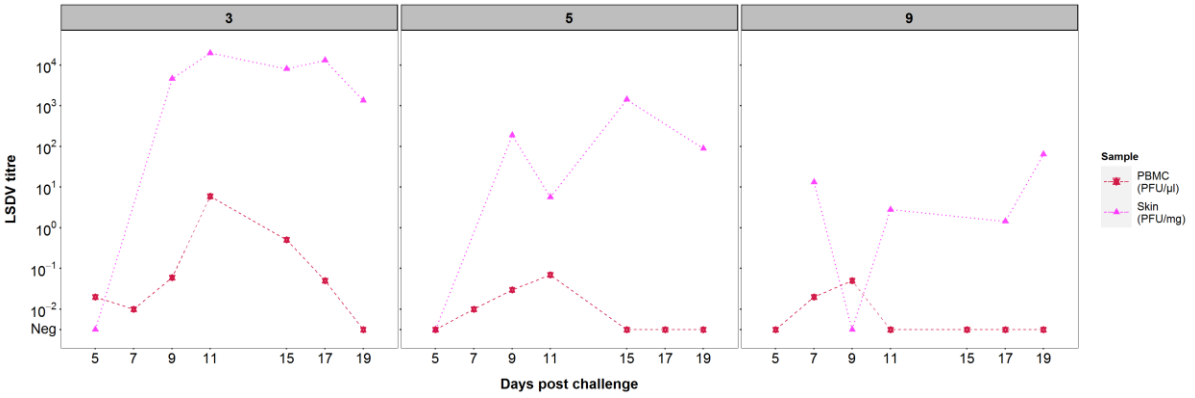

Figure 2. LSDV titres vary between three clinical animals but are consistently higher in the skin compared to blood. Levels of infectious lumpy skin disease virus (LSDV) in skin biopsies (PFU/mg of skin) (magenta triangles) and peripheral blood mononuclear cell (PBMC) fractions (PFU/µl suspension) (red stars) were quantified by titration on MDBK cells. Generalised skin lesions were first noted in all three animals at 7 days post challenge.

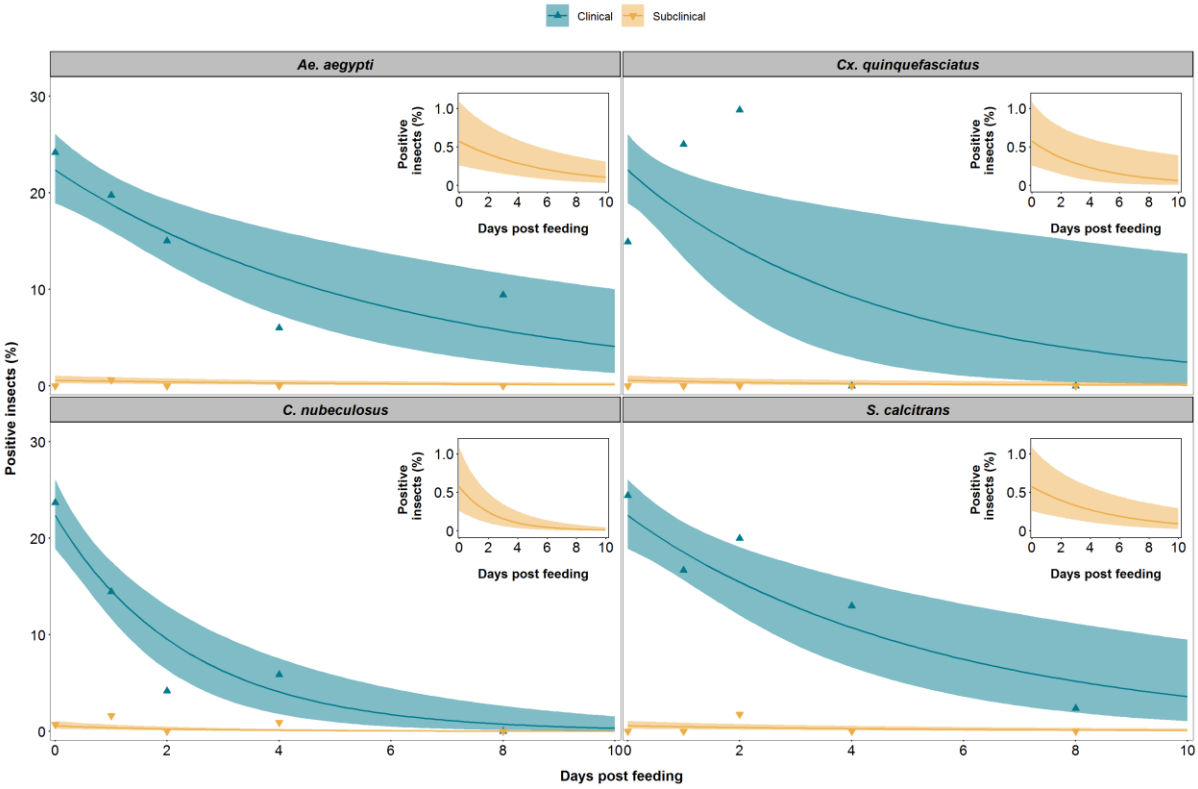

Figure 3. LSDV is retained in blood-feeding insects for up to 8 days post feeding. The proportion of blood-feeding insects positive for lumpy skin disease viral DNA after feeding on a clinical (green) or subclinically (yellow) animal is shown for the four species of insect: *Aedes aegypti*; *Culex quinquefasciatus*; *Culicoides nubeculosus*; and *Stomoxys calcitrans*. Each plot shows the observed proportion of positive insects (triangles) and the expected proportion of positive insects (posterior median (line), and 2.5th and 97.5th percentiles of the posterior distribution (shading)). The inset shows the expected proportion of positive insects after feeding on a subclinical animal.

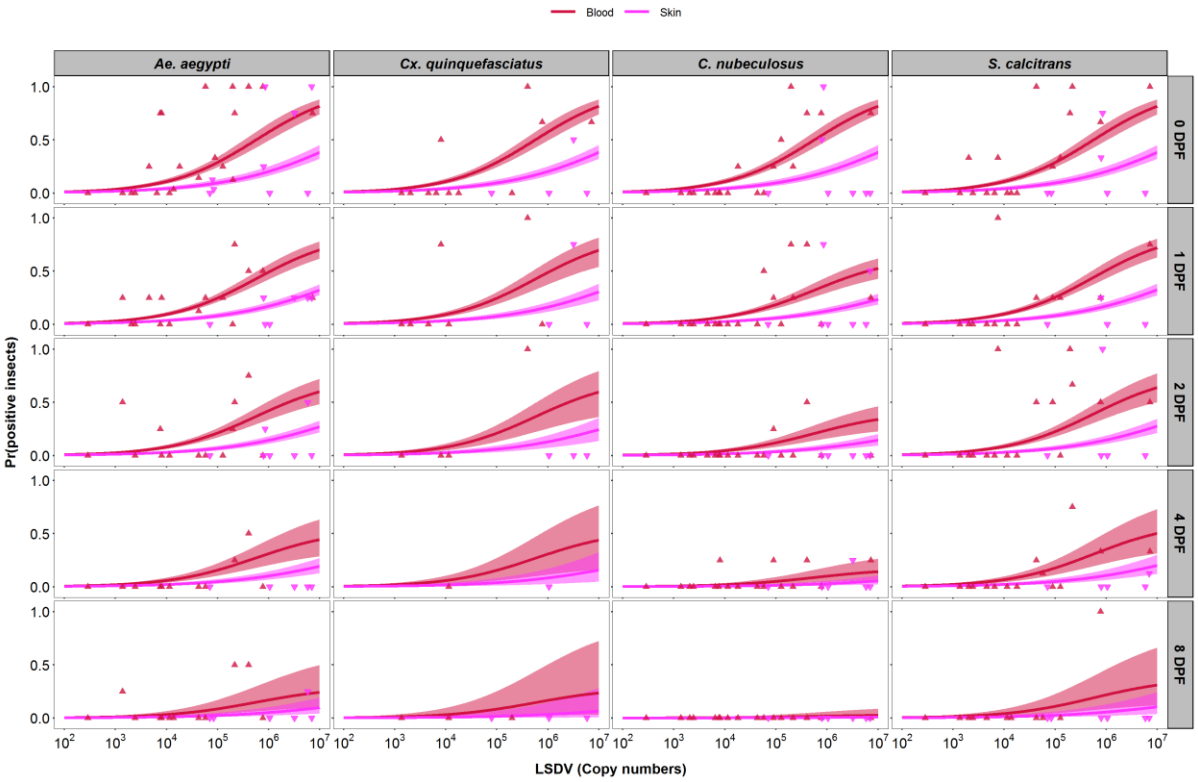

Figure 4. Levels of lumpy skin disease viral DNA in blood or skin are proxy measures of infectiousness. Each plot show the dose-response relationship between the probability of an insect being positive for lumpy skin disease virus (LSDV) DNA and the level of viral DNA in the blood (log_{10} copies/ml; red) or skin (log_{10} copies/mg; magenta) of the calf on which they fed. Four species of insect, *Aedes aegypti* (first column), *Culex quinquefasciatus* (second column), *Culicoides nubeculosus* (third column) or *Stomoxys calcitrans* (fourth column) were tested at 0, 1, 2, 4 and 8 days post feeding (rows). Plots show the observed proportion of positive insects (blood: red up triangles; skin: magenta down triangles) and the estimated probability of an insect being positive (posterior median (line) and 2.5th and 97.5th percentiles of the posterior distribution (shading: blood, red; skin: magenta)).

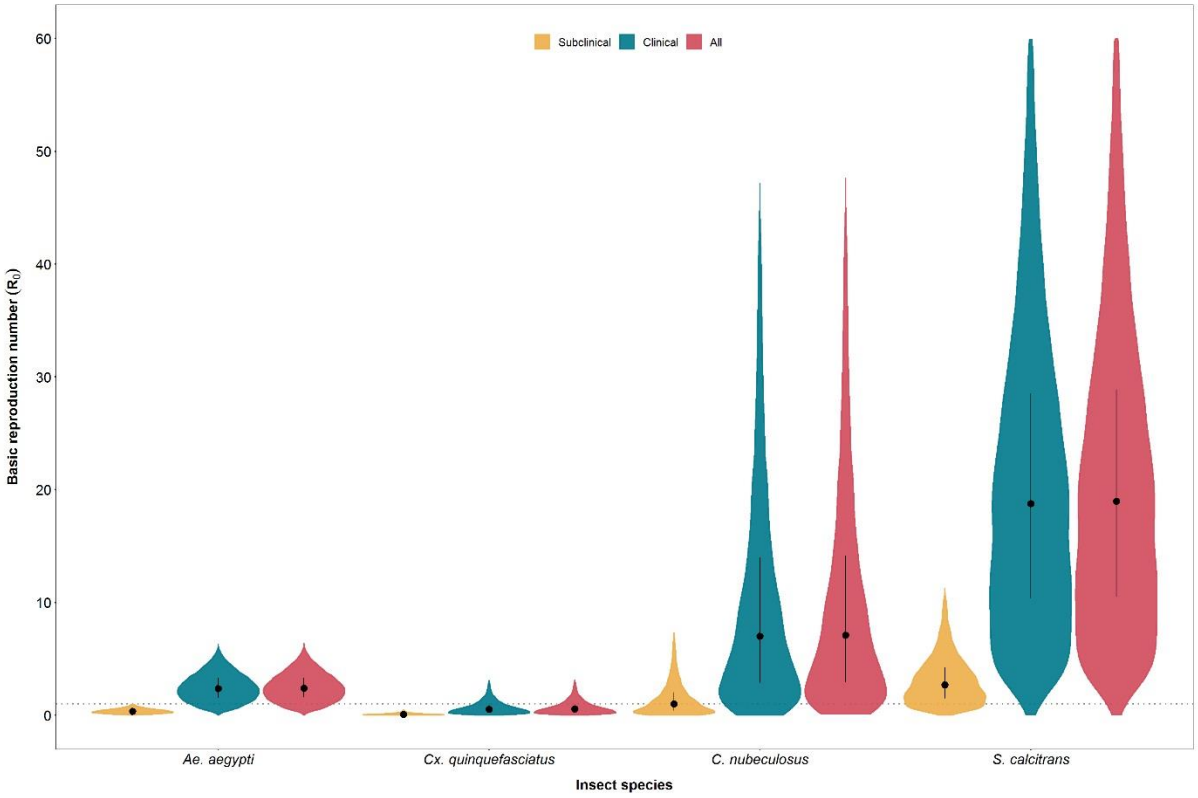

Figure 5. Basic reproduction number (R_0) for lumpy skin disease virus (LSDV) in calves when transmitted by *Aedes aegypti*, *Culex quinquefasciatus*, *Culicoides nubeculosus* or *Stomoxys calcitrans*. For each species, R_0 was calculated for subclinical calves only (yellow), clinical calves only (green) and both combined (red). Violin plots show the posterior median (black circle), interquartile range (black vertical line) and density (shape) for R_0 based on replicated Latin hypercube sampling (100 replicates with the range for each parameter subdivided into 100 steps).

Tables

Table 1. Parameters for the transmission of lumpy skin disease virus by four species of biting insect.

parameter	symbol	estimate*
probability of transmission from bovine to insect†		
clinical donor	β	0.22 (0.19, 0.26)
subclinical donor	$\rho\beta$	0.006 (0.003, 0.011)
relative risk of transmission from a subclinical compared to clinical bovine†	ρ	0.03 (0.01, 0.05)
virus inactivation rate (/day)	γ	
Ae. aegypti		0.17 (0.07, 0.29)
Cx. quinquefasciatus		0.22 (0.05, 0.51)
C. nubeculosus		0.42 (0.26, 0.64)
S. calcitrans		0.18 (0.08, 0.31)
mean duration of virus retention (days)	$1/\gamma$	
Ae. aegypti		5.9 (3.5, 13.4)
Cx. quinquefasciatus		4.5 (2.0, 22.0)
C. nubeculosus		2.4 (1.6, 3.9)
S. calcitrans		5.5 (3.2, 12.3)
probability of transmission from insect to bovine	b	
Ae. aegypti		0.56 (0.11, 0.98)
Cx. quinquefasciatus		0.11 (0.004, 0.73)
C. nubeculosus		0.19 (0.007, 0.91)
S. calcitrans		0.05 (0.02, 0.15)
basic reproduction number	R_0	
Ae. aegypti		2.41 (0.50, 5.22)
Cx. quinquefasciatus		0.55 (0.06, 2.37)
C. nubeculosus		7.09 (0.24, 37.10)
S. calcitrans		19.09 (2.73, 57.03)

* posterior median (95% credible interval)

† parameter does not differ amongst species

Table 2. Parameters for the dose-response relationship between levels of viral DNA in blood or skin and the probability of transmission of lumpy skin disease virus from bovine to insect.

parameter	estimate*	
	level of viral DNA in blood	level of viral DNA in skin
dose-response parameters		
intercept (d_0)	-6.89 (-7.74, -6.11)	-6.70 (-7.81, -5.76)
slope (d_1)	1.20 (1.03, 1.38)	0.89 (0.75, 1.06)

* posterior median (95% credible interval)

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