1   **The mechanical arthropod vector *Stomoxys calcitrans* influences the outcome of lumpy**

2   **skin disease virus infection in cattle**

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17   **Short title:** Arthropod-borne transmission of lumpy skin disease virus

18   Abstract

19   The poxvirus lumpy skin disease virus (LSDV) is the etiological agent of lumpy skin disease

20   (LSD), a severe disease of cattle and water buffalo that is characterised by numerous

21   necrotic cutaneous nodules. LSD is a rapidly emerging disease, spreading into and across the

22   Middle East, eastern Europe, and Asia in the past decade. The disease causes substantial

23   production and economic losses in rural communities and affected regions. LSDV is

24   mechanically transmitted by haematophagous arthropods including stable flies (*Stomoxys*

25   *calcitrans*), however our understanding of this mechanical transmission method is sparse. A

26   secreted saliva collection methodology using a modified artificial membrane feeding system

27   was optimised for *S. calcitrans* and used to collect and characterise secreted *S. calcitrans*

28   saliva. Saliva was mixed with LSDV and shown not to affect virus growth in primary bovine

29   fibroblasts. *S. calcitrans* saliva or spot-feeding by *S. calcitrans* was then incorporated into a

30   bovine in vivo experimental model of LSD to determine if either influenced disease

31   pathogenesis. *S. calcitrans* saliva resulted in fewer animals developing disease, however this

32   difference was not statistically significant. Spot-feeding with *S. calcitrans* prior to inoculation

33   did not alter the number of animals that developed disease or the overall severity of disease

34   however disease progression was accelerated as demonstrated by the appearance of

35   cutaneous nodules, detection of viral DNA in the blood stream, and production of

36   neutralising antibodies. This shows that *S. calcitrans* influence disease kinetics through co-

37   incident bite trauma and/or saliva deposition. This increases our understanding of LSDV

38   pathogenesis and highlights the overlooked importance of mechanical vectors in pathogen

39   transmission.

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41 Author summary

42 Insect vectors are important conduits for the transmission of pathogens that cause diseases

43 such as Zika, dengue, malaria, and lumpy skin disease. Biological vector-borne transmission

44 incorporates a replication phase for the pathogen in the insect, whereas no replication

45 occurs in the vector during mechanical transmission. When the insect bites the host it

46 inoculates a pathogen whilst also delivering arthropod-derived factors such as saliva

47 components and causing tissue trauma through biting and probing. Arthropod saliva and/or

48 bite trauma have been shown to enhance the speed and severity of disease following

49 inoculation with a range of biologically transmitted viruses. This study examined if this was

50 true also for the mechanically transmitted pathogen lumpy skin disease virus (LSDV). LSDV is

51 a neglected pathogen that causes severe systemic disease in cattle and is transmitted

52 mechanically by the stable fly *Stomoxys calcitrans*. Using an experimental bovine model of

53 LSD, we found that disease occurred more rapidly when virus was delivered in association

54 with the bites of uninfected flies. This work has increased our knowledge of lumpy skin

55 disease virus transmission, and the discovery that disease outcome can be impacted by

56 previously overlooked mechanical insect vectors should prompt further investigation into

57 this mechanism of transmission.

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Introduction:

Arthropod-borne diseases such as dengue, Zika, leishmaniasis, malaria, bluetongue, Rift Valley fever and lumpy skin disease exert a major burden on human and animal health. A number of these arthropod-borne diseases are increasing in incidence and geographical range and moving into temperate zones, representing a growing threat to a warming world (1, 2, 3).

Lumpy skin disease (LSD) is an emerging, high consequence disease of cattle and water buffalo. The causative agent is lumpy skin disease virus (LSDV), genus *capripoxvirus*, family *Poxviridae*. LSD is characterised by multifocal necrotic skin lesions, fever, weight loss, milk drop and death. Morbidity is approximately 10% and mortality 1% (4, 5, 6, 7, 8). LSD contributes to food insecurity and rural poverty and causes substantial economic loss to affected regions through loss of food and draught animal power, lost production, cost of control measures, and trade restrictions (9, 10, 11). LSD was first described in cattle populations in southern Africa in the early 20th century, from where it has spread steadily northwards into northern Africa and the Middle East (12). In 2013 the disease began a rapid period of spread known as the Eurasian LSD epidemic, spreading through cattle populations in Europe, Russia and Asia. Despite its swift emergence and broad impact LSDV remains a neglected pathogen, with fundamental knowledge gaps preventing the design and implementation of effective control programmes.

LSDV is transmitted by haematophagous arthropod vectors. A number of other poxviruses are also transmitted by blood-feeding vectors including the leporipoxviruses myxoma virus (13, 14) and Shope fibroma virus (15, 16), the avipoxvirus fowlpox virus (17, 18), and the capripoxviruses goatpox virus and sheeppox virus (19, 20). LSDV has been transmitted from

82   a clinical to naïve animal under experimental conditions by *Aedes aegypti* mosquitoes (21,

83   22), *Amblyomma hebraeum* and *Rhipicephalus appendiculatus* ticks (23, 24), and *S.*

84   *calcitrans* flies (22, 25, 26). LSDV can be acquired from cutaneous nodules by a range of

85   insect species, then retained on the vector mouthparts for days before being deposited in a

86   recipient animal (27, 28). Poxviruses, including LSDV, are believed to be transmitted

87   mechanically with no viral replication taking place in the arthropod vector. This is in contrast

88   to biologically transmitted pathogens which undergo replication in the vector.

89   The bite of an arthropod vector inoculates a pathogen into the host whilst also delivering

90   arthropod-derived factors such as saliva components, and causing tissue trauma through

91   biting and probing. Arthropod saliva and/or bite trauma have been shown to enhance the

92   kinetics and severity of disease caused by a range of biologically-transmitted pathogens

93   including West Nile virus (29, 30, 31), dengue virus (32, 33), bunyamwera virus (34), Semliki

94   Forest virus (34, 35) and leishmania (36). The mechanisms underpinning arthropod disease

95   enhancement include the *Aedes aegypti* venom allergen-1 which enhances viral replication

96   in macrophages and dendritic cells (37), and *Ae. aegypti* protein sialokinin which increases

97   blood vessel permeability and availability of virus permissive cells (38). While arthropod

98   enhancement of disease has been shown for a number of biologically-transmitted

99   pathogens, this potentiation has not been described for disease caused by mechanical

100   vectors.

101   *S. calcitrans*, also known as stable flies, are telmophagous filth flies and a common

102   arthropod pest particularly of cattle. They are pool feeders with coarse labellar teeth that

103   create a haematoma in the skin from which they feed (39). Stable flies are found across the

104   world (40) and are most active from spring to mid-autumn with populations peaking in

105   summer (41, 42, 43, 44). *S. calcitrans* tend to remain in areas with a high concentration of

106   cattle, however research has shown that they have the capacity for long range flight up to

107   225 km (45). *S. calcitrans* feed quickly, engorging after 3-4 minutes although feeds are often

108   interrupted (46). Their interrupted feeding and refeeding behaviour make *S. calcitrans* ideal

109   vectors for pathogen transmission. They are known to be involved in the transmission of

110   equine infectious anaemia virus (47), West Nile virus (48) and trypanosomes (49). They are

111   also suspected to be carriers of Rift Valley fever virus (50) and bovine herpes virus (51). *S.*

112   *calcitrans* have also been proposed as vectors of LSDV (22, 25, 26, 27, 52, 53).

113   The interactions of LSDV and the bovine skin in the context of an arthropod bite are

114   unexplored. We hypothesised that, in an analogous fashion to biologically transmitted

115   arthropod pathogens, the saliva and/or bite trauma of a *S. calcitrans* fly would enhance the

116   development of LSD after inoculation of virus into the skin of an animal.

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119   Results:

120   **Collection and characterisation of *S. calcitrans* secreted saliva**

121   Previous research has described studies of *S. calcitrans* salivary gland extract (SGE) (54, 55,

122   56). SGE often contains non-salivary structural contaminants therefore we developed a

123   method for collecting secreted *S. calcitrans* saliva to minimise contamination. The method

124   adapted a protocol for collecting secreted saliva from *Culicoides* midges using a heated

125   membrane feeding system (Hemotek™) incorporating a polyvinylidene fluoride (PVDF)

126   membrane (57, 58). A similar artificial membrane feeding system containing 1mM

127   ATP/endotoxin free water solution was used to collect *S. calcitrans* secreted saliva (**Figure**

128   **1**). A pot of 25 flies was fed on the PVDF membrane within the feeding system, with each

129   feeding session lasting 10-15 min. Each PVDF membrane was fed on by 3-4 pots of flies

130   sequentially before being retrieved and stored at 4°C in PBS. Up to 144 PVDF membranes

131   were fed on during each saliva collection procedure. At the end of the procedure the

132   secreted saliva was collected from the membranes by washing, and concentrated to

133   between 107-380 µg/mL in a total volume of approximately 1000 µL.

134   Proteins in the concentrated *S. calcitrans* secreted saliva were separated by SDS-PAGE and

135   visualised using silver stain. Six protein bands were consistently visible in each preparation

136   (**Figure 2A**), with the most abundant protein detected at 23-24kDa. Mass spectrometry was

137   used to identify the proteins present within the *S. calcitrans* secreted saliva (**Table 1**). This

138   revealed that the secreted saliva contained types of proteins typical of haematophagous

139   arthropod sialomes, such as endonucleases, serine proteases and carboxylic ester

140   hydrolases (59, 60, 61, 62, 63). The most abundant proteins (determined by count of

141   spectra) were an SCP domain-containing protein and antigen-5. These two proteins have a

142   very high amino acid sequence similarity and may be the same protein. Antigen-5 is a

143   member of the CAP (cysteine-rich secretory proteins, antigen-5 and pathogenesis-related

144   protein 1) superfamily and in the saliva of haematophagous insects has been shown to

145   function as antioxidant, to inhibit collagen-induced platelet aggregation and neutrophil

146   oxidative burst in hosts (64). Salivary antigen-5 was also found to be highly antigenic and to

147   induce antibody-mediated immune responses in repeatedly-exposed hosts (65), and specific

148   immune responses and immunoglobulin binding in cattle (56).

149   Table 1. Proteins identified in secreted *S. calcitrans* saliva. Proteins identified in at least two out of
150   three independent preparations were included. Hits were sorted by average abundance determined
151   by spectra counts. Duplicate hits with alternative UniProt accessions are marked in red.

UniProt Accession	UniProt Annotation	Vectorbase Annotation	Average protein mass (Da)	Average abundance (Spectra)
A0A1I8PC31	SCP domain-containing protein		29069	443
Q7Z0B5	Antigen 5		28940	441
A0A1I8Q1A7	Uncharacterized protein		142581	57
A0A1I8PUN2	Uncharacterized protein	Serine protease 53-like	79843	46
A0A1I8PL64	Uncharacterized protein	Protein starmaker-like	55798	33
A0A1I8Q6W6	Alpha-amylase		55421	28
A0A1I8PV60	Uncharacterized protein		45452	22
A0A1I8P4Z0	Uncharacterized protein		35563	22
A5WXR8	Putative 15.6 kDa secreted salivary gland protein		17541	14
A0A1I8PSA1	Lipocln_cytosolic_FA-bd_dom domain-containing protein		21441	12
A0A1I8PU36	Peptidase S1 domain-containing protein		26684	12
A0A1I8PR12	Transferrin		71124	11
A0A1I8Q544	Peptidase S1 domain-containing protein		27388	11
A5WXR7	Putative 13.7 kDa secreted salivary gland protein		15838	9
A0A1I8NWI4	Uncharacterized protein		18501	9
A0A1I8P7C1	Carboxylic ester hydrolase		64867	8

A0A1I8Q0V7	Lipase domain-containing protein		42932	8
A0A1I8PQD6	Uncharacterized protein	Murinoglobulin-2-like	165805	8
A0A1I8PQI3	Uncharacterized protein	Murinoglobulin-2-like	166711	8
A0A1I8PU39	Uncharacterized protein	Actin-5C	41822	8
A0A1I8NR78	Uncharacterized protein	Transmembrane protease serine 9	52998	6
A0A1I8PGD9	Uncharacterized protein	Venom serine protease	44853	6
A0A1I8Q114	Carboxylic ester hydrolase		64782	6
A0A1I8PI66	Uncharacterized protein		13078	5
A0A1I8Q1H9	P/Homo B domain-containing protein		172722	5
A0A1I8Q1I4	P/Homo B domain-containing protein		177770	5
A0A1I8QE72	Endonuclease_NS domain-containing protein		43497	5
A0A1I8NTR2	Peptidase S1 domain-containing protein		27598	5
A0A1I8NQD5	C-type lectin domain-containing protein		21272	4
A0A1I8PZ42	Uncharacterized protein	Protein yellow	48527	4
A0A1I8PZ57	Uncharacterized protein	Protein yellow	40878	4
A0A1I8PLH0	Uncharacterized protein	Glycerophosphocholine phosphodiesterase GPCPD1	77514	4
A0A1I8PLL7	Uncharacterized protein	Glycerophosphocholine phosphodiesterase GPCPD1	75000	4
A0A1I8PLN8	Uncharacterized protein	Glycerophosphocholine phosphodiesterase GPCPD1	77642	4
A0A1I8PLP5	Uncharacterized protein	Glycerophosphocholine phosphodiesterase GPCPD1	74872	4
A0A1I8PQF4	Uncharacterized protein	Murinoglobulin-2-like	166546	4
A0A1I8PQL1	Uncharacterized protein	Murinoglobulin-2-like	165871	4
A0A1I8PUP6	C-type lectin domain-containing protein		19344	4
A0A1I8P599	Peptidase S1 domain-containing protein		28478	3
A0A1I8Q0V1	Uncharacterized protein	Tyrosine-protein phosphatase non-receptor type 23	39376	3
A0A1I8Q5Q1	Uncharacterized protein	Protein 5NUC-like	63674	3
A0A1I8P1G4	Peptidase S1 domain-containing protein		27229	2

152

153 To further characterise the composition of the *S. calcitrans* sialome functional enrichment

154 analysis was performed (Table 2). In accordance with the biological function of saliva,

155 overrepresented molecular functions were serine-type peptidase activity-related functions.

156 "Extracellular region" was overrepresented as cellular component, indicating that the

157 dataset contained several secreted proteins. Once characterised, the *S. calcitrans* secreted

158 saliva was taken forward to characterise the influence of *S. calcitrans* on LSDV *in vitro* and *in*

159 *vivo*.

160 Table 2. Functional enrichment analysis of proteins identified in *S. calcitrans* saliva. UniProt accession
161 numbers listed in Table 1 were queried against the *S. calcitrans* genome using the g:GOSt on the
162 g:Profiler server. Alternative accession numbers for the same protein were automatically removed
163 prior to analysis (highlighted in red in Table 1). The g:SCS algorithm in g:GOSt was selected for multiple
164 testing correction of p-values with an experiment-wide p-value of 0.05 being considered statistically
165 significant.

GO:MF				
Term Name	Term ID	p (adj)	Enrichment score [$-\log_{10}$ p (adj)]	Sialome proteins
serine-type endopeptidase activity	GO:0004252	4.18E-05	4.379	9
serine-type peptidase activity	GO:0008236	8.32E-05	4.080	9
serine hydrolase activity	GO:0017171	8.32E-05	4.080	9
endopeptidase activity	GO:0004175	0.004	2.388	9
hydrolase activity	GO:0016787	0.014	1.861	17
GO:CC				
Term Name	Term ID	p (adj)	Enrichment score	Sialome proteins
extracellular region	GO:0005576	0.011	1.966	3

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168 **S. calcitrans saliva does not influence the replication of LSDV *in vitro*.**

169 Arthropod saliva has been shown to influence the infectivity of viruses in mammalian cell

170 culture, for example *Culicoides* saliva proteins reduced BTV-1 infectivity 2-6 fold in BHK cells

171 but increased infectivity in KC cells (66), Vesicular stomatitis virus growth in *Ae. triseriatus*

172 SGE-treated mouse fibroblast cells was significantly increased compared to untreated

10

173   controls (67), and crude mosquito saliva inhibited DENV infection of human dendritic cells

174   (68). Therefore, the impact of *S. calcitrans* saliva on LSDV viability *in vitro* was assessed using

175   a one-step growth curve on bovine primary fibroblasts. Varying concentrations of secreted

176   *S. calcitrans* saliva or PBS were combined with LSDV, incubated at room temperature (RT)

177   for 2 h, and then used to infect bovine primary fibroblasts at a MOI of 5. As shown in **Figure**

178   **2B**, the addition of *S. calcitrans* saliva to LSDV did not result in any difference in the

179   replication of LSDV when compared to the PBS control. This indicates that *S. calcitrans* saliva

180   does not influence the kinetics or magnitude of LSDV replication in vitro. The influence of *S.*

181   *calcitrans* in vivo was then studied.

182   **Spot-feeding by *S. calcitrans* prior to viral inoculation influences the kinetics of LSD**

183   An experimental bovine model of LSD was used to investigate whether *S. calcitrans* saliva or

184   bite trauma influences the infectivity, kinetics, or severity of LSD. Thirty male Holstein-

185   Friesian calves were randomised into three groups of 10 then challenged with LSDV using

186   three different inoculation routes. Group 1 were inoculated with $1{\times}10^6$ pfu of LSDV

187   inoculated intradermally into the skin via 20 "microdoses" of 100 µL. Ten microdoses were

188   injected into both the left and right dorsal flanks of the calf just caudal to the scapula. These

189   100 µL "microdoses" were designed to imitate inoculation of virus by *S. calcitrans*. Group 2

190   were inoculated with $1{\times}10^6$ pfu of LSDV mixed with 10 µg of *S. calcitrans* secreted salivary

191   protein, delivered in a total volume of 2 mL in the same 20 x 100 µL intradermal microdose

192   format (**Figure 3A**). Group 3 were spot-fed with approximately 25 adult virus-negative,

193   laboratory reared *S. calcitrans* flies on the left and right craniodorsal flanks of the calves for

194   5 min (**Figure 3B**), before $1{\times}10^6$ pfu LSDV was inoculated intradermally in 10 microdoses into

195   each feeding site. Examination of the flies immediately post-feeding revealed reddened

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196   abdomens (**Figure 3C**), consistent with biting and intake of blood. Calves in all three groups

197   were monitored and samples collected for 28 days after inoculation.

198   Clinical disease, defined as the development of one or more cutaneous nodules distant from

199   the site of inoculation, occurred in 16 of the 30 calves. Six out of ten animals inoculated with

200   virus alone (group 1), three out of ten animals inoculated with a mixture of virus and *S.*

201   *calcitrans* saliva (group 2), and seven out of ten animals spot-fed by flies then inoculated

202   with virus (group 3) developed clinical disease (**Figure 4**). These frequencies do not differ

203   between the groups (Fisher exact test: P=0.27). The gross pathology was similar in all three

204   groups with firm, slightly raised cutaneous lesions first detected 5-7 dpi and developing

205   from papule to vesicle to nodule before becoming necrotic by approximately 12 dpi. This

206   was consistent with previous bovine experimental models of LSD (69). Severely affected

207   calves developed over 100 cutaneous nodules. The clinical calves also developed

208   lymphadenopathy and pyrexia. Severely affected calves were euthanased between 9-13

209   dpi.

210   Fourteen of the 30 calves inoculated with LSDV did not develop any cutaneous nodules

211   distant from the inoculation site and were classified as non-clinical. These calves did exhibit

212   an increase in size of pre-scapular and pre-femoral lymph nodes from 3-15 dpi and, briefly, a

213   raised temperature around 5-9 dpi. Overall, this study shows that the "microdose" method

214   of inoculating LSDV produces lumpy skin disease in 60-70% of challenged calves.

215   We then examined whether the addition of *S. calcitrans* saliva or spot feeding by *S.*

216   *calcitrans* altered the infectivity of LSDV or the course of disease. The outcome of

217   inoculation – disease or no disease – differed between the groups as described above, with

218   only 3 calves in group 2 developing clinical disease, compared to 6 in group 1 and 7 in group

219   3. However this trend did not reach statistical significance (using the log-rank test, *P*=0.154,

220   **Figure 4**). No significant differences were noted between the three groups when the

221   temperature, lymphadenopathy, heart rate, respiration rate, and demeanour were

222   compared (data not shown). However significantly more cutaneous nodules per clinical

223   animal in group 3 were present at 7 dpi compared to group 1 (mixed effects model, *P*=0.013,

224   **Figure 5**), indicating that spot-feeding ahead of virus inoculation accelerates the appearance

225   of disease. This accelerated disease was also evident in a clinical scoring system that takes

226   into account the key signs of disease (**Table 3**). The clinical animals in group 3 reached a high

227   severity score more quickly than groups 1 and 2, and significantly higher than group 1 at

228   7dpi (*P*=0.010, mixed effects model, **Figure 6**). The clinical calves in group 2 had a lower

229   disease severity over the course of the experiment, but the variation between the three

230   clinical cattle in this group prevented any statistical significance from being reached. Overall,

231   these results indicate that fly feeding prior to viral inoculation accelerates the kinetics of

232   LSD.

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234   **Table 3**. Clinical scoring system for lumpy skin disease

Clinical sign	Score
Nodules	
0	0
1-20	1
21-40	2
41-60	3
61-80	4
80+	5
Lymph nodes	
Average of all lymph nodes rounded to nearest whole number	
0	0
>0.5-1	1
>1-2	2
>2-3	3
Temperature	
At or below baseline	0
>0 to 0.5 above baseline	1
>0.5 to 1 above baseline	2
>1 to 1.5 above baseline	3
>1.5 above baseline	4
Behaviour (isolation/slow/lethargic/abnormal posture)	
No abnormal behaviour	0
Animal exhibits one abnormal behaviour	1
Animal exhibits multiple abnormal behaviours	2
Anorexia	
No anorexia	0
Animal not eating	2
Oedema/lameness	
No oedema	0
Oedema in one area	1
Oedema in two or more areas	2
Ocular/nasal discharge/salivation	
No discharge	0
Discharge	1

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236   **Spot feeding results in quicker viraemia and more virus in blood**

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237   Fly feeding prior to inoculation of LSDV resulted in cutaneous nodules appearing more

238   rapidly, while inoculation of *S. calcitrans* secreted saliva along with the virus reduced the

239   number of calves that developed disease. To explore if these changes correlated with LSDV

240   replication the presence of virus in the blood and skin was quantified.

241   The amount of viral DNA in the blood of calves in each treatment group was determined by

242   PCR assay targeting viral gene LSDV074. As expected, all 16 clinical animals developed

243   viraemia with increasing amounts of viral DNA (inferred by decreasing cycle threshold

244   numbers) detected in the blood between 5 and 11 dpi. In contrast, viral DNA was detected

245   in the blood of non-clinical animals only intermittently and at low amounts (**Figure 7** A-C).

246   Median C_T values were significantly (P<0.05) lower for clinical compared with non-clinical

247   calves at 9 and 11 dpi (group 1), at 7, 11 and 13 dpi (group 2), and at 7, 9 and 11 dpi for

248   group 3. Statistical analysis of the data revealed that median C_T values did not differ

249   significantly (*P*>0.19) between non-clinical calves infected by different methods at any time

250   point.

251   The amount of viral DNA in the blood of clinical calves was compared across the three

252   groups. Viraemia was detected earlier in the calves in group 3 at 3 and 5dpi compared to 5

253   and 7 dpi for the calves in groups 1 and 2. One calf from group 3 was positive at 3 dpi, and 5

254   calves from group 3 were positive at 5 dpi. In comparison no calves from group 1 were

255   positive on either 3 or 5 dpi, and only one calf from group 2 was positive at 5 dpi. C_T values

256   were significantly (*P*=0.02) lower at 5 dpi for clinical calves from group 3, infected by

257   microdoses of LSDV after feeding by uninfected *S. calcitrans* (median C_T=38.8) compared

258   with those in group 1 that had been infected by microdoses of LSDV only (median C_T=42).

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259   This reveals that spot feeding with *S. calcitrans* flies accelerates the development of

260   viraemia in LSD.

261   To quantify the amount of infectious virus present in the blood of the inoculated calves,

262   viral titrations were carried out on the 63 blood samples that contained viral DNA. Of these,

263   52 samples were from clinical calves and 11 samples from nonclinical calves. Live virus was

264   detected in eighteen samples, all from clinical animals (animals marked (C) in **Figure 7D**).

265   The amount of virus detected was low, ranging from 10-1000 pfu/mL, consistent with

266   previous experimental studies of LSD (69, 70). Live virus was recovered from the blood of

267   two calves from group 1, two calves from group 2, and six calves from group 3. The

268   maximum amount of virus detected in blood samples from groups 1 and 2 was 100 pfu/mL,

269   however higher titres were present in the blood samples from calves in group 3, up to 1000

270   pfu/mL.

271   At 5, 11 and 16 dpi a 2mm skin biopsy was taken from each of the calves. The biopsies were

272   taken from normal skin of all calves at 5 dpi, from cutaneous nodules on the clinical animals

273   at 11 and 16 dpi, and from normal skin of nonclinical animals at 11 and 16dpi. DNA was

274   extracted from the biopsy and viral genomic DNA quantified using a qPCR targeting

275   LSDV068. This PCR incorporated a standard curve to enable quantification of the amount of

276   viral DNA present. No viral DNA was detected in any of the skin samples collected at 5dpi,

277   and no viral DNA was detected in any of the biopsy samples taken from the skin of a

278   nonclinical calf at 11 and 16 dpi. In contrast, all samples taken from the nodules of clinical

279   calves at 11 dpi were positive (5/5 clinical animals in group 1, 2/2 clinical animals in group 2,

280   and 6/6 clinical animals in group 3). The viral genome copy numbers present in each

281   microbiopsy at 11 dpi were similar across the inoculation groups (**Figure 8**), with the

282   exception of CV7 which contained a lower amount of viral DNA. Overall, there was no

283   evidence to suggest that the amount of viral DNA in the nodules of clinical animals was

284   impacted by *S. calcitrans* saliva or spot feeding.

285   ***S. calcitrans* saliva and fly feeding does not influence the blood cell population changes in**

286   **response to LSDV inoculation**

287   The immune response of the challenged calves was studied by examining changes in blood

288   cell populations over time and in the production of neutralising antibodies. The number of

289   mononuclear leukocytes in the venous blood of the calves at -2, 3, 7 and 9 dpi were

290   examined using a flow cytometry panel. The panel incorporated counting beads to enable

291   quantification of absolute numbers of monocytes, T cells, B cells and γδ T cells (**Figure 9**).

292   The cell counts were then presented as a percentage change from the preinfection baseline.

293   Changes in cell numbers were broadly similar across the three groups of calves. A reduction

294   in all four cell subsets was seen at 3 and 7 dpi when compared to -2 dpi. At 9 dpi the number

295   of γδ T cells continued to drop, however the T and B cell numbers increased. At 9 dpi the

296   number of monocytes dropped in group 1, plateaued in group 2, and increased in group 3.

297   There were no statistically significant differences in the number of blood cells between the

298   three groups at any time point.

299   The data were then re-analysed, comparing the clinical and nonclinical calves from all three

300   groups. The absolute numbers of monocytes, T cells, B cells and γδ T cells were remarkably

301   similar in both groups of calves, with the only difference being the number of monocytes at

302   9 dpi. At 7 dpi the number of monocytes in the clinical calves was 62.3% of the -2 dpi

303   number, and 62.6% of the -2 dpi number in the non-clinical calves. At 9 dpi the number of

304   monocytes in the clinical calves had reduced even further to 45.9% of pre-challenge

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305   numbers, but had increased in the non-clinical calves to 81.5%. There were no significant

306   differences ($P>0.39$) in percentage of CD14$^+$ cells at any time point with the exception of 9

307   dpi ($P=0.009$), where the median percentage was lower in clinical (33.8%) compared with

308   non-clinical (62.2%) calves.

309   **Fly-fed and inoculated animals produced antibodies more rapidly.**

310   A fluorescent virus neutralisation test (FVNT) was used to analyse the ability of sera

311   collected at -2, 7, 11, 16 and 27 dpi to completely neutralise LSDV (71). The highest dilution

312   of sera which completely neutralised the virus is shown in **Table 4** with calves that

313   developed clinical disease highlighted in red. The data is plotted in **Figure 10**. There were no

314   significant differences amongst calves infected by different methods at any time point

315   ($P>0.23$), except 11 days post infection (dpi) ($P=0.007$). At this time point (11 dpi) the

316   median titre was significantly ($P<0.04$) higher for group 3 calves that had been infected by

317   microdoses of LSDV following feeding by uninfected *S. calcitrans* (median FVNT=$10^{1.3}$) than

318   for those group 2 calves infected by microdoses of mixed LSDV and secreted *S. calcitrans*

319   saliva (median FVNT=0) or group 1 calves infected by microdoses of LSDV only (median

320   FVNT=0). These data indicate that spot feeding with *S. calcitrans* ahead of LSDV inoculation

321   stimulates a more rapid humoral immune response.

322

323   Table 4. Total neutralisation titres of sera

	Complete Neutralisation (FVNT$_{100}$) Days Post Infection				
	-2	7	11	16	27
CV1	neg	neg	neg		
CV2	neg	neg	neg	15	40
CV3	neg	neg	neg	80	80
CV4	neg	neg			
CV5	neg	neg	neg	80	320
CS1	neg	neg	neg		
CS2	neg	neg	neg	320	320
CS3	neg	neg	neg	40	80
CS4	neg	neg	10	320	320
CS5	neg	neg			
CF1	neg	neg	10	40	
CF2	neg	neg	10		
CF3	neg	neg			
CF4	neg	neg	neg		
CF5	neg	neg	10	320	>1280*

	Complete Neutralisation (FVNT$_{100}$) Days Post Infection				
	-2	7	11	16	27
CV6	neg	neg	neg		
CV7	neg	neg	10		
CV8	neg	neg	neg		
CV9	neg	neg	neg		
CV10	neg	neg	neg	80	240
CS6	neg	neg	neg	>1280*	>1280*
CS7	neg	neg	neg	neg	15
CS8	neg	neg	30		
CS9	neg	neg	neg	neg	10
CS10	neg	neg	neg	480	>1280*
CF6	neg	neg	neg		
CF7	neg	neg	60		
CF8	neg	neg	10	120	640
CF9	neg	neg	40		
CF10	neg	neg	20		

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Discussion

Poxviruses including myxoma virus, fowlpox virus and LSDV are transmitted mechanically by a range of blood-feeding arthropod vectors. LSDV is an unusual poxvirus because haematogenous arthropod vectors are its only route of transmission, with direct transmission playing little if any role in the epidemiology of LSD (27, 72, 73, 74). This study investigates the influence of the stable fly *S. calcitrans* on the replication and spread of LSDV in vitro, and the kinetics and severity of LSD in vivo. *S. calcitrans* saliva was found to have no effect on LSDV replication in vitro, however addition of secreted *S. calcitrans* saliva to LSDV inoculum in a bovine experimental model of LSD reduced the proportion of calves that developed clinical disease. In contrast, spot feeding by *S. calcitrans* prior to intradermal viral inoculation *in vivo* accelerated the kinetics of clinical signs of disease, the appearance of viraemia, and the production of neutralising antibodies. This indicates that arthropod-induced enhancement is a factor in disease caused by mechanically-transmitted as well as biologically-transmitted pathogens.

Previous studies of *S. calcitrans* saliva have used salivary gland extract (SGE) which involves dissection of the salivary glands from insects followed by maceration and filtration to minimise cellular debris. SGE of *S. calcitrans* has been used to study the proteome and transcriptome (54) of the salivary gland of the fly and the effect of the components of SGE on the bovine immune response (55, 56). The disadvantage of using SGE is the presence of structural components of the salivary gland which are not usually found in saliva. In contrast, the "secreted" methodology described here minimises contaminants and produces more representative and reproducible *S. calcitrans* saliva. Mass spectrometry of the secreted *S. calcitrans* saliva revealed putative immunomodulatory proteins such as antigen-

350 5 as well as enzymatic components of saliva such as peptidases, endonucleases, and

351 amylases, and furthers our knowledge of the fly saliva. This is consistent with previously

352 published arthropod sialomes. This method for obtaining minimally contaminated *S.*

353 *calcitrans* saliva will facilitate studies of LSDV and other pathogens that are transmitted by

354 this abundant vector.

355 We found that the addition of *S. calcitrans* secreted saliva did not influence the replication

356 or spread of LSDV in bovine primary fibroblasts, indicating the saliva did not directly impact

357 the infectivity of the virus, or induce an effective antiviral effect in the bovine fibroblasts.

358 The influence of arthropod saliva on virus replication in cell culture is inconsistent with

359 some studies reporting an impact (66, 67, 68), but others not (38).

360 Addition of arthropod saliva enhances disease caused by multiple biologically transmitted

361 viruses (29, 30, 31, 32, 36, 38, 67). We hypothesised that adding saliva to a mechanically

362 transmitted pathogen, LSDV, would likewise enhance disease. However, fewer calves

363 inoculated with virus mixed with saliva (group 2) developed clinical disease (3/10) compared

364 to calves in group 1 which received virus alone (6/10), suggesting that *S. calcitrans* saliva

365 inhibits the ability of LSDV to cause disease. Whilst a strong trend, this difference did not

366 reach statistical significance. The amount of saliva added to the inoculum was 0.5 μg of *S.*

367 *calcitrans* saliva in each microdose, comparable with other studies which have examined the

368 influence of arthropod saliva on disease outcome (29, 38). This may not accurately

369 represent the amount of saliva inoculated during a *S. calcitrans* bite. Likewise the amount of

370 virus in the inoculum, 5 x 10^4 pfu per microdose, may be greater than the amount

371 inoculated by an arthropod in the field. The dose of LSDV inoculated during an arthropod

372 bite is very small and difficult to quantify because LSDV is a mechanically transmitted

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373   pathogen and does not replicate within the arthropod vector. Future studies could examine

374   the impact of varying the amount of *S. calcitrans* saliva or the amount of virus in the

375   inoculum on disease outcome.

376   Spot-feeding is often used to model arthropod-enhancement of disease as both arthropod

377   saliva inoculation and bite trauma occur (30, 33, 34). *S. calcitrans* is a telmophagous feeder

378   with slashing mouthparts that create a haemorrhagic pool from which to feed (39), and are

379   therefore likely to modulate the skin microenvironment at the bite site. We found that spot

380   feeding prior to LSDV inoculation accelerated the pathogenesis of LSDV in our model of

381   disease, with earlier development of cutaneous nodules, earlier appearance of viral DNA in

382   the blood stream, and earlier production of neutralising antibodies. Characterisation of the

383   changes to the mononuclear blood cell population of the calves in response to LSDV

384   inoculation did not reveal a mechanism to explain the difference seen, with no differences

385   detected between the three groups of calves studied. Possible mechanisms for the influence

386   of spot feeding on the development of LSD are the recruitment of inflammatory cells which

387   promote viral survival, as seen in the recruitment of neutrophils to the skin in response to

388   sand fly bites promoting leishamaniasis (75), or by promoting oedema to retain the virus at

389   the inoculation site, as seen with *Aedes aegypti* mosquito bites (34). Since we only examined

390   the influence of spot feeding by *S. calcitrans* it is not clear if the acceleration in disease

391   development is specific to this arthropod species or more broadly applicable to other

392   species known to transmit LSDV such as mosquitoes. In fact, the mechanism may not be

393   specific to arthropods at all but could be due to any trauma, such as scarification which has

394   been shown to influence the immune response to the poxvirus vaccinia virus (76).

395 Understanding the impact of *S. calcitrans* saliva and spot feeding on the kinetics of LSD

396 provides an insight into the mechanical transmission of poxviruses. By studying the

397 influence of *S. calcitrans* on the pathogenesis of a bovine disease in its natural host species,

398 this work has revealed that the mechanical transmission of pathogens by arthropod vectors

399 is more sophisticated and intricate than previously thought, and worthy of deeper

400 investigation.

401

402

Materials and Methods.

Viruses: LSDV was propagated on MDBK cells (ATCC code CCL-22) in high-glucose Dulbecco's Modified Eagle's medium (Life Technologies No. 41965) supplemented with 2.5% fetal bovine serum (Antibody Production Services Ltd), and 1% penicillin-streptomycin (Life Technologies No. 15140122) at 37°C in a 5% CO_2 atmosphere. LSDV was semi purified through a 36% sucrose cushion prior to titration. Infectious virus titre expressed as number of plaque or foci forming units/millilitre (PFU/mL), was determined by titrating virus on MDBK cells as previously described (77).

Isolation of primary bovine fibroblasts: Bovine primary fibroblasts (BPF) from the distal ear pinna of skin were isolated and expanded as previously described (77). Briefly, the dermis was dissociated from a section of skin before pieces were then placed in a tissue culture plate in growth medium overnight at 37°C, in 5% CO_2 atmosphere. More media was then added and the skin pieces removed two days later. Adherent fibroblast colonies were then seeded into cell culture vessels and grown at 37°C, 5% CO_2.

Infection of fibroblasts: Fibroblasts were grown to 90% confluency in 6-well tissue culture plates in BPF media. The cells were infected with LSDV at a high MOI (5) or mock infected for 1h at 37°C in 5% CO_2. The inoculum was then removed, the cells washed twice with media, then incubated at 37°C in 5% CO_2. Cellular and supernatant fractions were collected together at timepoints up to 7 days post infection (dpi) and infectious virus titres determined by plaque assay on MDBK cells.

Saliva collection: The feeding reservoirs of the Hemotek™ membrane feeding system were prepared by securing an unstretched layer of Parafilm™ with a rubber ring, sealing the inner chamber before the reservoir was filled with endotoxin free water (Fisher Scientific). A

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426   Durapore™ PVDF 0.2 µm membrane (Millipore) was dipped briefly in 1mM ATP/endotoxin

427   free water solution before being laid over the parafilm. Two thinly stretched layers of

428   Parafilm™ were laid on top of the PVDF to allow for penetration by the proboscis but

429   minimise contamination of the membrane by non-salivary proteins during feeding (Figure

430   1). Excess Parafilm™ was trimmed away from the completed reservoir insert before it was

431   screwed into the main Hemotek™ apparatus and allowed to warm to 37°C. Laboratory-

432   reared male and female *S. calcitrans* at an average of 14.5 days post-eclosion (range: 10-19

433   days) were starved the day before feeding, then placed into 53mm diameter plastic pots

434   closed with mesh netting and placed onto the membrane feeding system (MFS) for

435   approximately 15 min per pot. After the MFS was fed on 3-4 times by a pot of flies the

436   Durapore™ membrane was placed into PBS and stored at 4°C.

437   **Saliva concentration**: Once saliva collection from the flies was completed, the membranes

438   were cut into four irregular pieces and washed in PBS at 4°C overnight under constant

439   rotation. The eluted proteins were then sterilised by passing the eluate through a 0.2 µm

440   filter (Merck Millipore), then concentrated using a 3kDa Vivaspin 20 centrifugal

441   concentrator column (Sartorius) to give a final volume of approximately 1000 µL. The

442   protein concentration in the saliva was calculated using the Pierce™ BCA Protein Assay Kit

443   (ThermoFisher Scientific).

444   **SDS-PAGE and silver stain/Coomassie**: Whole cell lysates were prepared by reduction and

445   denaturation with dithiothreitol (DTT) and heating for 5 mins at 95°C. 15% SDS-PAGE gels

446   were then used to separate *S. calcitrans* salivary proteins by size with proteins visualised by

447   Coomassie brilliant blue dye or silver staining (Pierce™ Silver Stain Kit, ThermoFisher

448   Scientific) according to manufacturer's instructions.

449 **Proteomic analysis of *S. calcitrans* secreted saliva:**

450 *Sample digestion*

451 In-gel digestion was essentially as described by Shevchenko et al. (78). Pre-excised and

452 stained bands were cut into smaller pieces (approx. 1mm^3). Gel pieces were then de-stained

453 and in gel digestion performed as described below.

454 Slices were de-stained with 25mM ammonium bicarbonate/ 50 % acetonitrile (v/v). Proteins

455 were reduced for 10 minutes at 60°C with 10 mM dithiothreitol (Sigma) in 25 mM

456 ammonium bicarbonate and then alkylated with 55 mM iodoacetamide (Sigma) in 50 mM

457 ammonium bicarbonate for 30 minutes in the dark at room temperature. Gel pieces were

458 washed for 15 minutes in 50 mM ammonium bicarbonate and then dehydrated with 100%

459 acetonitrile. Acetonitrile was removed and the gel plugs rehydrated with 0.01 µg/µL

460 proteomic grade trypsin (Thermo Fisher Scientific) in 25 mM ammonium bicarbonate.

461 Digestion was performed overnight at 37°C. Peptides were extracted with 50% (v/v)

462 acetonitrile, 0.1% trifluoroacetic acid (TFA) (v/v) and the extracts were reduced to dryness

463 using a centrifugal vacuum concentrator (Eppendorf) and re-suspended in 3 % (v/v)

464 methanol, 0.1 % (v/v) TFA for analysis by MS.

465 *Mass spectrometry*

466 LC-MS/MS analysis was similar to that described by Aljabr et al. (79). Peptides were

467 analysed by on-line nanoflow LC using the Ultimate 3000 nano system (Dionex/Thermo

468 Fisher Scientific). Samples were loaded onto a trap column (Acclaim PepMap 100, 2 cm × 75

469 µm inner diameter, C18, 3 µm, 100 Å) at 9 µL /min with an aqueous solution containing 0.1

470 % (v/v) TFA and 2% (v/v) acetonitrile. After 3 min, the trap column was set in-line an

471 analytical column (Easy-Spray PepMap® RSLC 50 cm × 75 µm inner diameter, C18, 2 µm, 100

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472   Å) fused to a silica nano-electrospray emitter (Dionex). The column was operated at a

473   constant temperature of 35°C and the LC system coupled to a Q-Exactive mass spectrometer

474   (Thermo Fisher Scientific). Chromatography was performed with a buffer system consisting

475   of 0.1 % formic acid (buffer A) and 80 % acetonitrile in 0.1 % formic acid (buffer B). The

476   peptides were separated by a linear gradient of 3.8 – 50 % buffer B over 30 minutes at a

477   flow rate of 300 nl/min. The Q-Exactive was operated in data-dependent mode with survey

478   scans acquired at a resolution of 70,000 at m/z 200. Scan range was 300 to 2000m/z. Up to

479   the top 10 most abundant isotope patterns with charge states +2 to +5 from the survey scan

480   were selected with an isolation window of 2.0Th and fragmented by higher energy

481   collisional dissociation with normalized collision energies of 30. The maximum ion injection

482   times for the survey scan and the MS/MS scans were 250 and 50ms, respectively, and the

483   ion target value was set to 1E6 for survey scans and 1E4 for the MS/MS scans. MS/MS

484   events were acquired at a resolution of 17,500. Repetitive sequencing of peptides was

485   minimized through dynamic exclusion of the sequenced peptides for 20s.

486   *Protein Identification*

487   Spectral data were analysed using the PEAKS studio X+ software (Bioinformatics Solutions

488   Inc., Waterloo, ON, Canada (80)). Tandem MS data were searched against the *Stomoxys*

489   *calcitrans* protein database (UniProt) and a contaminant database (cRAP, GPMDB, 2012).

490   Search parameters were as follows; precursor mass tolerance set to 15 ppm and fragment

491   mass tolerance set to 0.02 Da. Trypsin, was set as the protease. Two missed cleavage events

492   were permitted. Carbamidomethylation (C) was set as a fixed modification. Oxidation (M)

493   and acetylation (N-term) were set as variable modifications. The search was specific. The

494   false discovery rate was set at 1%.

495   *Sialome Functional Enrichment Analysis*

496   Functional enrichment analysis of proteins represented in at least two out of three saliva

497   collections was conducted using the g:GOSt algorithm available on the g:Profiler web server

498   (81). UniProt accession numbers in a ranked list as shown in Table 1 were queried against

499   the *S. calcitrans* Ensemble genome version GCA_001015335.1/SCIU1.6 (82) integrated in

500   g:GOSt. Where alternative accession numbers for the same protein existed, these were

501   removed prior to analysis. The g:SCS algorithm in g:GOSt was employed for computing

502   multiple testing correction for p-values gained from the GO and pathway enrichment

503   analysis with an experiment-wide p-value of 0.05 (confidence interval level of 95%).

504   **Animal study:** The animal study was performed in the high-containment animal facilities at

505   The Pirbright Institute under project license P2137C5BC from the UK Home Office according

506   to the Animals (Scientific Procedures) Act of 1986, and was approved by The Pirbright

507   Institute Animal Welfare and Ethical Review Board. Thirty male Holstein-Friesian calves 4-6

508   months old (weight range 133- 167kg) were included in the study. The study was carried out

509   as two replicates each of 15 animals. In each replicate animals were stratified according to

510   weight then randomly assigned to the three treatment groups. The animals were sourced

511   from a commercial high health herd and confirmed as negative for bovine viral diarrhoea

512   virus (BVDV) via PCR prior to study commencement. The animals were housed in 3 rooms

513   (22m^2) in a high-containment (SAPO4) animal facility at the Pirbright Institute. Bedding

514   material was provided (https://www.mayofarmsystems.co.uk/mayo-mattress-stable-mat/),

515   light/dark cycle was 12:12 h, temperature was held between 10°C to 24°C, and humidity

516   40% to 70%. Animals were fed concentrated rations twice daily and given ad lib access to

517   hay and water. Environmental enrichment was provided, including rubber toys and a hollow

518   ball stuffed with hay. Calves were treated for three co-morbidities during the study

519   (ringworm, bronchopneumonia, and orchitis / epididymitis secondary to castration) as

520   detailed in the supplementary data.

521   Calves in group 1 were inoculated with 1×10^6 PFU/mL LSDV intradermally. The inoculum was

522   delivered as ten 100 μl "microdoses" into an 8cm diameter region of skin on the left and

523   right craniodorsal flank, just caudal to the scapula. Calves in group 2 were challenged with

524   1×10^6 PFU/mL LSDV that had been mixed with 10μg *S. calcitrans* salivary proteins. Inoculum

525   was delivered as in group 1. Calves in group 3 had two pots, each pot containing

526   approximately 25 *S. calcitrans* flies, placed sequentially on the skin of the left and right

527   craniodorsal flank for 5 min each pot. Each animal was then challenged with 1×10^6 PFU/mL

528   LSDV with the inoculum delivered in microdoses (as described for groups 1 and 2), into the

529   site where the flies had fed.

530   Following inoculation each animal was clinically examined regularly including heart rate,

531   temperature, respiratory rate, swelling at inoculation site, number of nodules (and their

532   locations), anorexia, lameness, behavioural changes (including isolation, lethargy, abnormal

533   posture), and lymphadenopathy. Venous blood samples and skin microbiopsies were

534   collected at regular intervals up to 28 dpi.

535   **Clinical scoring system.** A scoring system for experimental LSD developed by Carn and

536   Kitching (83) was modified to include the full range of LSD clinical signs seen after microdose

537   inoculation. The scoring system included cutaneous nodules, enlargement of lymph nodes,

538   temperature, unusual behaviour, anorexia, oedema and lameness, ocular/nasal discharge

539   and salivation. These criteria were then weighted to account for severity of some clinical

540   signs (Table 3).

29

541 **PCR of blood:** Nucleic acid extraction and PCR was conducted by the Non-Vesicular

542 Reference Laboratory at The Pirbright Institute. DNA was extracted using the MagMAX™

543 CORE Nucleic Acid Purification Kit (Applied Biosystems) on a Kingfisher flex automated

544 extraction robot (ThermoFisher Scientific). The starting sample volume was 200 μL, and the

545 DNA was eluted into 90 μL elution buffer in the final step. The real-time PCR assay for CPPV

546 (Bowden et al., 2008) targeted an 89 bp region within the LSDV074 ORF. Each assay used a

547 dual labelled fluorogenic (TaqMan®) probe that allowed the assay to be performed in a

548 closed-tube format minimising the potential for cross-contamination of post-PCR products.

549 **Titration of blood**: PCR positive blood samples were serially diluted in calcium-free DMEM,

550 before infectious virus titre (number of plaque-forming units/millilitre, PFU/mL) was

551 determined by plaque assay on MDBK cells (77).

552 **Skin biopsy PCR**: Experiment was carried out according to the protocol used by Sanz-

553 Bernardo et al. (84). Viraemia was analysed in duplicate, and results presented as mean

554 copies per 2mm biopsy.

555 **Flow cytometry**: Antibody cocktails were prepared with 0.5% bovine serum albumin

556 (BSA)/PBS as diluent. 100 μL heparinised whole blood from each animal was aliquoted in

557 triplicate into empty Round-bottom polystyrene 5ml (Falcon 5ml) tubes and mixed with 30

558 μL antibody cocktail, made of CD3 (MM1a) conjugated with AF488 (Alexa Fluor™ 488

559 Antibody Labeling Kit), CD14 (CC-G33, BioRad) conjugated with AF647 (Alexa Fluor™ 647

560 Antibody Labeling Kit), CD40 (IL-A158, Immunological Toolbox) conjugated with PE

561 (Lightning Link, Novus Biologics), and γδ TCR (GB21a, Washington State University)

562 conjugated with PB (Pacific Blue Antibody Labeling Kit). Blood from CV1 and CV6 cattle were

563 used as single colour (SC), fluorescence minus one (FMO) and unlabelled controls. The blood

564   samples and antibodies were incubated for 30 min at RT in the dark. 2 mL eBioscience™

565   Fix/Lyse Solution (10×) diluted 1:10 in sterile water was added to each tube to lyse the red

566   blood cells. The tubes were incubated at RT for 30 min in the dark. 100µl of vortexed

567   Invitrogen™ 123count eBeads™ (Fisher Scientific) were added to each tube. Samples were

568   read on a 4 Laser LSR Fortessa (405nm, 488nm, 561nm and 640 nm). AF488 signal was

569   detected with BP530/30, after excitation with the 488nm laser. AF647 signal was detected

570   with BP670/14, after excitation with the 640nm laser. PE signal was detected with

571   BP582/15, after excitation with the 561nm laser. PB signal was detected with BP450/50,

572   after excitation with the 405nm laser. Compensation was run on single colour controls. Cells

573   were gated according to **Figure 11**, and data was analysed in FCS Express 7 (De Novo

574   software).

575   **Fluorescent Virus Neutralisation Test**: The neutralisation titre was determined as described

576   previously (71). Briefly, MDBK cells were seeded (3×10^5/mL) in 96 well plates. Sera were

577   heat-inactivated at 56°C for 30 min then diluted 1:10 in cell culture medium (DMEM, 2.5%

578   heat-inactivated FBS, 100 units/mL penicillin, and 100µg/mL streptomycin). Two-fold serial

579   dilutions from 1:10 to 1:1280 were prepared. Recombinant EGFP-095-LSDV Neethling virus

580   (7×10^2 pfu/mL) was added to each dilution and incubated for 1h at 37°C in a 5% CO_2

581   atmosphere. Samples were added to the MDBK cells and incubated at 37°C in a 5% CO2

582   atmosphere for 5 days. To interpret the results, fluorescent foci were counted using a

583   fluorescent UV light microscope (Olympus CKX53). The complete neutralising antibody titre

584   was calculated as the highest dilution at which no foci were identified. All samples and

585   controls were tested in duplicate.

586   **Statistical methods**

587   **Clinical scoring:** Mixed effects models were conducted to determine statistical significance

588   between the treatment groups and clinical outcomes. p-values < 0.05 were considered to be

589   statistically significant.

590   **Nodule number:** Mixed effects models were conducted to determine statistical significance

591   between the treatment groups and clinical outcomes. p-values < 0.05 were considered to be

592   statistically significant.

593   **FVNT**: Because of non-normality of errors and non-equal variances, the median log FVNT

594   were compared amongst calves infected by each method (Group 1, Group 2 or Group 3) at

595   each timepoint. If the Kruskal-Wallis test was significant (P<0.05), pairwise Wilcoxon rank-

596   sum tests (two-sided, unpaired) were used to compare responses between groups.

597   **% CD14+ cells**: Because of non-normality of errors and non-equal variances, the median

598   percentage CD14+ cells were compared amongst clinical and non-clinical calves (regardless

599   of infection method) at each timepoint using a Wilcoxon rank-sum test (two-sided,

600   unpaired).

601   **C_T values in blood**: Because of non-normality of errors and non-equal variances, median CT

602   values in blood were compared amongst groups at each timepoint. Calves were divided into

603   six groups based on the infection method (Group 1, Group 2 or Group 3) and clinical status

604   (clinical or non-clinical). If the Kruskal-Wallis test was significant (P<0.05), pairwise Wilcoxon

605   rank-sum tests (two-sided, unpaired) were used to compare responses between groups,

606   specifically: (i) clinical and non-clinical cattle infected by the same method; (ii) clinical cattle

607   infected by different methods; and (iii) non-clinical cattle infected by different methods.

608   A sample with no C_T value was given an arbitrary value of 42 for the purposes of analysis. In

609   addition, samples were tested in duplicate. The mean of the duplicate values for each

610   sample was used in the analysis, unless one result produced a C_T value and the other did

611   not, in which case only the (positive) C_T value was used.

612   The analyses of clinical scores and nodule numbers were implemented in GraphPad Prism 8,

613   while the analyses of FVNT, % $CD14^+$ and C_T values were implemented in R (version 4.1.3)

614   (85).

615

616

617   **Acknowledgements**

620

621   **Funding**

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630

34

631 **Author contributions:**

Conceptualization	Ideas; formulation or evolution of overarching research goals and aims.	CGC, KD, PMB
Data Curation	Management activities to annotate (produce metadata), scrub data and maintain research data (including software code, where it is necessary for interpreting the data itself) for initial use and later reuse.	CGC, SG, SA
Formal Analysis	Application of statistical, mathematical, computational, or other formal techniques to analyze or synthesize study data.	CGC, SG
Funding Acquisition	Acquisition of the financial support for the project leading to this publication.	PMB, KD, DRG
Investigation	Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection.	CGC, HM, PCF, NW, KM, SA, PMB
Methodology	Development or design of methodology; creation of models	CGC, HM, PCF, KD, PMB
Project Administration	Management and coordination responsibility for the research activity planning and execution.	PMB
Resources	Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools.	KM, SA
Software	Programming, software development; designing computer programs; implementation of the computer code and supporting algorithms; testing of existing code components.	
Supervision	Oversight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team.	PMB, KD, DRG
Validation	Verification, whether as a part of the activity or separate, of the overall replication/reproducibility of results/experiments and other research outputs.	
Visualization	Preparation, creation and/or presentation of the published work, specifically visualization/data presentation.	CGC, SG, PMB
Writing – Original Draft Preparation	Creation and/or presentation of the published work, specifically writing the initial draft (including substantive translation).	CGC, PMB, KD
Writing – Review & Editing	Preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre- or post-publication stages.	CGC, HM, PCF, NW, KM, SG, SA, DRG, KD, PMB

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858   Figure 1
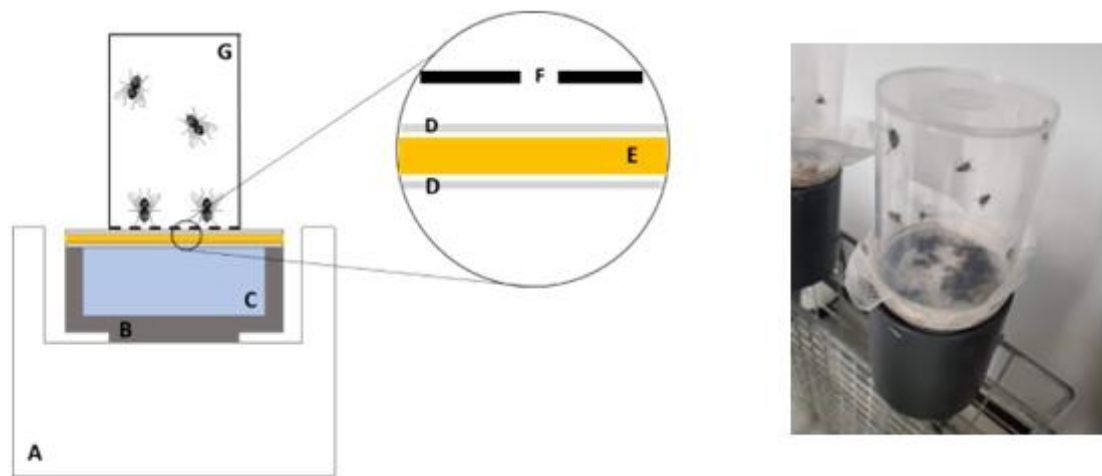
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862   **Figure 1. Method for collecting secreted *S. calcitrans* saliva**. Left: A: Hemotek™ base heats

863   the reservoir to 37°C; B: screw-in reservoir; C: distilled water to conduct heat to the

864   membrane; D: Parafilm to prevent contamination of the membrane with non-salivary

865   proteins and seal water in the reservoir; E: Durapore PVDF 0.2 µm membrane soaked in ATP

866   solution; F: fine netting to allow flies to feed; G: transparent 4cm diameter pot containing *S.*

867   *calcitrans* flies. Right: a pot of flies feeding on the apparatus.

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873   Figure 2

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875

876   **Figure 2 (A) The protein profile of *S. calcitrans* saliva.** Secreted saliva from *S. calcitrans* was

877   collected and concentrated then separated on a 15% SDS/PAGE gel before being visualised

878   by silver staining (lane 2). PageRuler Plus was used as the ladder (lane 1). Image is

879   representative of the three collections. (**B). Secreted saliva does not impact LSDV kinetics**

880   **in BPF.** LSDV was incubated with either secreted *S. calcitrans* saliva or PBS for 2h at RT, then

881   inoculated onto confluent monolayers of BPFs at an MOI of 5. Cells and supernatant were

882   collected together at the time points indicated, sonicated, and titred on MDBK cells. Data

883   shown as mean ± SEM, and are representative of two independent experiments.

42

884   Figure 3

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886

887   **Figure 3**. **LSDV inoculation methodology.** Cattle were inoculated using three different

888   methods. (A) Cattle in group 1 were inoculated intradermally with 1 x10^6 pfu/ml LSDV in 10

889   microdoses of 100µl on the left and right craniodorsal flank, just caudal to the scapula.

890   Cattle in group 2 were inoculated intradermally in the same manner in 10 microdoses of

891   100µl, but with 1x10^6 pfu/ml LSDV mixed with 10µg *S. calcitrans* salivary proteins. The

892   image shows microblebs from an inoculation site of a calf in group 2, with the site outlinned

893   in black ink. (B) Cattle in group 3 were fed on by pots of *S. calcitrans* flies on the left and

894   right craniodorsal flank, as shown in the image, then inoculated intradermally with 1 x10^6

895   pfu/ml LSDV in 10 microdoses of 100µl (the same as group 1 and 2) into the sites of feeding.

896   (C) Post-feeding the flies that fed on the cattle in group 3 showed evidence of feeding (red

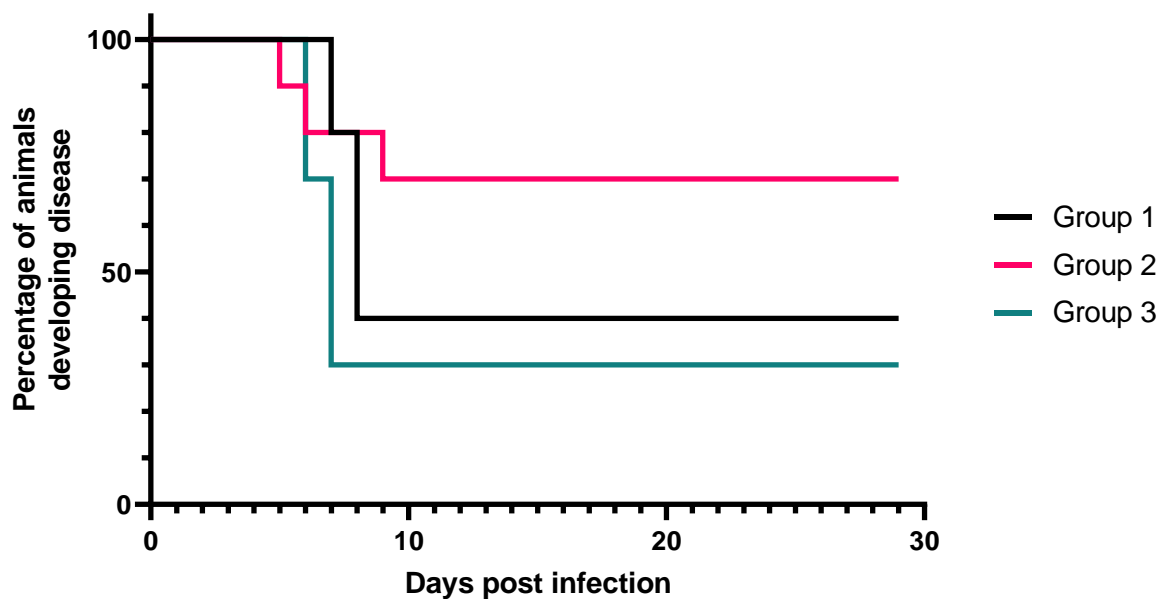897   abdomens).

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900 Figure 4

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902

Figure 4. Clinical outcome of different inoculation strategies of LSDV. Disease development was tracked in each animal by the initial onset of cutaneous nodule development and shown as a Kaplan-Meier graph. Those that developed nodules were scored as "1" and those that did not were scored as "0". Data were tested for significant differences between the groups using the log-rank test, but were not significant (P=0.154).
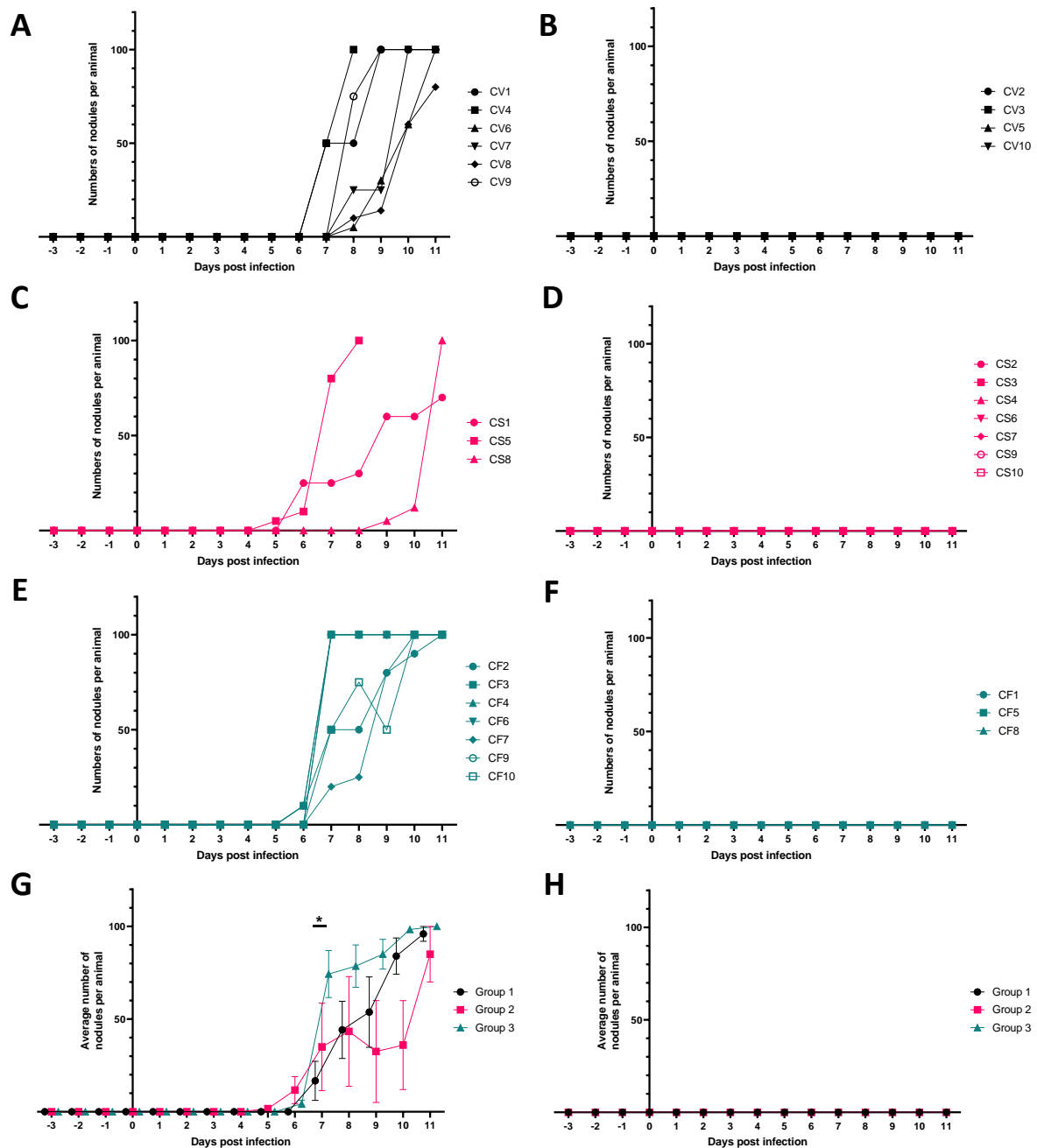
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Figure 5

Figure 5. Cutaneous nodules appear faster in the calves in group 3 that were spot-fed before virus inoculation. Nodules were measured by manual examination of each animal, up to a maximum of 100 nodules per animal. Calves with cutaneous nodules distant from the site of inoculation were designated clinical (left column) and calves that did not develop cutaneous nodules distant from the site of inoculation were designated nonclinical (right

45

938   column). (A and B) number of nodules on each calf from group 1 (inoculated with LSDV

939   alone), (C and D) number of nodules on each calf from group 2 (inoculated with LSDV and

940   secreted *S. calcitrans* saliva), (E and F) number of nodules on each calf from group 3

941   (inoculated with LSDV into area that had been previous spotfed by *S. calcitrans* flies). (G and

942   H) Average number of nodules per animal (black for group 1, pink for group 2, and blue

943   group 3). Data shown as mean ± SEM. Graphs show data collected from -3dpi to 11dpi.

944   Significance was determined by a mixed effects model.
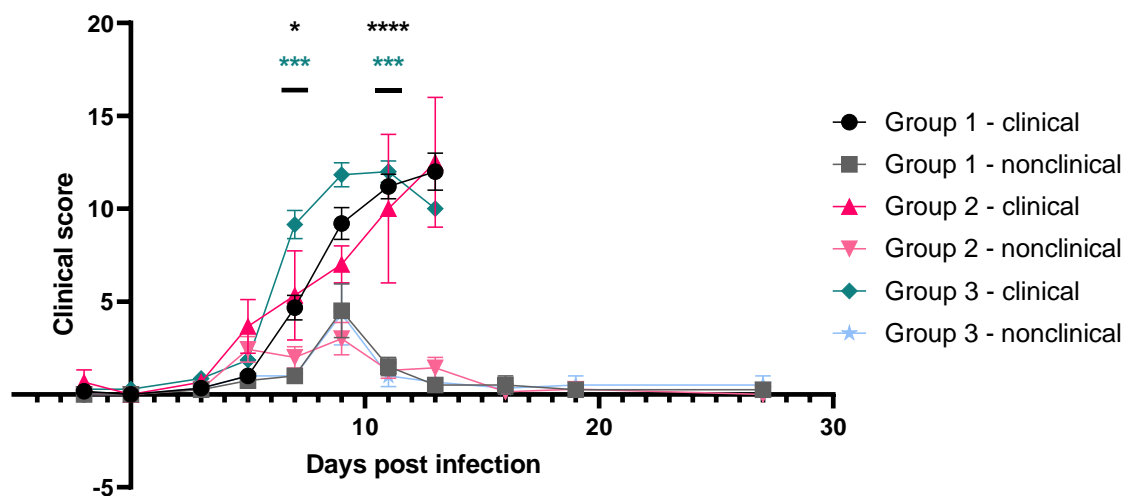
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946   Figure 6

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950   **Figure 6. Spot feeding prior to LSDV inoculation results in more rapid development of**

951   **disease.** Clinical scores for each animal were separated according to group (1-3) and into

952   clinical or nonclinical outcomes and averaged. Data shown as mean ± SEM. Data are shown

953   to 13 dpi for clinical animals and 27 dpi for non-clinical animals. The score of the group 3
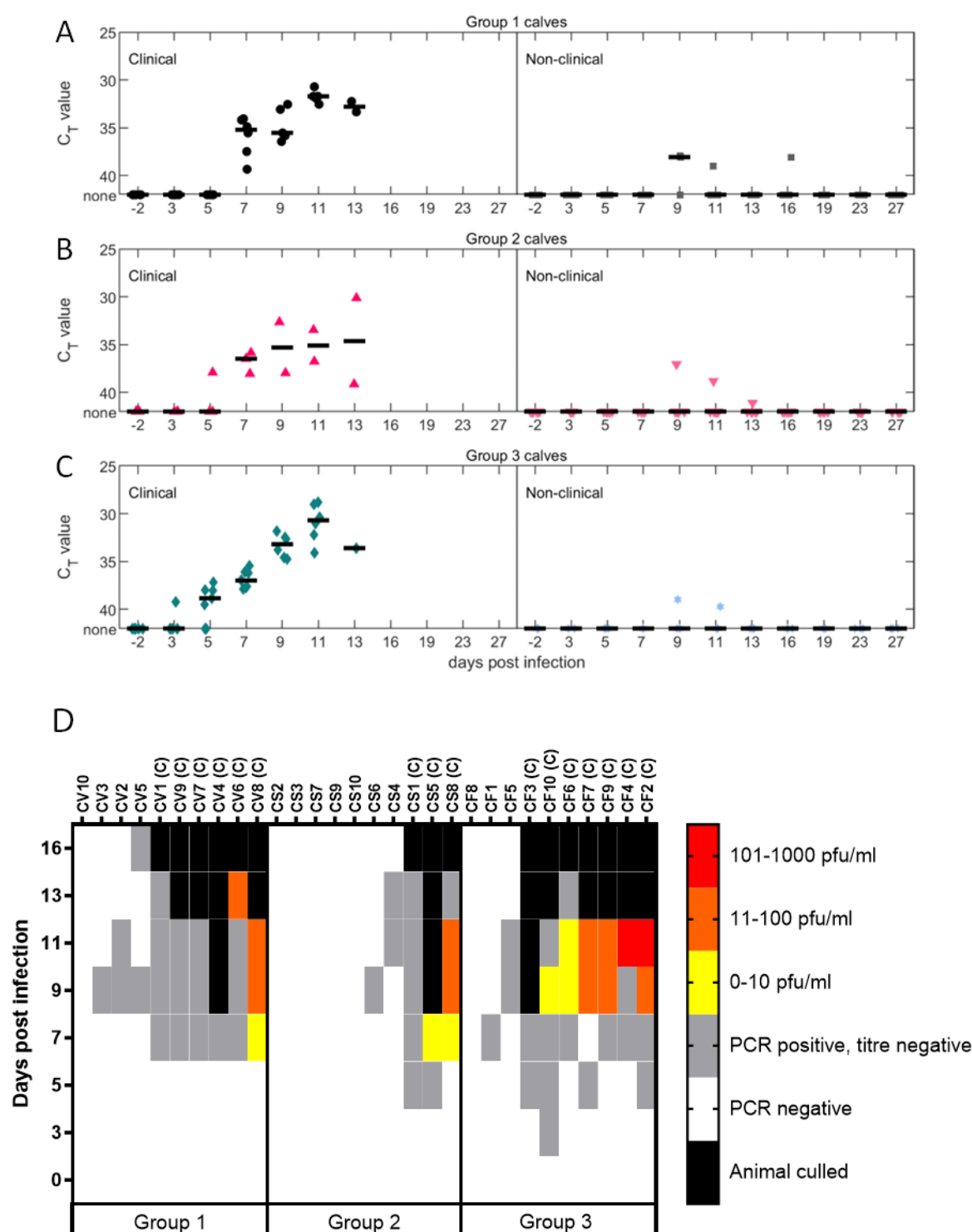
954   clinical animals was significantly higher than the score of the group 1 clinical animal at 7dpi

955   (p=0.0104, mixed effects model). A significant difference was also detected between clinical

956   and non- clinical animals in group 1 (black) and between the clinical and non-clinical animals

957   in group 3 (blue/green) at both 7dpi and 11dpi (mixed effects model).

958

47

959   Figure 7

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961

962   **Figure 7. Clinical animals develop a higher viraemia than non-clinical animals.** Comparison

963   of C_T values in blood at different days post infection in calves in group 1 (A), group 2 (B) and

964   group 3 (C). The left-hand plots show the C_T values for clinical calves and the right-hand

965   plots those for non-clinical calves. (D). Blood samples that were positive on PCR were titred

966   on MDBK cells, and viral foci counted manually. A letter C after the animal identification

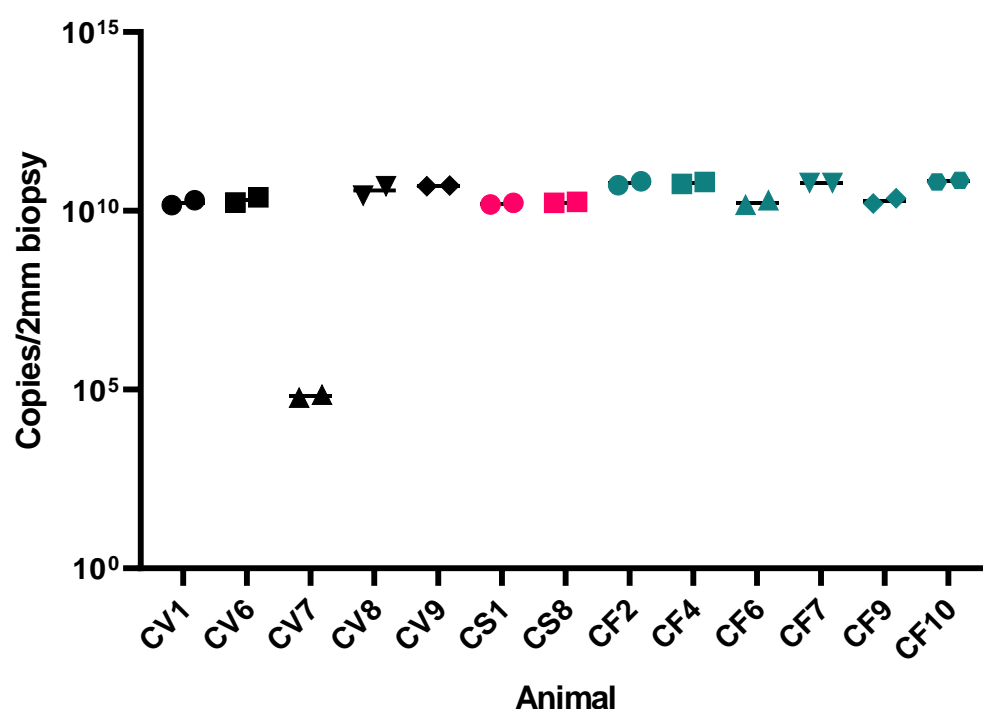967   number denotes a clinical animal.

968

969   Figure 8

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972

973   **Figure 8. Virus is present in the nodules of clinical animals at 11dpi.** Skin samples were

974   taken from using a 2mm biopsy punch at 5dpi, 11dpi and 16dpi. Viral DNA was extracted

975   from the tissue and quantified using qPCR in duplicate. Viral DNA was detected only in 11dpi

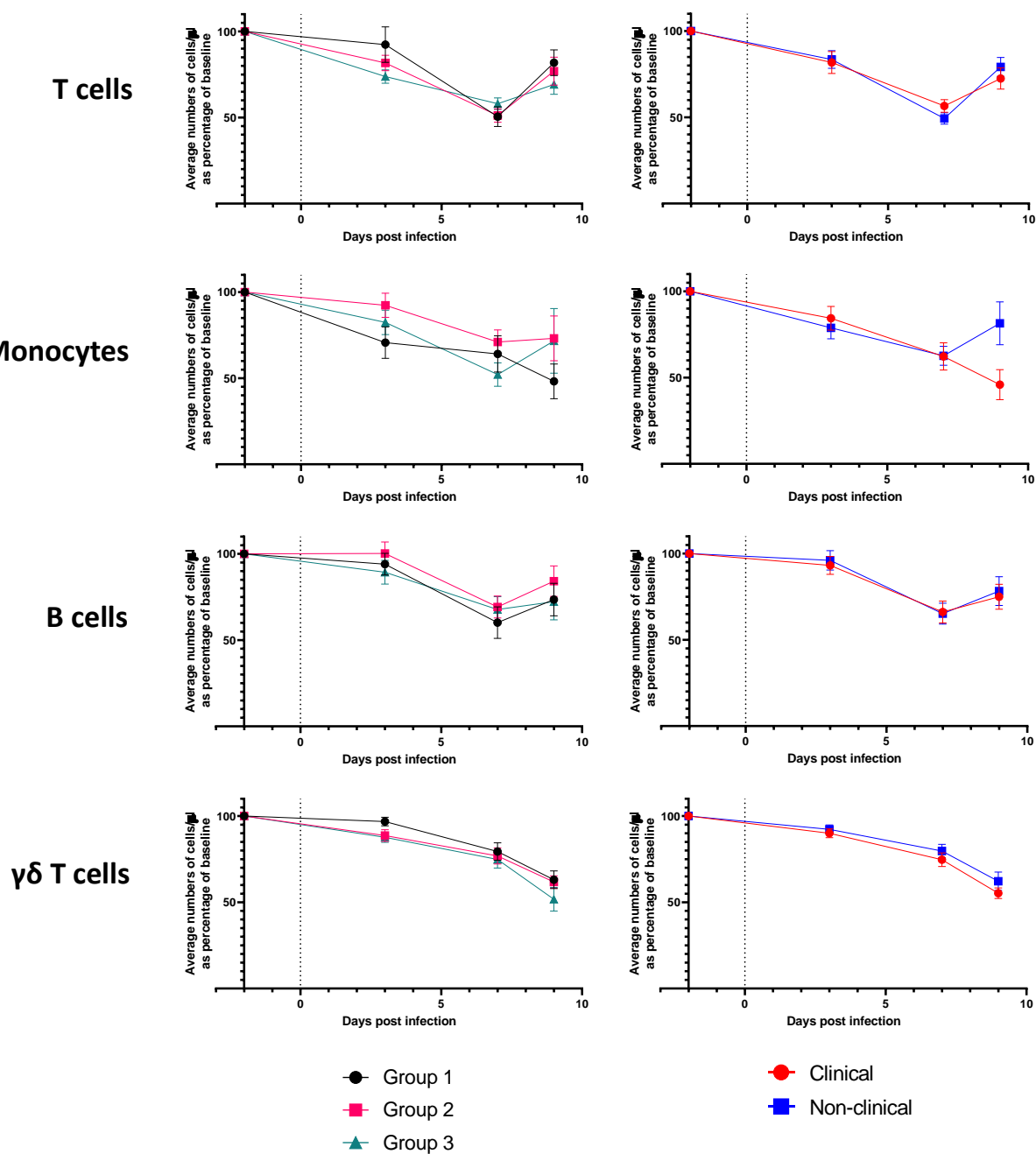976   samples and shown here. Data shown as mean ± SEM.

977

Figure 9

Figure 9. LSDV infection of cattle induces a lymphopenia in clinical and non-clinical animals. For each cell type the triplicate counts of cells/µl for each animal were averaged and converted to a percentage of the cell count at -2 dpi for each animal. These proportions were then pooled into the three groups of calves and averaged (group 1 black, group 2 pink

51

1006   and group 3 green) on the left, and into clinical and non-clinical outcomes on the right.

1007   Animals that became clinical are shown in red, and those that remained non-clinical are

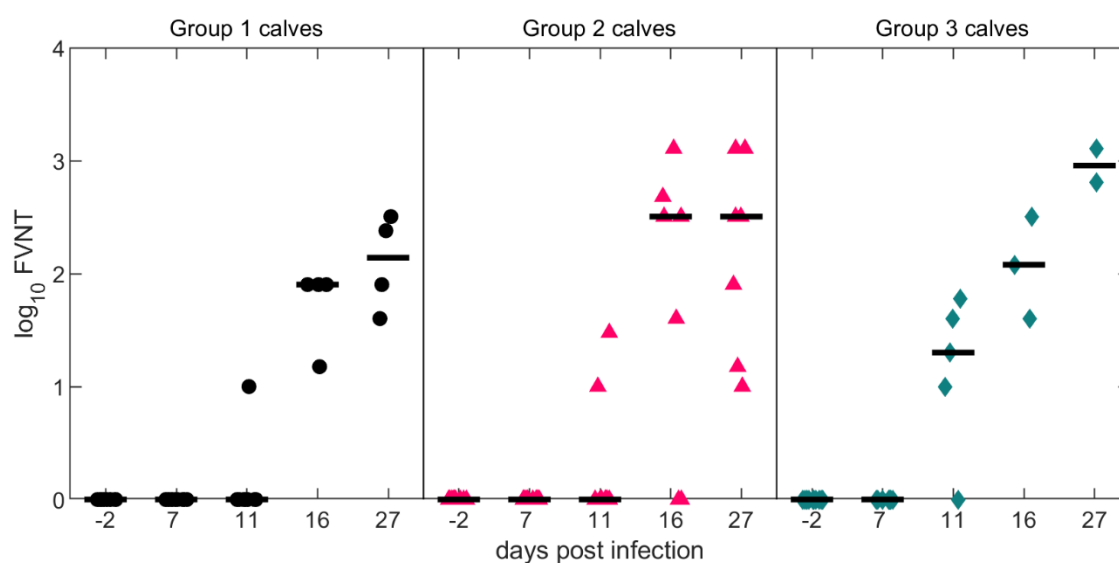1008   shown in blue. Data shown as mean ± SEM. The dashed line indicates the day of challenge.

1009

1010   Figure 10

1011

1012

Figure 10. Comparison of log$_{10}$ FVNT over time in calves infected with lumpy skin disease

virus (LSDV). Difference colours indicate different infection methods: calves infected via

microdoses of LSDV following feeding by uninfected *Stomoxys calcitrans* (group 1, red);

calves infected with microdoses of a mixture of LSDV and secreted *S. calcitrans* saliva (group

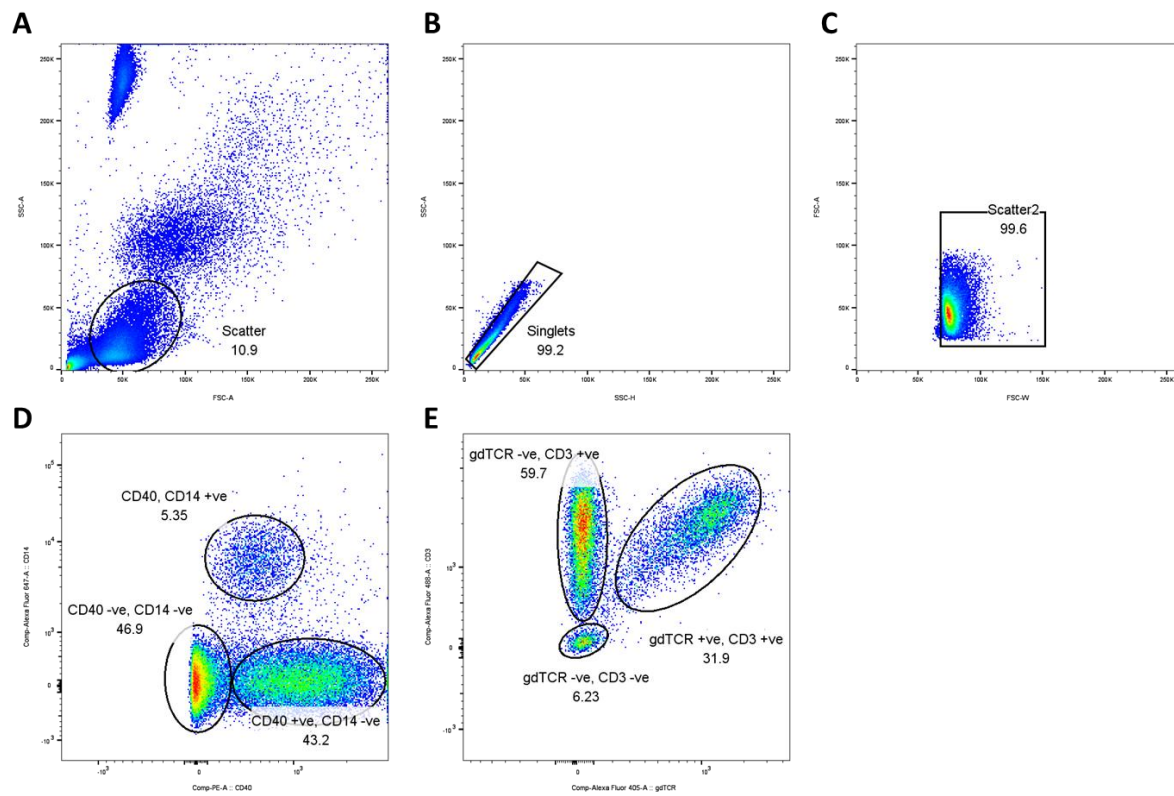2, magenta); and calves infected with microdoses of LSDV only (group 3, blue).

1018

1019

1020   Figure 11

1021

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1023

1024   **Figure 11. The whole blood flow cytometry panel gating strategy.** Cells were plotted on a

1025   forward vs side scatter plot (A) to isolate the mononuclear cell population, then gated to

1026   exclude doublets via a side scatter area vs height plot (B). Further debris was excluded by

1027   gating on a forward scatter area vs width plot (C). Monocyte (CD14 positive, CD40 negative)

1028   and B cell (CD14 negative, CD40 positive) populations were selected by plotting markers for

1029   CD14 vs CD40 (D). The CD40/CD14 negative population was plotted on CD3 vs gamma delta

1030   T cell receptor (γδ TCR) cell marker plot, to isolate the T cell population (CD3 positive, γδ

1031   TCR negative) and gamma delta T cells (CD3 positive, γδ TCR positive) (E).

1032

1033

1034

1035   Figure captions

1036   **Figure 1. Method for collecting secreted *S. calcitrans* saliva**. Left: A: Hemotek™ base heats

1037   the reservoir to 37°C; B: screw-in reservoir; C: distilled water to conduct heat to the

1038   membrane; D: Parafilm to prevent contamination of the membrane with non-salivary

1039   proteins and seal water in the reservoir; E: Durapore PVDF 0.2 µm membrane soaked in ATP

1040   solution; F: fine netting to allow flies to feed; G: transparent 4cm diameter pot containing *S.*

1041   *calcitrans* flies. Right: a pot of flies feeding on the apparatus.

1042

1043   **Figure 2 (A) The protein profile of *S. calcitrans* saliva.** Secreted saliva from *S. calcitrans* was

1044   collected and concentrated then separated on a 15% SDS/PAGE gel before being visualised

1045   by silver staining (lane 2). PageRuler Plus was used as the ladder (lane 1). Image is

1046   representative of the three collections. (**B). Secreted saliva does not impact LSDV kinetics**

1047   **in BPF.** LSDV was incubated with either secreted *S. calcitrans* saliva or PBS for 2h at RT, then

1048   inoculated onto confluent monolayers of BPFs at an MOI of 5. Cells and supernatant were

1049   collected together at the time points indicated, sonicated, and titred on MDBK cells. Data

1050   shown as mean ± SEM, and are representative of two independent experiments.

1051

1052   **Figure 3**. **LSDV inoculation methodology.** Cattle were inoculated using three different

1053   methods. (A) Cattle in group 1 were inoculated intradermally with 1×10^6 pfu/ml LSDV in 10

1054   microdoses of 100µl on the left and right craniodorsal flank, just caudal to the scapula.

1055   Cattle in group 2 were inoculated intradermally in the same manner in 10 microdoses of

1056   100µl, but with 1×10^6 pfu/ml LSDV mixed with 10µg *S. calcitrans* salivary proteins. The

56

1057   image shows microblebs from an inoculation site of a calf in group 2, with the site outlinned

1058   in black ink. (B) Cattle in group 3 were fed on by pots of *S. calcitrans* flies on the left and

1059   right craniodorsal flank, as shown in the image, then inoculated intradermally with 1 x10^6

1060   pfu/ml LSDV in 10 microdoses of 100µl (the same as group 1 and 2) into the sites of feeding.

1061   (C) Post-feeding the flies that fed on the cattle in group 3 showed evidence of feeding (red

1062   abdomens).

1063

1064   **Figure 4. Clinical outcome of different inoculation strategies of LSDV**. Disease development

1065   was tracked in each animal by the initial onset of cutaneous nodule development and

1066   shown as a Kaplan-Meier graph. Those that developed nodules were scored as "1" and

1067   those that did not were scored as "0". Data were tested for significant differences between

1068   the groups using the log-rank test, but were not significant (P=0.154).

1069

1070   **Figure 5. Cutaneous nodules appear faster in the calves in group 3 that were spot-fed**

1071   **before virus inoculation.** Nodules were measured by manual examination of each animal,

1072   up to a maximum of 100 nodules per animal. Calves with cutaneous nodules distant from

1073   the site of inoculation were designated clinical (left column) and calves that did not develop

1074   cutaneous nodules distant from the site of inoculation were designated nonclinical (right

1075   column). (A and B) number of nodules on each calf from group 1 (inoculated with LSDV

1076   alone), (C and D) number of nodules on each calf from group 2 (inoculated with LSDV and

1077   secreted *S. calcitrans* saliva), (E and F) number of nodules on each calf from group 3

1078   (inoculated with LSDV into area that had been previous spotfed by *S. calcitrans* flies). (G and

1079   H) Average number of nodules per animal (black for group 1, pink for group 2, and blue

1080   group 3). Data shown as mean ± SEM. Graphs show data collected from -3dpi to 11dpi.

1081   Significance was determined by a mixed effects model.

1082

1083   **Figure 6. Spot feeding prior to LSDV inoculation results in more rapid development of**

1084   **disease.** Clinical scores for each animal were separated according to group (1-3) and into

1085   clinical or nonclinical outcomes and averaged. Data shown as mean ± SEM. Data are shown

1086   to 13 dpi for clinical animals and 27 dpi for non-clinical animals. The score of the group 3

1087   clinical animals was significantly higher than the score of the group 1 clinical animal at 7dpi

1088   (p=0.0104, mixed effects model). A significant difference was also detected between clinical

1089   and non- clinical animals in group 1 (black) and between the clinical and non-clinical animals

1090   in group 3 (blue/green) at both 7dpi and 11dpi (mixed effects model).

1091

1092   **Figure 7. Clinical animals develop a higher viraemia than non-clinical animals.** Comparison

1093   of C_T values in blood at different days post infection in calves in group 1 (A), group 2 (B) and

1094   group 3 (C). The left-hand plots show the C_T values for clinical calves and the right-hand

1095   plots those for non-clinical calves. (D). Blood samples that were positive on PCR were titred

1096   on MDBK cells, and viral foci counted manually. A letter C after the animal identification

1097   number denotes a clinical animal.

1098

1099   **Figure 8. Virus is present in the nodules of clinical animals at 11dpi.** Skin samples were

1100   taken from using a 2mm biopsy punch at 5dpi, 11dpi and 16dpi. Viral DNA was extracted

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1116   2, magenta); and calves infected with microdoses of LSDV only (group 3, blue).

1117

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1126