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Putative roles of mosquitoes (Culicidae) and biting midges (*Culicoides* spp.) as mechanical or biological vectors of lumpy skin disease virus

Anca I. Paslaru[1,2,3] | Lena M. Maurer[1] | Andrea Vögtlin[2,3] | Bernd Hoffmann[4] | Paul R. Torgerson[5] | Alexander Mathis[1] | Eva Veronesi[1]

[1]National Centre for Vector Entomology, Institute of Parasitology, Vetsuisse Faculty, University of Zürich, Zürich, Switzerland

[2]Diagnostics department, Institute of Virology and Immunology (IVI), Mittelhäusern, Switzerland

[3]Department of Infectious Diseases and Pathobiology, Vetsuisse Faculty, University of Bern, Bern, Switzerland

[4]Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany

[5]Section of Epidemiology, Vetsuisse Faculty, University of Zürich, Zürich, Switzerland

Correspondence
Alexander Mathis, National Centre for Vector Entomology, Institute of Parasitology, Vetsuisse Faculty, University of Zürich, Winterthurerstr. 266A, 8057 Zürich, Switzerland.
Email: alexander.mathis@uzh.ch

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Abstract

The stable fly *Stomoxys calcitrans* (Diptera: Muscidae) is considered as the main mechanical vector of the lumpy skin disease virus (LSDV). In addition, the mosquito species *Aedes aegypti* (Diptera: Culicidae) was shown to transmit the virus from donor to receptor animals. Retention of the virus for several days was shown for two additional tropical mosquito species and the biting midge *Culicoides nubeculosus* (Diptera: Ceratopogonidae). In the present study, viral retention for 10- or 7-days post feeding on virus-spiked blood through a membrane was shown for field-collected *Aedes japonicus* and laboratory-reared *Culex pipiens*, two widely distributed mosquito species in temperate regions. Viral DNA could be detected from honey-coated Flinders Technology Associates (FTA) cards and shedded faeces for 1 or 4 days after an infectious blood meal was given to *Ae. aegypti*. Virus increase over time and virus dissemination was observed in laboratory-reared *C. nubeculosus*, but the virus could be isolated from field-collected biting midges only from the day of exposure to the blood meal. Thus, mosquitoes might serve as mechanical vectors of LSDV in case of interrupted feeding. A putative biological virus transmission by Culicoides biting midges, as suspected from field observations, deserves further investigations.

KEYWORDS
Aedes aegypti, *Aedes japonicus*, *Culex pipiens*, *Culicoides nubeculosus*, field-collected *Culicoides*, transmission

INTRODUCTION

Lumpy skin disease (LSD) is caused by a virus in the genus Capripoxvirus from the Poxviridae family, affecting mainly cattle. The disease is characterized by skin nodules, which may cover the whole body, and by similar lesions in the internal mucosa of the digestive and respiratory tracts. Systemic effects include pyrexia, anorexia, dysgalactia, and pneumonia. Though case fatality is low, morbidity is high, making LSD an economically important disease which is categorized as notifiable by The World Organization of Animal Health (OIE) (Davies, 1991; Sanz-Bernardo et al., 2020; Tuppurainen & Oura, 2012). LSD used to be endemic to Sub-Saharan Africa but first epidemics occurred in the Middle East in the 1980s (Tuppurainen & Oura, 2012). In 2015, the disease was diagnosed in south-eastern Europe (Greece, the Balkans) where it rapidly spread but was controlled by the implementation of concerted measures

(Tuppurainen et al., 2020). Flying blood-feeding insects have been incriminated as vectors by circumstantial evidence (seasonality of transmission, lack of transmission in insect-proof environments) and mathematical models (Gubbins et al., 2020; Kahana-Sutin et al., 2017; Magori-Cohen et al., 2012; Sprygin et al., 2019). The main suspected vector is the biting stable fly *Stomoxys calcitrans* (Linnaeus, 1758) (Diptera: Muscidae), and experimental transmission of LSD virus (LSDV) from infected to susceptible animals via blood-fed *S. calcitrans* was proven (Issimov et al., 2020; Sohier et al., 2019). A very recent study demonstrated the retention of LSDV in *S. calcitrans* for 4 days (7/47 flies positive for LSDV DNA in quantitative polymerase chain reaction [qPCR] on homogenized whole insects) and in rare cases (1/41) up to day 8 after feeding on an infected animal (Sanz-Bernardo et al., 2021). In another recent study that addressed the mode of LSDV transmission by *S. calcitrans*, flies were fed with virus-spiked blood through a membrane, and heads, bodies, regurgitated blood, and faeces were investigated by qPCR and virus isolation in cell culture. Thus, viral DNA was detected in all specimens up to the full duration of the experiment of 3 days, and viable virus up to 2 days (Paslaru et al., 2021). Viral loads did not increase over time, consolidating *S. calcitrans* as mechanical vector.

Additional blood-feeding insects have experimentally been investigated for their potential role as LSDV vectors, namely laboratory colonies of mosquitoes (Diptera: Culicidae; *Aedes aegypti* [Linnaeus, 1762], yellow fever mosquito; *Anopheles stephensi* Liston, 1901, Asian malaria vector; *Culex quinquefasciatus* Say, 1823, southern house mosquito) and *Culicoides* biting midges (Diptera: Ceratopogonidae; *Culicoides nubeculosus* [Meigen, 1830], Western Palearctic species), collectively termed 'model vector species' by Sanz-Bernardo et al. (2021). They were fed on lesions of LSDV-infected donor animals or virus-spiked blood through membranes, and virus transmission to recipient animals was investigated (Chihota et al., 2001, 2003). LSDV could be isolated from all six recipient animals exposed to *Ae. aegypti* between 2 and 6 days after feeding on donor animals, and 5/6 displayed clinical signs. In contrast, no animals challenged with *An. stephensi*, *Cx. quinquefasciatus* or *C. nubeculosus* became infected. In another recent study, viral retention in insects after feeding on infected cattle was analysed (Sanz-Bernardo et al., 2021). The virus was detected in homogenates of whole insects by qPCR up to 2 (*Cx. quinquefasciatus*), 4 (*C. nubeculosus*) or 8 days (*Ae. aegypti*) after feeding.

Culicidae were incriminated as LSDV vectors in a single report due to high populations at areas of the first disease outbreaks in Kenya, (Burdin & Prydie, 1959, cited in Sprygin et al., 2019). *Culicoides* biting midges were recently considered as putative vectors, because the explosive spread of LSDV in the Balkan countries was reminiscent of the bluetongue epizootic in Western Europe whose causative virus is efficiently spread by *Culicoides* spp. Indeed, LSDV DNA was recorded in field-collected, non-engorged *Culicoides punctatus* (Meigen, 1804) during the 2014–2015 LSDV outbreak in Turkey (Sevik & Dogan, 2017).

Finally, ixodid ticks were experimentally demonstrated to act as mechanical vectors of LSDV, but their lifecycle does not fit with the rapid spread of LSD as described above. Vertical transmission of LSDV was observed in ticks, and thus their epidemiological significance might be a role as reservoirs (summarized in Berg et al., 2015; Sprygin et al., 2019).

The present study expands on the findings of the insect 'model vector species'. The LSDV vector suitability of further species was investigated using an artificial feeding method under laboratory conditions. The species included were *Cx. pipiens* Linnaeus, 1758 (northern house mosquito, laboratory-reared), *Aedes japonicus* (Theobald, 1901) (Asian bush mosquito, invasive to Europe and Northern America and locally highly abundant [Koban et al., 2019], field-collected) in addition to laboratory-reared *Ae. aegypti*, *C. nubeculosus*, and field-collected *Culicoides* spp.

MATERIALS AND METHODS

Insects

Laboratory strains of the mosquito species *Ae. aegypti* (AAE_IPNC strain; generation 22 in our laboratory) and *Cx. pipiens* (CPI_BRW strain; maintained in our laboratory since 2017, generations not recorded) were initially provided by the Institut Pasteur, New Caledonia, and The Pirbright Institute UK, respectively. Eggs of *Ae. japonicus* mosquitoes were collected with ovitraps in the field in the Zürich area (Switzerland) as described (Verhulst et al., 2020). Mosquitoes were reared according to standard protocols published earlier by our group (Glavinic et al., 2020; Verhulst et al., 2020; Wagner et al., 2018).

Culicoides nubeculosus were originally provided by The Pirbright Institute and reared as described (Kaufmann et al., 2015). Live *Culicoides* spp. were collected at a farm in the outskirts of Zürich with OVI UV light traps as earlier described in detail (Paslaru et al., 2018).

Oral exposure of insects to virus

The LSDV (Macedonia 2016, field strain) (Möller et al., 2019) was propagated five times on Madin-Darby bovine kidney epithelial cells (MDBK NBL-1; CCL-22 line, ATCC), reaching a final titre of 6.25 $\log_{10}$TCID$_{50}$/ml. The virus was mixed in a ratio of 1:9 with heparinised bovine blood (obtained from a local abattoir, Zürich), yielding a final titre of 5.75 $\log_{10}$TCID$_{50}$/ml in the infectious blood meals.

Mosquitoes (7–9 days old) were exposed to infectious blood meals for 45 min as earlier described (Veronesi et al., 2018) but by using Parafilm M (Sigma–Aldrich, Buchs, Switzerland) instead of pig intestine as a membrane for the Hemotek feeders (Hemotek Ltd., Lancashire, UK). Field-collected *Culicoides* and 2–3 days old *C. nubeculosus* were exposed to infectious blood meals for 30–45 min at 25 ± 4 °C precisely as described (Paslaru et al., 2018). Freshly engorged insects were collected after immobilization by exposure to −20 °C for 4–5 min. Ten were immediately stored at −80 °C (Day 0 specimens) for further tests whereas the remaining ones were incubated in batches of approximately 30 under a fluctuating temperature

regime (14–28 °C, mean 22 °C; 80 %–85 % relative humidity) in a climate chamber. At different time intervals post-incubation, the live insects of a batch were collected after immobilization as described above, individually transferred into 1.5 ml Eppendorf tubes, and stored at −80 °C until molecular analysis. Also, 1 ml aliquots of the infectious inoculums were collected immediately after the blood-feeding (Day 0 samples).

All the feeding, manipulation, and incubation of LSDV-exposed insects were done under biosafety containment level 3 (BSL3) conditions.

Processing of insects

Heads and bodies (abdomens and thoraxes) were chopped by using sterile needles 26 G × ½″ (Fine-Ject, Tuttlingen, Germany) and separately transferred into 1.5 ml Eppendorf tubes containing either 180 µl TE (Tris ethylenediaminetetraacetic acid) buffer (heads) or 400 µl Glasgow Minimum Essential Media (GMEM) (Gibco, Thermo Fisher Scientific, Reinach, Switzerland) supplemented with 2 % antibiotics and fungizone (1000 IU/ml penicillin/streptomycin; 4 µg/ml amphotericin; Gibco) (bodies). In an additional experiment with *C. nubeculosus*, heads, wings, and abdomens were separately homogenized in 400 µl GMEM. Samples were manually homogenized for 30 s using sterilized polypropylene pestles (Sigma–Aldrich, Gillingham, UK) mounted to a motorized grinder (Micro Handrührer, Carl Roth, Karlsruhe, Germany), centrifuged for 5 min at 13,000*g* and stored at −80 °C until further use.

Collection of saliva and faeces of *Ae. aegypti*

Aedes aegypti females ($n = 20$) exposed to LSDV-spiked blood as described above were individually incubated in cardboard boxes (Adelphi healthcare packaging 4 oz., APD Deep, Haywards Heath, UK), covered with nets at both sides. Every box was provided with a Flinders Technology Associates card (FTA®, Sigma–Aldrich, Darmstadt, Germany) coated with Manuka honey (nu3 Manuka-Honig MGO 400, Alnatura, Zürich, Switzerland) and blue food colouring (Dr Oetker-Lebensmittelfarben-Set, Coop, Zürich, Switzerland) to collect expectorated saliva (Hall-Mendelin et al., 2010). Also, a filter paper (Whatman 1541-185, Grade 541 Ashless Fast Filter Paper, Tisch Scientific, Cleves, OH, U.S.A.) was provided at the bottom of each box to collect the mosquitoes' faeces. The FTA cards and filter papers were exchanged at Days 1, 4, 7, and 10 post exposure to the infectious blood meals.

Elution of saliva from FTA cards and faeces from the filter papers was performed basically as described (Ritchie et al., 2013). Briefly, they were incubated at 4 °C for 20 min in a 2 ml Eppendorf tube with 400 µl GMEM supplemented with 2 % antibiotics (as above). After vortexing for 15 s, the supernatants were transferred into 1.5 ml Eppendorf tubes and stored at −80 °C for further analyses (DNA extraction and virus isolation, see below).

LSDV DNA detection

Extraction of DNA from 180 µl aliquots of the insect homogenates, the elutes or the virus-spiked blood meals was carried out using the QIAamp viral DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions (elution volume 100 µl). LSDV DNA was detected by qPCR with the primers and the probe described earlier (Stubbs et al., 2012) by using the EXPRESS qPCR SuperMix Universal (Invitrogen, Zug, Switzerland). Amplification using 5 µl of DNA solution was done in QuantiStudioFlex 7 (Applied Biosystems, Thermo Fischer, Zug, Switzerland) using the following program: 50 °C for 2 min (UDG incubation), 95 °C for 2 min (UDG inactivation, Taq polymerase activation), followed by 50 cycles of 15 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C.

A standard curve was generated using 10-fold serial dilutions of the viral inoculum used in the experiments, revealing a cut-off of Cq ≤ 34.6 (Figure S1). The virus-spiked blood meals had Cq values ranging between 17.3 and 29.

Virus isolation

Aliquots (200 µl) of the supernatants of GMEM sample homogenates or elutes were added to 25 cm^2 flasks seeded with 7×10^5 cells (MDBK NBL-1; CCL-22 line) 24 h before inoculation. The flasks were incubated for 1 h at 37 °C with 5 % CO_2 after which growth medium (GMEM with 2.5 % horse serum [Biowest S0910, Lubio Science GmbH, Zürich, Switzerland] supplemented with 2 % antibiotics and fungizone) was added. The incubation was continued at the same conditions for 7 days, and the cells were examined daily for cytopathic effect (CPE).

Statistical analysis

A binomial (logistic) regression model was applied to assess whether the presence or absence of LSDV varied according to species and incubation period. The analyses were performed using R (https://www.r-project.org/). For quantitative analysis, the reciprocal of the Cq values were analysed. For Cq values above the cut-off value of 34.6, the reciprocal was allocated a value of zero which was consistent with no virus particles in the sample. These transformed data were then analysed with a tweedie generalized linear model which can model data that have a positive mass at zero but is otherwise continuous.

RESULTS

Three mosquito species (*Ae. aegypti*, *Ae. japonicus*, *Cx. pipiens*) as well as biting midges (*C. nubeculosus*, field collected *Culicoides* spp.) were exposed to LSDV-spiked blood and examined for the presence of the virus in bodies and head or wings (as proxy for virus dissemination) at

TABLE 1 Analyses of different body parts of three mosquito species for the presence of lumpy skin disease virus DNA by quantitative polymerase chain reaction (qPCR)[a] or viable virus by cell culture inoculation at different time points after exposure to infectious blood meals.

Species	Day post infectious blood meal	qPCR (head) No. positive/no. tested	qPCR (body[b])	CPE in cell culture (body)
Aedes aegypti	0	3/10	5/5	nd
	1	1/10	4/5	2/4
	2	0/10	4/5	3/4
	4	0/10	1/5	1/1
	7	0/10	0/5	nd
	10	0/10	0/5	nd
Aedes japonicus	0	0/10	5/5	5/5
	1	0/10	0/5	nd
	2	0/10	2/5	0/2
	4	0/10	4/5	4/4
	7	0/10	0/5	nd
	10	0/10	1/5	1/1
Culex pipiens	0	2/10	5/5	nd
	1	0/10	3/5	3/3
	2	1/10	5/5	3/5
	4	0/10	2/5	1/2
	7	1/10	1/5	1/1
	10	0/10	0/5	nd

Abbreviations: CPE, cytopathic effect (all qPCR-positive bodies examined); nd, not done.
[a]Positive: Cq ≤ 34.6.
[b]Body: thorax and abdomen (bodies investigated from all specimens qPCR-positive in the head samples, complemented with randomly chosen samples).

different time points after feeding. In addition, saliva expectorated onto FTA cards and faeces shed by exposed *Ae. aegypti* were accordingly investigated.

Mosquitoes

Mosquitoes of three species (*Ae. aegypti*, *Ae. japonicus*, *Cx. pipiens*) were exposed to LSDV-spiked blood, and the heads of 10 specimens per time point (0, 1, 2, 4, 7, 10 days post feeding) examined by qPCR for the presence of viral DNA (Table 1, Table S1). In the freshly blood-fed mosquitoes (d 0) viral DNA was detectable in few (2, 3) heads of two species. All heads examined from *Ae. japonicus* tested negative, whereas single additional heads of the two other species were positive (d 1 for *Ae. aegypti*; d 2, 7 for *Cx. pipiens*). Five bodies (thorax and abdomen) per time point were examined, including the bodies from all specimens with positive qPCR in the head samples. Viral DNA was detected in all Day 0 body samples of all three mosquito species, and up to Day 4 in *Ae. aegypti*, Day 10 in *Ae. japonicus* and Day 7 in *Cx. pipiens*. Overall, from the total 25 body samples tested from the time points d 1–10 of each species, 9, 7, and 11, respectively, were positive. qPCR-positive body samples were investigated for viable virus by cell culture. CPE caused by the presence of infectious virus particles was observed in all specimens from Day 0 (only *Ae. japonicus* samples investigated) and in total 19 of the 27 samples (d 1–10), originating from all three species, including one sample from d 10 (Table 1).

Saliva and faeces from 20 *Ae. aegypti* orally exposed to LSDV were collected with FTA cards and filter papers provided in the cages. qPCR with extracted DNA yielded Cq values below 50 up to 10 days post-infection (Figure 1). However, when applying the cut-off value generated from the standard curve (Cq ≤ 34.6; Figure S1) only 1 FTA card from Day 1, and 7 (d 1: 5; d 4: 2) filter papers up to Day 4 can be considered positive. No CPE was observed with these specimens.

Culicoides

Similar to the experiments with the three mosquito species, the presence of LSDV was investigated in body parts of *C. nubeculosus* that fed on virus-spiked blood, with an additional time point investigated (d 5 after blood meal; Table 2a, Figure 2; Table S1). The number of heads positive for virus DNA tended to be higher with increasing time span after the blood meal, with 3/10 being positive at both d 7 and d 10 post exposure (for statistical significance see below). qPCR with DNA from bodies of these head-positive specimens, complemented to 5 samples by randomly chosen other insects, revealed no virus DNA in the biting midges at time points d 4 and d 5, but high positivity rates in the early phase of the experiment (d 0, d 1, d 2) and

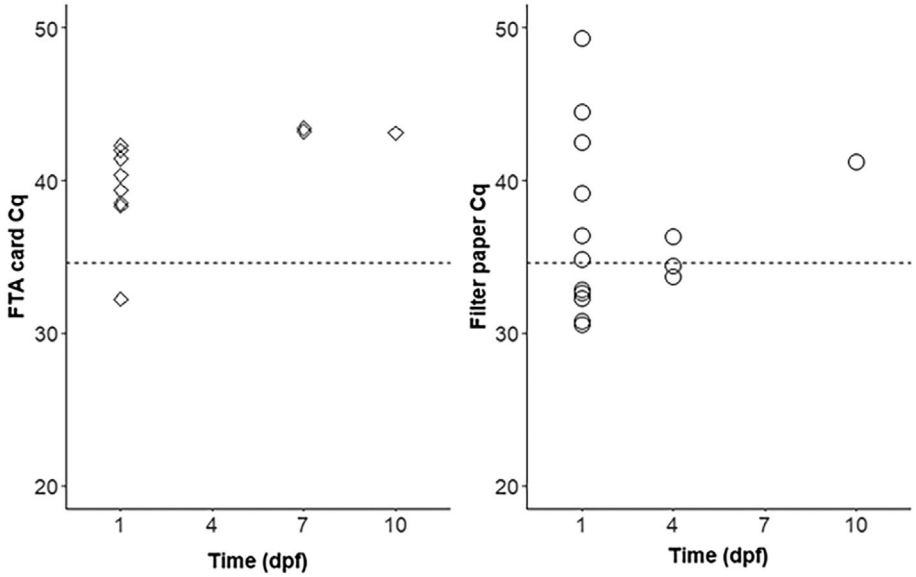

FIGURE 1 Detection of lumpy skin disease virus DNA by quantitative polymerase chain reaction from honey-coated Flinders Technology Associates (FTA) cards and filter papers provided to 20 individually kept *Aedes aegypti* mosquitoes at different days post feeding (dpf) on virus-spiked blood. The Cq values below 50 are shown (dotted line: threshold 34.6).

TABLE 2 Analyses of different body parts of *Culicoides nubeculosus* for the presence of lumpy skin disease virus DNA by quantitative polymerase chain reaction (qPCR)[a] or viable virus by cell culture inoculation at different time points after exposure to infectious blood meals.

(a) First experiment

Day post-infectious blood-meal	qPCR (head)	qPCR (body[b])	CPE$^+$ in cell culture (body)
	No. positive/no. tested		
0	1/10	5/5	4/5
1	0/10	5/5	5/5
2	1/10	4/5	1/4
4	2/10	0/5	nd
5	1/10	0/5	nd
7	3/10	3/5	0/3
10	3/10	2/5	2/2

(b) Second experiment

Day post-infectious blood-feeding	qPCR (head)	qPCR (abdomen)	qPCR (wings)	CPE in cell culture (head)	CPE in cell culture (abdomen)	CPE in cell culture (wings)
	No. positive/no. tested					
1	3/10	2/5	nd	0/3	0/2	nd
10	1/38	1/20	1/16	1/1	1/1	1/1

Abbreviations: CPE, cytopathic effect (all qPCR-positive specimens examined); nd, not done.
[a]Positive: Cq ≤ 34.6.
[b]Body: thorax and abdomen. Bodies (a) or abdomens only (b) investigated from all specimens qPCR-positive in the head samples, complemented with randomly chosen samples.

moderate ones towards the end (d 7, d 10). Viable virus was isolated by cell culture also from the body samples from d 10 (Table 2a). A second experiment focused on analysing more insects only at a later stage (d 10) and refining the analyses by investigating the abdomens and wings separately in addition to the heads (Table 2b). One of the 38 biting midges analysed at d 10 showed a disseminated infection, that is, qPCR and cell cultures were positive for all samples analysed.

In the corresponding experiment with field-collected *Culicoides* spp., all surviving individuals per time point were analysed (n = 10–24; Table 3). qPCR was positive only with specimens of d 1 after blood

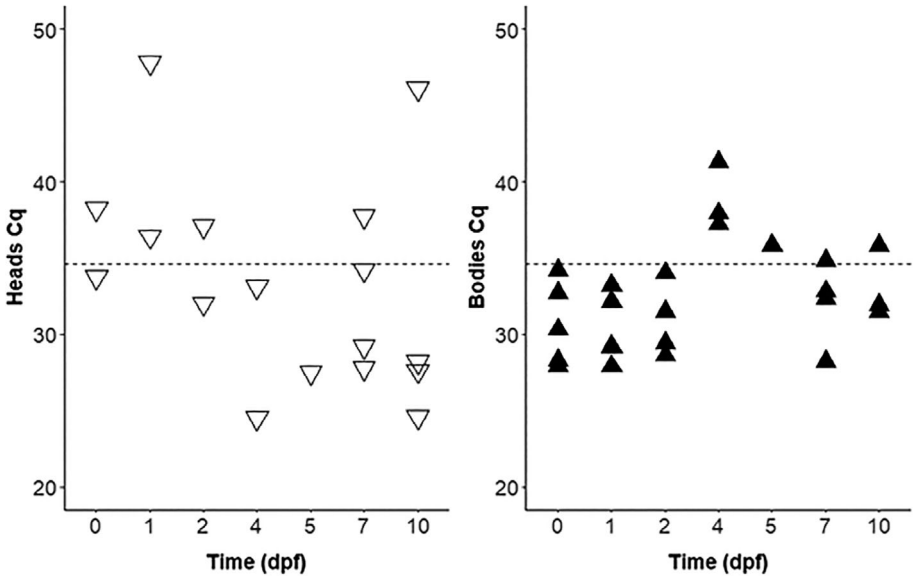

FIGURE 2 Detection of lumpy skin disease virus DNA by quantitative polymerase chain reaction (qPCR) in *Culicoides nubeculosus* heads and bodies at different days post feeding (dpf) on virus-spiked blood. Ten heads were investigated per time point, and the bodies from all specimens qPCR-positive in the head samples, complemented with randomly chosen samples to total 5. The Cq values below 50 are shown (dotted line: threshold 34.6).

TABLE 3 Analyses of different body parts of field-collected *Culicoides* spp. for the presence of lumpy skin disease virus DNA by quantitative polymerase chain reaction (qPCR)[a] or viable virus by cell culture inoculation at different time points after exposure to infectious blood meals.

Day post infectious blood meal	qPCR (head) No positive/ no tested	qPCR (body[b])	CPE in cell culture (body)
0	0/10	3/5	3/3
1	3/24	0/5	nd
3	0/15	0/5	nd
5	0/15	0/5	nd
7	0/20	0/5	nd
10	0/15	0/5	nd

Abbreviations: CPE, cytopathic effect (all qPCR-positive bodies examined); nd, not done.
[a]Positive: Cq ≤ 34.6.
[b]Body: thorax and abdomen (bodies investigated from all specimens qPCR-positive in the head samples, complemented with randomly chosen samples).

feeding; homogenates from bodies were positive by DNA analyses and cell culture only from insects obtained at the day of feeding (d 0).

Statistical analysis

There was neither a significant difference detected between species of arthropods tested and the presence of LSDV nor a change over time in the proportion of insects from which virus could be detected by PCR. The only exception to the latter is the second experiment with *C. nubeculosus* (Table 2b; i.e., there were significantly less insect heads infected at d 10 as compared to d 1, $p = 0.0245$, Fisher test). The reciprocal of the Cq values significantly increased with time only in *C. nubeculosus* (d 68: $t = 2.20$, $p = 0.0312$). Therefore, the Cq values decreased, and viral load increased over time.

DISCUSSION

With the present study, two additional mosquito species (*Ae. japonicus*, *Cx. pipiens*) were shown to harbour viable LSDV in their bodies upon oral exposure for an extended period of time (10 and 7 days, respectively). Thus, retention of LSDV in mosquito vectors might be a general feature but the mechanism remains unknown (see below). The retention times of LSDV in different insects varied among the studies (Chihota et al., 2001, 2003; Sanz-Bernardo et al., 2021), ranging from 4 to 9 days for *Ae. aegypti* and 0 to 10 days for *C. nubeculosus*. This might be due to the use of different virus strains and different feeding methods, that is, through membrane on infectious blood versus on lesion of infected animals. It was shown that LSDV was much more efficiently acquired by different vector species when feeding on clinical animals as compared to subclinical ones (Sanz-Bernardo et al., 2021). By contrast with earlier studies, virus detection in the present study was done on homogenates of heads and body parts (thorax and abdomen; wings) (Figure S2) rather than on whole insects, allowing for estimating the dissemination of LSDV.

In all three mosquito species investigated, many more body samples were qPCR-positive than head samples, indicating that the virus was not efficiently retained in the mouthparts and that there was no virus dissemination (Table 1). This is particularly exemplified with *Ae. japonicus*, of which all d 0 heads examined were negative and all bodies from this timepoint positive by PCR and, for confirmatory reasons, also by virus-cell isolation. In *Ae. aegpyti*, PCR on heads and on FTA cards, containing expectorated saliva (Hall-Mendelin et al., 2010) was only positive from Day 1 after feeding. Thus, mechanical transmission of LSDV by this species seems feasible in case of interrupted feeding; also, by considering that the LSDV minimum infective dose when inoculated intradermally is as low as 10^1 TCID50/ml to establish an infection (Carn & Kitching, 1995). Interrupted feeding is well known for the main vector *S. calcitrans*, whose bites are painful, triggering fierce defensive behaviour of hosts (Baldacchino et al., 2013). Such a behaviour against mosquitoes (Matherne et al., 2018) and therefore interrupted feeding is also evident for mosquitoes as, for example, substantiated by revealing mixed blood meals in engorged mosquitoes (Hernandez-Colina et al., 2021; Schönenberger et al., 2016). Faecal shedding of LSDV from *Ae. aegypti* was observed until Day 4 after feeding. The significance of this finding is unclear. It might merely contribute to transfer the virus to naïve hosts upon interrupted feeding as mosquitoes, by contrast with *S. calcitrans* (Paslaru et al., 2021), do not regurgitate (i.e., reflux of gut content) (Day, 1955). However, such a transmission, corresponding to a transmission by contact of animals, is considered ineffective (Sprygin et al., 2019). Thus, mechanical transmission in the course of interrupted feeding seems possible for *Ae. aegypti*. Interestingly, this species was shown to transmit LSDV to recipient animals up to Day 6 after feeding in infected animals (Chihota et al., 2001). However, all animals involved in this study were exposed to infected mosquitoes, that is, there was no control animal involved (challenged with non-infected mosquitoes). Though several lines of evidence suggest that direct or indirect transmission (e.g., through fomites, feed, and water contaminated with secretions from infected animals) do not play a significant role in LSDV epidemiology they may also be transmission routes (Berg et al., 2015). Animal-to-animal transmission experiments with *S. calcitrans*, involving LSDV (Issimov et al., 2020; Sohier et al., 2019) were done by allowing the flies to partially (10 min) feed on infected animals and then transferring them immediately to naïve animals. Such experiments with a re-feeding after 24 h were unsuccessful (Chihota et al., 2003). In a study with *Aedes vexans* (Meigen, 1830), transmission of the porcine reproductive and respiratory syndrome virus was achieved after interrupted feeding for 1 min on viremic donor pigs and immediate transfer to healthy pigs (Otake et al., 2002).

From the mosquito species so far investigated with regard to LSDV retention, *Ae. aegypti* and *An. stephensi* preferably bite humans (Scott & Takken, 2012) and are of little significance for virus transmission under field conditions. The other investigated mosquito species (*Ae. japonicus*, *Cx. pipiens*, *Cx. quinquefasciatus*) are opportunistic feeders and also bite large mammalian hosts (Becker et al., 2010; Schönenberger et al., 2016).

From experiments on biologically transmitted pathogens, it is known that insect populations from laboratory colonies and from field-collections differ with regard to vector competence (Silaghi et al., 2017). To the best of our knowledge, nothing is known with this regard on mechanical transmission of animal pathogens. By contrast with the mechanical transmission of plant pathogens (designated non-circulative), which involves specific interactions of the pathogens with receptors on mouthparts or in the alimentary tract, no such interaction is known for animal viruses (Blanc & Gutierrez, 2015). Consequently, no difference in mechanical transmission between field-collected and laboratory-reared populations are to be expected. Nevertheless, with the present study, the suitability of field-collected mosquitoes (*Ae. japonicus*) to retain viable LSDV for an extended period of time (10 d, Table 1) and potentially serve as a vector was shown.

Other mosquito species that feed on cattle and which can be highly abundant in Europe (Schönenberger et al., 2016), for example, the flood-plain species *Ae. vexans* and *Aedes sticticus* (Meigen, 1838), and also *Aedes cantans* (Meigen, 1818)/*Aedes annulipes* (Meigen, 1830), *Anopheles maculipennis* s.l. Meigen, 1818, *Coquillettidia richiardi* (Ficalbi, 1898), should be considered as putative LSDV vectors.

By contrast with the earlier study including *C. nubeculosus*, in which only insects at the day of feeding on an LSDV-infected steer were PCR positive (Chihota et al., 2003), viable virus was isolated from homogenized bodies up to the end of the experiments (Day 10 post-infectious blood meal) in the present study (Table 2a). Interestingly, Cq values decreased over time, and a disseminated infection at d 10 post feeding was identified in one insect (Table 2b). Considering the postulated absence of salivary gland barriers in *Culicoides* spp. (Fu et al., 1999), these findings indicate that the laboratory-reared *C. nubeculosus* might behave as biological vectors of LSDV under laboratory conditions.

By contrast with these *C. nubeculosus*, no persistence of LSDV was observed in field-collected biting midges (Table 3). However, only relatively few field-collected biting midges could be investigated due to their inherent low feeding rates under laboratory conditions (Paslaru et al., 2018). *Culicoides punctatus*, the incriminated LSDV vector in Turkey (Sevik & Dogan, 2017) is widely distributed in the Northern hemisphere and can reach high abundances (e.g., The Netherlands; around 30% of specimens; Meiswinkel et al., 2008). This species, however, is virtually absent at the collection location of the biting midges used in the present study where the Culicoides fauna is nearly exclusively composed of *Culicoides obsoletus* (Meigen, 1818) and *Culicoides scoticus* Downes & Kettle, 1952 (Paslaru et al., 2018). Given the very high population densities that biting midges can reach, even low vector capabilities could be contributing to the transmission of pathogens. Experimentally investigating LSDV vector competence of *Culicoides* from locations with other species compositions is highly desired.

AUTHOR CONTRIBUTIONS

Anca I. Paslaru: Methodology; formal analyses; investigation; writing—original draft. **Lena M. Maurer**: Investigation; writing—review and editing. **Andrea Vögtlin**: Resources; writing—review and editing; funding acquisition. **Bernd Hoffmann**: Resources. **Paul R. Torgerson**:

Formal analyses; writing—review and editing. **Alexander Mathis**: Conceptualization; writing—original draft; supervision; funding acquisition. **Eva Veronesi**: Conceptualization; methodology; formal analyses; writing—original draft; supervision; project administration. All authors edited, read, and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Alexander Mathis https://orcid.org/0000-0002-5499-531X

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Table S1. Lumpy skin disease virus (LSDV) qPCR values of heads, bodies (abdomen[1] and thorax), and wings[2] of all the investigated arthropods. At least 10 individual's heads and 5 bodies were tested for each time point. All the surviving field-collected *Culicoides* were tested.

Figure S1. Standard curve for LSDV Macedonia 2016 field strain (Möller et al., 2019). Conversion of viral DNA (Cq values) into log10TCID50/ml was determined.

Figure S2. Cytopathic effect in MDBK cell cultures of homogenates of *Culicoides nubeculosus* head (a), thorax/abdomen (b), and wings (c) 10 days post feeding.

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