1 **Natural reassortment of a segmented RNA arbovirus illustrates plasticity of phenotype in**

2 **the arthropod vector and mammalian host *in vivo***

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28   **ABSTRACT** Segmented RNA viruses are a taxonomically diverse group of 11 families that can
29   infect plant, wildlife, livestock and human hosts. A shared feature of these viruses is the
30   ability to exchange genome segments during co-infection of a host by a process termed
31   'reassortment'. Reassortment enables rapid evolutionary change, but in the case of
32   segmented RNA viruses utilising an arthropod vector is set against the constraint of
33   purifying selection and genetic bottlenecks imposed by replication in two evolutionarily
34   distant hosts. In this study, we use an *in vivo* host: arbovirus: vector model to investigate the
35   impact of reassortment on two phenotypic traits: vector competence and virulence in the
36   host. Bluetongue virus (BTV) (*Reoviridae*) is the causative agent of bluetongue (BT), an
37   economically important disease of domestic and wild ruminants and deer. The genome of
38   BTV is comprised of 10 linear segments of dsRNA and the virus is transmitted between
39   ruminants by *Culicoides* biting midges (Diptera: Ceratopogonidae). Five strains of BTV
40   representing three serotypes (BTV-1, BTV-4 and BTV-8) were isolated from naturally
41   infected ruminants in Europe and parental/reassortant lineage status assigned through full
42   genome sequencing. Each strain was then assessed in parallel for the ability to infect
43   *Culicoides* and to cause BT in sheep. Our results demonstrate that two reassortment strains,
44   which themselves became established in the field, had obtained high replication ability in *C.
45   sonorensis* from one of the parental virus strains which allowed inferences of the genome
46   segments conferring this phenotypic trait.

47   **IMPORTANCE**

48   Reassortment between strains can lead to major shifts in the transmission parameters and
49   virulence of segmented RNA viruses with consequences for spread, persistence and impact.
50   The ability of these pathogens to change their phenotypes rapidly in response to selection
51   pressure in new environments presents a major challenge in understanding factors driving
52   emergence. Utilising a natural mammalian host-insect vector infection and transmission
53   model, we demonstrated for the first time the genetic basis for a phenotypic trait of BTV
54   within strains directly isolated from the field and, hence, selected and relevant for natural
55   transmission.

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61   **KEYWORDS**

62   Orbivirus; Reassortant; Bluetongue Virus; Emerging Diseases; Diptera; Ruminant

63   **INTRODUCTION**

64   Segmented RNA viruses include a diverse array of species classified across eleven taxonomic
65   families that infect a wide range of hosts that include plants, animals, fungi, bacteria and
66   marine protists (1). A key feature of segmented RNA viruses is their ability to exchange
67   complete segments of RNA during coinfection of a single host cell by two or more virus
68   strains, producing hybrid progeny. This form of recombination is termed 'reassortment'. In
69   the case of arboviruses (arthropod-borne viruses), selection of reassortant strains with
70   advantageous phenotypic traits can occur through replication bottlenecks within both the
71   hosts and biological vectors of the virus. When compared to genetic drift through mutation,
72   reassortment can lead to more rapid changes in the phenotypic characteristics of progeny
73   viruses and can lead to an increased transmissibility (2), increased pathogenicity (3, 4) and
74   the potential for avirulent vaccine strains to revert to virulence in the field (5, 6).

75   Bluetongue virus (BTV) (*Reoviridae*) is the causative agent of bluetongue (BT), an
76   economically important disease of domestic and wild ruminants (7). The virus is primarily
77   spread between ruminants by *Culicoides* biting midges which act as biological vectors (8, 9).
78   Severe clinical signs of bluetongue (BT) are most commonly observed in specific breeds of
79   sheep (7) and are characterised by injury to the vascular and lymphatic endothelium. This
80   can result in haemorrhage and vascular leakage that in acute cases result in fever, oedema,
81   coronitis, oral and nasal erosion, cyanosis of the tongue and death (7, 10, 11). Cattle
82   typically show only mild clinical signs of BT following infection, but are important reservoirs
83   of the virus and recent outbreaks in naïve populations have documented more severe
84   clinical signs in this species caused by specific BTV strains (12, 13).

85   Since the turn of the century, there has been an unprecedented shift in the epidemiology of
86   BTV in Europe, involving the incursion of multiple strains into regions with no recorded
87   history of transmission (8, 14). These epidemics have persisted in some cases and BTV has
88   become endemic in several European countries, with major consequences for livestock
89   production and trade (15, 16). Full-genome sequencing of BTV has demonstrated that
90   reassortment occurs at a high frequency in the field (6, 17, 18) and this has been highlighted
91   as a potential driver of virus emergence and spread in the region (6). BTV has a linear dsRNA
92   genome consisting of 10 segments encoding seven structural (VP1-7) and at least four non-
93   structural (NS1-4) proteins (19, 20).

94   Under experimental conditions, reassortment of genome segments between BTV strains
95   during co-infection has been reported in the insect and ruminant hosts, where strains have
96   been introduced into hosts, vectors or cell culture simultaneously (21-24). Arbovirus species
97   and strain are also known to influence vector competence (25) and reassortment has been
98   used to generate viral strains that express different levels of infection rate in *Culicoides*
99   vectors in the laboratory (26). The proportion of a vector population able to become
100   infected with BTV following oral exposure has been demonstrated to be under a

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101 combination of genetic and environmental control in *Culicoides* (9, 27, 28). To date,
102 however, little is known regarding the impact of field-based reassortment of segmented
103 RNA viruses on transmission by vectors and whether vector susceptibility to infection can be
104 affected by this process.

105 Previous studies have also demonstrated variation in the clinical severity of BTV virus strains
106 *in vivo* (7, 29-31), but the molecular basis for pathogenicity of BTV strains is poorly
107 understood (32, 33). Pathogenicity and disease outcome appear to be highly complex and
108 cannot be explained by the presence or absence of a single genome segment or specific
109 combinations of segments (32-34). Reassortant bluetongue viruses generated from wild
110 type and attenuated strains using reverse genetics demonstrated impacts on pathogenicity
111 *in vitro* and *in vivo*, albeit with limited consistency between the different host systems used.
112 To date, specific virulence characteristics could not be assigned to specific gene segments
113 and, hence, no virulence markers for BTV strains exist (33, 35, 36). Field derived reassortant
114 BTV strains may therefore demonstrate differential pathogenicity or disease outcome to
115 that of their closely related strains or those which they share serotype. Studies with field-
116 derived reassortant strains complement those carried out using BTV strains generated
117 synthetically through reverse genetics, which have a far greater level of artificial selection
118 through multiple cell passage and plaque purification (33, 37).

119 The aim of this study is to define phenotypic transmission characteristics of five BTV strains
120 of three serotypes isolated from the Mediterranean Basin and Europe and to examine the
121 impact of reassortment on virus phenotype. We identified a history of reassortment
122 between these strains during their co-circulation in the field, which enabled the opportunity
123 to examine the impact of this process on BTV phenotype in both host and vector. This
124 represents the first fully comparative analysis of parental and reassortant strain phenotype
125 under highly controlled conditions using a natural BTV-sheep-*Culicoides* transmission model.

126 **RESULTS**

127 **Phylogenetic lineage of parental and reassortant strains can be traced.**

128 The genetic relationship between BTV strains used in the study (Table S1) was defined using
129 sequence comparison between segments (Table S2), phylogenetic analyses (Fig S1) and a
130 range of additional detection methods (Fig 1). Three strains were identified as parental
131 (BTV-1 MOR2004/01, BTV-4 MOR2004/02 and BTV-8 NET2006/06), while two strains were
132 identified as reassortant strains (BTV-4 MOR2009/07 and BTV-4 MOR2009/10) derived from
133 the lineages of the three parental strains.

134 The detection of the reassortant BTV-4 MOR2009/07 was supported by seven different
135 detection methods within the recombination detection program: RDP (1.00×10^{-10}),
136 GENECONV (7.42×10^{-110}), Bootscan (6.62×10^{-131}), Maximum Chi Square (4.02×10^{-28}),
137 CHIMAERA (2.03×10^{-29}), SISCAN (5.26×10^{-31}) and 3SEQ (3.22×10^{-100}). BTV-4 MOR2004/02 and

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138   BTV-1 MOR2007/01 sequences were identified as the major parent and the minor parent,
139   respectively (Fig 1). The detection of the second reassortant (BTV-4 MOR2009/10) was
140   supported by six different detection methods: GENECONV (4.55×10^{-136}), Bootscan (4.64×10^{-4}),
141   Maximum Chi Square (4.18×10^{-27}), CHIMAERA (5.92×10^{-36}), SISCAN (1.42×10^{-37}) and 3SEQ
142   (1.11×10^{-15}). BTV-4 MOR2009/10 was identified as a triple reassortant, sharing segments
143   from BTV-4 MOR2004/02 (Seg-2, 6 and 9), BTV-1 MOR2007/01 (Seg-4, 5, 7 and 10) and BTV-
144   8 NET2006/06 (Seg-1, 3 and 8) strains (Fig 1, Table S2). It is possible that the BTV-4
145   MOR2009/10 emerged from a single cell co-infection with two strains (BTV-4 MOR2009/07
146   and BTV-8 NET2006/06) rather than simultaneous co-infection with three different BTV
147   strains (BTV-1 MOR2007/01, BTV-4 MOR2004/02, and BTV-8 NET2006/06).

148   Two of the parental strains (BTV-1 MOR2007/01 and BTV-4 MOR2004/02) were isolated
149   from samples collected in Morocco, while the third parental strain (BTV-8 NET2006/06) was
150   isolated from a sample collected in the Netherlands. The field-derived reassortant strains,
151   BTV-4 MOR2009/07 and BTV-4 MOR2009/10 were also isolated from samples collected in
152   Morocco. The beginning and end recombination breakpoints (shown in Fig 1) corresponded
153   with the segment position in the sequence alignment and are for Seg-1 (1-3944), Seg-2
154   (3945-6910) Seg-3 (6911-9682), Seg-4 (9683-11663), Seg-5 (11664-13441), Seg-6 (13442-
155   15079), Seg-7 (15080-16235), Seg-8 (16236-17360), Seg-9 (17361-18411) and Seg-10
156   (18412-19233). Both reassortant strains had Seg-2 derived from BTV-4 MOR2004/02, and
157   therefore both belonged to the same genotype/serotype (Fig S1).

158   As both the BTV-4 MOR2009/07 and BTV-4 MOR2009/10 reassortants were derived from
159   the same parental strains, they were either identical or close to identical at the amino acid
160   level in Seg-2 (99.8%), 4 (100%), 5 (100%), 6 (100%), 7 (100%), 9 (100%) and 10 (100%).
161   However, BTV-4 MOR2009/07 and BTV-4 MOR2009/10 differed in the remaining segments:
162   Seg-1 (99.4%), Seg-3 (99.9%), and Seg-8 (97.7%) (Table S2).

163   **Infection of sheep with BTV by infected *Culicoides* is highly efficient, independent of BTV**
164   **strain.** A total of 2762 *C. sonorensis* were intrathoracically inoculated (ITI) with the 5 strains
165   of BTV, of which 1121 (40.6%) survived the incubation period and 486 (17.6%) successfully
166   blood fed on sheep (Table S3). All sheep in all replicates were successfully infected with BTV
167   despite ≤10 infected *C. sonorensis* taking a blood meal in 6 of 20 infection attempts and only
168   one individual *C. sonorensis* taking a blood meal from a sheep on two occasions. All five
169   strains of BTV were successfully transmitted on at least one occasion from the bites of 5 or
170   fewer infected *C. sonorensis* during the trials (Fig 2; Fig 3).

171   **Level of BTV viraemia in sheep varies with strain and number of infected *Culicoides***
172   **feeding.** There was no significant effect of BTV strain (P=0.47) or number of *Culicoides*
173   feeding (P=0.58) on the timing of peak viraemia (Fig 2; Fig 3). The level of peak viraemia
174   differed significantly amongst BTV strains (P=0.009), with a higher level of RNA detected (i.e.
175   lower C_T value) for BTV-1 MOR2007/01 compared with BTV-4 MOR2004/02 (P=0.02) and

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176   BTV-4 MOR2009/07 (P=0.02) (Fig 2; Fig 3). In addition, the level of peak viraemia increased
177   significantly (i.e. the C_T value decreased) with the number of *Culicoides* feeding (P=0.02, b=-
178   0.05, 95% confidence interval: -0.09 to -0.01) (Fig 2). At an individual replicate level, several
179   sheep produced a late and slowly progressing viraemia (Fig 3). In these three cases, BTV
180   RNA was not detected until 5 or 6 days post-infection. These cases involved BTV-4
181   MOR2009/07 transmitted by four *C. sonorensis* and two infections of BTV-4 MOR2009/10 by
182   one and three biting midges, respectively (Table S3).

183   **Clinical disease in infected sheep varies with BTV strain and illustrates multi-factorial**
184   **causation.** Infection with BTV-1 MOR2007/01 caused acute clinical disease in all four
185   exposed sheep necessitating euthanasia between 7-11 dpi for reaching pre-defined humane
186   endpoints (Fig.4; Fig S2 Tables S4 and S5). Clinical disease in sheep infected with BTV-1
187   MOR2007/01 was highly uniform in manifestation, while all other BTV strains exhibited
188   individual sheep variation in disease within infected cohorts. Three of the other strains
189   produced acute clinical disease necessitating euthanasia in two sheep and milder, chronic
190   disease in the other two (Fig 4; Fig S2; Table S5), while parental strain BTV-4 MOR2004/02
191   only led to acute disease in one sheep, which at post-mortem showed only mild pathological
192   damage. Although the overall severity score of the parental strain, BTV-4 MOR2004/02 was
193   comparable to those of the reassortment strains BTV-4 MOR2009/07 and BTV-4
194   MOR2009/10, the overall disease manifestation presented as more chronic, reflected in a
195   higher clinical index score accumulation based on signs such reddening of eyes, nasal
196   discharge and feet lesions (Figure 4; Fig S2; Table S5).

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198   At post-mortem all four sheep infected with BTV-1 MOR2007/01 demonstrated systemic
199   haemorrhages and oedema that was not only confined to the oral and nasal cavity, but also
200   generalised through the subcutaneous layer and skeletal muscles (Figure S3). These lesions
201   were less pronounced in BTV-8 NET2006/04 and further reduced in BTV-4 MOR2004/02.
202   Three of the four acutely affected sheep infected with either the reassortant strains BTV-4
203   MOR2009/07 or BTV-4 MOR2009/10 developed overt ulceration, either of the oral or labial
204   mucosa, or the muzzle, while only one sheep from the BTV-8 NET2006/04 cohort developed
205   mild ulceration among all the parental strains (Figure S3). The acutely affected sheep
206   infected with either reassortant strain also developed significant haemorrhages in the
207   oral/nasal cavities that was slightly less serve than BTV-1 MOR2007/01, but exceeding those
208   observed for BTV-4 MOR2004/02 and BTV-8 NET2006/04. All sheep which had exhibited
209   mild or moderate clinical disease and survived to the study end at 19/20 dpi only
210   demonstrated mild pathological signs of BTV infection at post-mortem, independent of the
211   BTV strain.

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213   **Susceptibility to infection in *Culicoides* varies significantly with BTV strain and can change**
214   **rapidly via natural reassortment.** A total of 5263 out of 6457 female *C. sonorensis* that
215   were fed directly on viraemic sheep survived eight days of incubation across the four

216   replicates (Fig 5; Table S6). In addition, a total of 2673 out of 4943 female *C. sonorensis* were
217   successfully membrane-fed on matched blood from the viraemic sheep and survived the
218   period of incubation (Table S6). The proportion of *Culicoides* in which BTV RNA was detected
219   varied significantly between strains tested (Fig 5). *Culicoides sonorensis* was close to
220   refractory to infection with parental strains BTV-1 MOR2007/01 (2.4%) and BTV-8
221   NET2006/04 (0.4%), but highly susceptible to infection with the third parental strain BTV-4
222   MOR2004/02 (21.6%). Both BTV-4 MOR2009/07 (28.3%) and BTV-4 MOR2009/10 (45.0%)
223   retained this infectivity following reassortment. The effect of blood-feeding-route (sheep vs
224   membrane-based system) differed amongst the strains, but broadly recapitulated the clear
225   difference in infectivity between BTV-1 MOR2007/01 and BTV-8 NET2006/04 in comparison
226   to the three other BTV strains (Fig 5; Fig 6; Table S7). In BTV-4 MOR2004/02, membrane
227   feeding was associated with decreased viral replication in the insect vector compared with
228   feeding on an infected sheep, while for BTV-4 MOR2009/07 it was associated with an
229   increase. For the other three strains, there was no significant difference in vector infection
230   efficiency between the two feeding routes (Fig 6; Table S7).
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232   **DISCUSSION**
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234   This is the first study to demonstrate the impact of natural reassortment on biological
235   transmission of a segmented RNA arbovirus between ruminant and arthropod hosts. Using a
236   highly repeatable and manipulatable *Culicoides*-BTV-sheep model and full genome
237   sequencing data, we show that inheritance of phenotype during reassortment was
238   explanatory of vector susceptibility to infection, while virus pathogenicity following
239   reassortment was unpredictable. These observations have fundamental consequences both
240   for understanding selective pressures operating on reassortment in the field and in the
241   ability to predict the potential for rapid shifts in virus phenotype within regions where virus
242   strains circulate.

243   While tracing a direct lineage between reassortant strains of BTV is challenging, our
244   sequencing data analyses suggested a high degree of similarity in segments inherited from
245   parental strains, indicating negligible genetic drift within segment sequences. This inference
246   was aided by a clear timeline in the isolation of strains used for the study and the use of
247   parallel, rather than sequential, comparison of both clinical signs in sheep and transmission
248   rates to *Culicoides* that underpinned phenotype definition. Comparisons of both clinical
249   severity of BTV strain infection in hosts (29, 31, 38) and transmission to vectors (39) have
250   been carried out previously. However, this is the first study to link both these parameters
251   with viruses of known reassortment lineage.

252   Transmission of parental and reassortant strains of BTV from ITI *Culicoides* to naïve sheep
253   was highly efficient across all five strains of BTV, confirming previous studies (40, 41).
254   Interestingly, severity of clinical infection in sheep was not broadly correlated to the number

255   of infective bites received, but, where very few fully infected *C. sonorensis* fed, this

256   occasionally led to a late and slowly progressing viraemia. During the 2006/7 epidemic of

257   BTV-8 in northern Europe (15), initial outbreaks of disease were classified as being mild

258   which was initially attributed to reduced exposure to *Culicoides* biting as the incursion

259   began in late summer (42). Our study suggests a decline in populations of *Culicoides* in

260   autumn is unlikely to change the severity of report cases and clinical surveillance should still

261   be effective (43, 44).

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263   Rates of viral replication in *C. sonorensis* for four of the five BTV strains were comparable

264   between membrane feeding on viraemic sheep blood and feeding directly from the sheep

265   itself. The exception to this was the BTV-4 MOR2009/07 strain which produced a significantly

266   higher rate of vector competence in membrane fed individuals. It is unclear at present why

267   this strain-specific variation occurred as blood meal titres across the testing were similar.

268   Moreover, this result was influenced by a high feeding rate on a single sheep and the results

269   should therefore be treated with caution. One potential explanation could be that as the

270   RNA quantities were estimated in blood taken from a superficial vein, whereas local virus

271   replication in the skin might lead to local differences from systemic titres (45), leading to

272   variation in vector competence when assessed. More broadly, however, it has been

273   demonstrated here that the systemic level of BTV infection even when determined in blood

274   samples taken at the jugular vein is a valid predictor of vector infection.

275   No specific pattern was demonstrated in gene segment inheritance for the severity of clinical

276   disease within infected sheep, in part due to the wide variation in observations within

277   cohorts. This finding was consistent with previous studies attempting to elucidate the

278   genetic basis of BTV strain virulence, which also could not identify specific genome segments

279   determining virulence in ruminants (32, 33). It is interesting to note that virulence still

280   presented as a multi-segmented trait in this study, even when low-passage field isolates

281   were transmitted to the sheep host via the most natural route of blood-feeding infected

282   *C. sonorensis*. Infection with BTV-1 MOR 2007/01 did, however, result in a uniform

283   manifestation of what would be considered to be classical clinical BT as described from field

284   observations (7). In contrast all other strains led to a far more typical intra-cohort variation

285   in BT severity using the clinical scoring approach. BTV-8 NET 2006/06 was somewhat less

286   severe than expected from previous field reports (12, 13), although this is consistent with

287   previous experiments (16, 31). Anecdotal observation had previously reported that BTV-4

288   strains increased in severity throughout time following co-circulation with BTV-1 and BTV-8.

289   While the acute clinical presentation of sheep infected with the early parental BTV-4

290   MOR2004/02 strain was milder in comparison to the two BTV-4 reassortant strains

291   MOR2009/07 and MOR2009/10, this was not reflected in the overall average severity score

292   (Figure 3, Figure S2 and Table S5), partly through the more chronic presentation of disease in

293   sheep infected with BTV-4 MOR2004/02 still leading to an overall significant clinical impact.

294   Although the pathological damage caused by BTV-4 MOR2004/02 was clearly less severe,

295 this is also compounded by the fact that sheep euthanised during peak clinical disease
296 presented as vastly more acute during post-mortem examination compared to those
297 examined later at study end. It is evident, however, that neither of the two BTV-4
298 reassortant strains inherited the full virulence of its primary parental strain BTV-1
299 MOR2007/01 despite six (BTV-4 MOR2009/07) or four (BTV-4 MOR2009/10) segments
300 originating from this viral lineage.

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302 Infection rates of *Culicoides* fed on viraemic sheep, in contrast to virus virulence, varied
303 consistently and provided a clear marker of BTV strain phenotype during the studies.
304 *Culicoides sonorensis* was almost entirely refractory to two of the parental strains (BTV-1
305 MOR 2007/01 and BTV-8 NET2006/06), while the third gave among the highest rates of
306 infection recorded (BTV-4 MOR2004/02). This extremely high rate of infection was retained
307 within both of the reassortant progeny (BTV-4 MOR2009/07 and BTV-4 MOR2009/10).
308 To investigate the impact of amino acid variations of each viral protein (encoded by the
309 specific genome segments) on vector competence, we investigated the BTV genomes for
310 amino acid positions that were identical for all three BTV-4 strains, but different for both
311 BTV-1 and BTV-8 parental strains.

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313 Seg-7 was the most conserved across all five BTV isolates. Four out of five strains shared
314 100% amino acid similarity in this segment, whereas BTV-4 MOR2004/02 differed by one
315 very conserved amino acid substation (Table S2). Therefore Seg-7 does not influence the
316 infection rate of these five BTV strains in *C. sonorensis*. Although there were some amino
317 acid variations in proteins encoded by segments -1 (99.0-99.7%), -3 (99.6-100%), -4 (98.4-
318 100%), -8 (97.7-100%), and -10 (94.7-100%) (Table S2), no single position was identified that
319 would be identical for all three BTV-4 strains, but different for both BTV-1 and BTV-8.
320 Therefore, these segments also seem to have limited impact on the infection rate of BTV in
321 *C. sonorensis* observed for these strains.

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323 Seg-2, 6 and 9 of the BTV genome, encoding for VP2, VP5 and VP6 respectively, were shared
324 across the three BTV-4 strains that exhibited a high rate of vector infection. For segment-9,
325 four amino acids substitutions have been identified in all three BTV-4 strains, but not in BTV-
326 1 MOR2007/01 and BTV-8 NET2006/06. Of these four amino acids substitutions, three were
327 classified as conservative and only one as radical (position 95, Glycine to Arginine). It is
328 unclear if this single amino acid substitution could have any effect on vector competence,
329 but this could be further investigated in the future by generating reverse-engineered mono-
330 reassortant strains.

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332 Most notably, numerous amino acid substitutions have been identified in Seg-2 and -6
333 between the three BTV-4 strains and the other two parental strains BTV-1 MOR2007/01 and
334 BTV-8 NET2006/06. It therefore seems highly like that VP2 and/or VP5 proteins derived

335   from the parental BTV-4 MOR2004/02 play a key role in determining the vector
336   competence, either individually or in combination, for the BTV strains used in this study.
337   Viral proteins including VP2 from the atypical BTV 26 have been shown to restrict infection
338   and therefore transmission in *Culicoides (46, 47)*. The BTV outer capsid protein VP2 is known
339   to be highly variable, containing the epitopes to the host's neutralising antibody response,
340   thereby determining the strain serotype (48). Both outer capsid proteins VP2 and VP5 are
341   responsible for cell entry (48), whilst VP5 also plays a role in the penetration of mammalian
342   and insect cells (49) during the release of the core particle from the endosome. The affinity
343   of VP2 for erythrocyte glycoprotein may facilitate transmission from mammalian blood to
344   the vector (48). Furthermore, VP2 is cleaved by proteases in the saliva of competent vector
345   *Culicoides* resulting in increased infectivity to *Culicoides* derived cells (50). The role of VP2 in
346   the binding, entry and infection of *Culicoides* mesoenteron gut cells, the barrier to infection
347   (51), is as yet undetermined.
348
349   This study has demonstrated that rapid phenotypic changes in co-circulating strains of BTV
350   can occur, driven by reassortment, even in the presence of purifying selection and genetic
351   bottlenecks imposed by utilising only distantly related hosts and vectors. From a policy
352   perspective, the emergence of reassortant strains of BTV possess additional complexity both
353   in the decision to implement vaccination campaigns and in the likelihood of spread in local
354   vector populations. Currently, low pathogenicity strains of BTV are often allowed to spread
355   where their impact is perceived as being less damaging than the cost of vaccination (15). In
356   addition, the factors underlying the spread of BTV strains between regions dominated by
357   different species of *Culicoides* are poorly understood and reassortment could allow these
358   barriers to be overcome.

359   A future step is to examine the genetic drivers of this process by sequencing of strains
360   exhibiting specific phenotypic characteristics in hosts and vectors. The application of next
361   generation sequencing will be informative in understanding how the virus populations
362   interacting in the process of reassortment are sustained and selected in both the ruminant
363   and insect host. Studies could also be extended to examine tropism of virus communities in
364   both the ruminant and insect host with a view to understanding dissemination. Subsequent
365   testing of viruses produced using reverse genetics and informed by sequencing could also
366   elucidate the genomic basis of a range of phenotypic responses including transmission
367   probability, temperature limits to replication and pathogenicity in the ruminant host.

368   **MATERIALS AND METHODS**

369   **Full genome sequencing of BTV strains:** Full genome sequences of BTV strains were
370   obtained by Sanger sequencing (BTV-4 MOR2004/02, BTV-1 MOR2007/01, BTV-4
371   MOR2009/07 and BTV-4 MOR2009/10), with the exception of BTV-8 NET2006/06 which was
372   additionally resequenced using High Throughput Sequencing (HTS) (BTV-8 NET2006/06).

373   Sanger sequencing was carried out as previously described (6). For HTS, total RNA was

374   extracted from cell culture pellets using TRIzol Reagent (Life Technologies, Paisley, UK) and

375   eluted in 100 µL of nuclease free water (Sigma-aldrich, Gillingham, UK). One microlitre of

376   RNase T1 enzyme was added into each tube and incubated at 37 °C for 30 min in a

377   thermocycler to remove ssRNA. DsRNA was purified using the RNA Clean and Concentrator

378   kit (Zymo, Irvine, CA, USA) according to the manufacturer's recommendations. The purified

379   dsRNA (8 µL) was denatured by heating at 95 °C for 5 min and the first cDNA strand

380   synthesised using SuperScript III RT (Life Technologies, Paisley, UK) while the second strand

381   was synthesised using NEBNext (New England BioLabs, Hitchin, UK) according to the

382   manufacturers' instructions. Double stranded cDNA was quantified using the Qubit dsDNA

383   HS Assay kit (Life Technologies) and then adjusted to 0.2 ng µL^{-1} with 10 mM Tris-HCl, pH

384   8.0 buffer. Library preparation was performed using the Nextera XT library preparation kit,

385   and paired end read sequencing (2 x150bp) was performed using MiSeq platform and

386   reagent kit v2 (Illumina, San Diego, CA, USA). For sequences obtained by HTS a pre-

387   alignment quality check was performed using the FASTQC program v0.11.8 and the Trim

388   Galore script (52) was used for quality and adapter trimming of FASTQ files along with

389   removal of short sequences (<50 bp). Subsequently, reads were mapped to a reference

390   sequence using the BWA-MEM tool (53) and then the DiversiTools software

391   [http://josephhughes.github.io/DiversiTools/; accessed 12/10/2019] was used to generate

392   the consensus sequence. Finally, the consensus sequence was used as a reference sequence

393   to increase the number of BTV reads mapped to the reference, and the final consensus

394   sequence was saved and used for further analysis. Genbank accession numbers for the BTV-

395   8 NET2006/06 strain are MW159097-MW159106.

396

397   **Reassortment analysis:** Reassortment analysis was performed using Recombinant Detection

398   Program version 4.95 (RDP4) (54) under default settings. Previously sequenced and

399   published strains were retrieved from GenBank: BTV-1 MOR2007/01 (KP820890, KP821010,

400   KP821132, KP821252, KP821372, KP821492, KP821614, KP821734, KP821855, KP821975),

401   BTV-4 MOR2004/02 (KP820941, KP821061, KP821183, KP821303, KP821423, KP821543,

402   KP821665, KP821785, KP821905, KP822026), BTV-4 MOR2009/07 (KP820942, KP821062,

403   KP821184, KP821304, KP821424, KP821544, KP821666, KP821786, KP821906, KP822027)

404   and BTV-4 MOR2009/20 (KP820945, KP821065, KP821187, KP821307, KP821427,

405   KP821547, KP821669, KP821789, KP821909, KP822030). Multiple sequence alignment was

406   performed separately for each viral segment using the Muscle algorithm in MEGA6 program

407   (55), coding regions were aligned on the amino acid level. Then, individual alignment files

408   were concatenated using SequenceMatrix software (56) and NEXUS files were used for RDP

409   analysis. The detection of potential recombination events was performed with the following

410   methods: RDP, GENECONV, Maximum Chi Square, CHIMAERA, BOOTSCANing, Sister

411   Scanning (SISCAN) and 3SEQ. Strains BTV-4 MOR2004/02, BTV-1 MOR2007/01 and BTV-8

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412   NET2006/06 were considered as parental sequences based on their year of detection, BTV-4

413   MOR2009/07 and BTV-4 MOR2009/10 were investigated as potential reassortant strains.

414

415   **Standardization of viral inoculum:** All isolates used were passaged once on *C. sonorensis*

416   derived KC cells to generate infectious tissue culture supernatant (TCS) that was derived in

417   the same cell culture system (Table S1). Briefly a T175 flask of KC cells in suspension of

418   growth media (Schneider's insect cell media (Sigma-Aldrich, Dorset, UK); 10% Gibco® heat-

419   inactivated fetal calf serum (ThermoFisher Scientific, MA, USA) and 1%

420   Penicillin/Streptomycin (Sigma-Aldrich, Dorset, UK)) was inoculated with the stock virus

421   from the Orbivirus reference collection and incubated for 7 days at 25°C. Following

422   incubation, cells and supernatants were collected and cells pelleted through centrifugation

423   for 10 min at 1000g and 4°C.

424   The supernatants of each BTV strain were titrated on KC cells using a 96-well end-point

425   dilution microtitration assay. 10-fold serial dilutions were added to the titration plates

426   containing 1×10^5 KC cells/well in suspensions of growth media. Titration plates were

427   incubated for 7 days at 25°C at which point supernatants were removed and cells were fixed

428   using 4% paraformaldehyde for 30-45 minutes, washed 3 x with PBS and stored under 100µl

429   PBS/well at 4°C. As KC cells do not develop cytopathic effects, BTV infection of each well was

430   determined through visualisation of BTV antigen by immunofluorescence microscopy. Cells

431   were permeabilised with 0.2% Trition for 15 min followed by incubation with anti-BTV

432   guinea pig hyperimmune serum (ORAB279) at 1:2000 in PBS-0.5%BSA for 1h at room

433   temperature (RT). Following another 3 washes with PBS plates were incubated for 1h with

434   secondary goat anti-guinea pig-AlexaFluor488 (Invitrogen, UK) at 1:500 in PBS-0.5%BSA.

435   After 3x final PBS washes plates were examined under the fluorescence microscope and the

436   presence or absence of fluorescence was recorded for each well.

437   The final infectious titre for each virus was calculated according to the Spearman-Karber

438   method. Serotype specificity and the absence of cross-contamination between the different

439   virus inocula was confirmed by serotype specific qRT-PCR (57). Viral inoculum characteristics

440   are summarised in Table S1.

441   **Infection of *Culicoides* with BTV strains.** Colony-derived adults of *Culicoides sonorensis*

442   Wirth & Jones, a BTV vector in North America, were used in the study (58). Maintenance

443   was as described previously (59), with the exception that the colony was sustained using a

444   Hemotek artificial feeder and horse blood from a commercial supplier (TCS Bioscience,

445   Botolph Claydon, UK). *Culicoides sonorensis* were intrathoracically (IT) inoculated with 0.2 µl

446   BTV tissue culture supernatant at a standardised titre of 5.75 $\log_{10}TCID_{50}$ for each BTV strain

447   using a pulled glass capillary needle (Narishige, Japan) and a Nanoject II microinjector

448   (Drummond Scientific, PA, USA). Between 50 and 200 *Culicoides* were inoculated for each

449   virus strain in each experimental replicate and were subsequently incubated at 25°C (Binder,

450   Tuttlingen, Germany) in card pillboxes with mesh screen for five to six days (60). *Culicoides*

451 were given access to 10% sucrose solution on cotton wool that was replenished daily
452 throughout this period.
453
454 **Animal experiment:** This animal experiment was carried out in accordance with the UK
455 Animal Scientific Procedure Act (ASPA) 1986 which transposes European Directive
456 2010/63/EU into UK national law. All animal procedures carried out were reviewed and
457 approved by the Animal Welfare and Ethics Review Board at the Pirbright Institute and
458 conducted in compliance with the relevant project licences granted by the UK Home Office.
459 The study was conducted under high biological containment at the Pirbright Institute in 4
460 sequential replicates, each consisting of 6 sheep with one sheep in each replicate exposed
461 to one parental or reassortant BTV strain and an associated uninfected control animal (24
462 sheep total). The sheep used were British mule crosses. All sheep were tested to confirm the
463 absence of anti-BTV antibodies by the UK BTV reference laboratory prior to arrival at The
464 Pirbright Institute using a commercial competitive anti-VP7 antibody ELISA (IDVet,
465 Montpellier, France). All animals were allowed to acclimatise to the new facilities for 6-8
466 days before onset of any procedures and were fed twice a day with grain pellets and with ad
467 libitum access to hay and water throughout the experiment (10).
468
469 *Infection of sheep:* Pots containing ITI *Culicoides* were placed on the inner thigh of a
470 restrained sheep to feed for a period of 10 minutes. The strain of BTV used to infect each
471 sheep and the sheep identification number were recorded. The *Culicoides* were
472 anaesthetised and examined under a light stereomicroscope for evidence of blood feeding.
473 Engorged, or partially engorged, individuals were placed in a microtube containing in 100 μl
474 of either Schneider's media (Experimental Replicates 1 and 2) or RPMI media (Experimental
475 Replicates 3 and 4) to which 2% Penicillin/Streptomycin and Amphotericin B (Sigma-Aldrich,
476 Dorset, UK) had been added. Samples were homogenised for two cycles of 30 seconds at
477 25Hz with a 3mm stainless steel bead in a Tissuelyser™ (Qiagen, Manchester, UK). The
478 homogenates were then diluted to a total volume of 1000 μl with RPMI media (Sigma-
479 Aldrich, Dorset, UK) and 2% antibiotics, the stainless steel bead removed and samples were
480 then vortexed at 13,000 rpm for 10 minutes and stored at +4°C. 50 μl of homogenate was
481 then used for nucleic acid extraction and analysis by rtRT-PCR to determine if the *Culicoides*
482 had supported a disseminated infection and that transmission of BTV to the sheep was likely
483 to have occurred.
484
485 *Monitoring of BTV infection in sheep:* Blood samples were taken from the jugular vein on the
486 day before the experiment began (day 0) and then on days 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14,
487 16, 18 and 19/20 following infection. Clinical signs of disease and rectal temperature were
488 recorded for each animal daily. A post-mortem of all sheep was carried out following
489 euthanasia at the study end (19/20 dpi) or when individuals reached a humane endpoint for
490 the protocol.

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Calculation of clinical scores: A cumulative daily clinical score was determined for each cohort of four sheep infected with a single BTV strain and the four control animals (Table S4). To account for sheep euthanized due to exceeding clinical endpoints, daily accumulated clinical scores for each cohort were divided by the number of sheep present. A total severity score (Figure S2 and Table S5) for each virus was obtained by dividing the overall accumulated total clinical score from all sheep by the combined days the entire cohort of four individuals stayed alive between 4-19 dpi (period for which any clinical signs were recorded).

Transmission of BTV strains from viraemic sheep to *Culicoides.* The quantity of BTV RNA in blood samples taken from sheep in the first ten days post-infection was tested on the day of collection. Preliminary data suggested that a blood sample with a sqPCR C_T value of less than 25 indicated that BTV infection would reach peak viraemia in the sheep within the following two days and this was used as a guide to time blood-feeding of *C. sonorensis* (40). *C. sonorensis* were allowed to feed on sheep in card boxes through a fine mesh lid at a density of approximately 250 individuals. The boxes were placed on the inner thigh of a restrained sheep for a period of 10 minutes and two boxes of *Culicoides* were fed on each sheep in each replicate. Replete female *C. sonorensis* were selected under light CO_2 anaesthesia using a stereomicroscope. Blood-fed *C. sonorensis* were placed in new card pill boxes and incubated at 25°C with access to a 10% sucrose solution on cotton wool pads that were replenished daily. After an eight day incubation period post-feeding, all surviving female *Culicoides* were selected under CO_2 anaesthesia and placed individually in microtubes (Qiagen) containing 100µl Schneider's media (Experimental Replicates 1 and 2) or RPMI (ThermoFisher Scientific) (Experimental Replicates 3 and 4) for homogenisation as described previously. Homogenates were then diluted with 9000µl of either Schneider's media or RPMI to a total of 1 ml, sealed and stored at +4°C.

Comparison of feeding methods on infection rate in *Culicoides.* Natural blood feeding of *Culicoides* on viraemic sheep was complemented by artificial feeding of *C. sonorensis* on sheep blood taken on the matched day of peak viraemia for each strain in each replicate. Identical boxes of *C. sonorensis* were fed on sheep blood collected the same day as direct feeding. *Culicoides* were fed through a Parafilm™ (SigmaAldrich, Dorset, UK) membrane over a reservoir (Hemotek Ltd, UK) filled with 3 ml of viraemic sheep blood and maintained at 37°C. Following a period of 30 mins exposure to the blood, replete females were selected out under CO_2 anaesthesia and treated as described for the *Culicoides* fed directly on sheep. Any individual *Culicoides* sample with a C_T value <40 when tested by PCR was considered BTV positive.

Nucleic acid extraction and semi-quantitative rtPCR. Nucleic acid extraction throughout the studies was carried out using extraction robots. In replicates 1 and 2 a Universal extraction

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531 robot (Qiagen, Germany) and associated nucleic acid extraction (Qiagen, Germany) kits were
532 used. In replicate 3 and 4, a Kingfisher Flex extraction robot (Thermo Fisher Scientific) and
533 associated kits (MagVet™ Universal Isolation Kit) were used. Validation of the Kingfisher Flex
534 against the Universal extraction robot demonstrated variation between machines was
535 similar to variation observed between repeat runs on the same equipment (+/- 0.5 C_T) (data
536 not shown). Analysis by sqPCR for all samples used a BTV group specific PCR against
537 segment 1 of the virus genome (61). For replicates 1 and 2 a Strategene Mx3005P PCR
538 machine (Agilent, USA) was used, in replicates 3 and 4, a Life Systems 7500 Fast PCR
539 machine (Thermo Fisher Scientific, UK) was used. In validation between these machines a
540 similar variation was observed to that between repeat runs on the same equipment (+/- 0.5
541 C_T). Confirmation of BTV serotype of viral stocks were carried out using a serotype specific
542 rRT-PCR assay (57).
543
544 In assessments of *Culicoides* infection rates following blood feeding on sheep or through the
545 membrane-based system, 50μl of pooled homogenate from 8 individually homogenised
546 *Culicoides* was added to a microtube. From this 400μl pool, nucleic acid was extracted from
547 a 50μl sample and viral RNA assessed by sqPCR as described above. Individuals contributing
548 to a pooled sample found to contain BTV RNA (≤40 Ct), were subsequently tested
549 individually by sqPCR.

550 **Statistical analysis.** The effect of strain and number of *Culicoides* feeding at infection on
551 timing and magnitude of peak viraemia (i.e. the lowest C_T value) was assessed using linear
552 models with time of peak viraemia or C_T value at peak viraemia as the response variable and
553 strain and number of *Culicoides* feeding and an interaction between them as explanatory
554 variables. Model selection proceeded by stepwise deletion of non-significant (*P*>0.05) terms
555 as judged by *F*-tests.
556
557 The vector competence (i.e. the probability of a *Culicoides* midge having a C_T value) after
558 feeding on blood infected with strain *s* by route *f* (either on a sheep (*f*=0) or via a membrane
559 (*f*=1)) when the C_T value of the infected blood meal is *c* is given by;

560
$$\log\left(\frac{p_s(c,f)}{1-p_s(c,f)}\right) = b_0^{(s)} + b_1^{(s)}c + b_2^{(s)}f, \tag{1}$$

561 where the b_is are strain-specific parameters. Differences amongst strains were incorporated
562 by assuming hierarchical structure for the model parameters, so that the parameters for
563 strain *s* are drawn from higher-order normal distributions, namely,

564
$$b_i^{(s)} \sim \mathrm{N}(\mu_{b_i}, \sigma_{b_i}^2),$$

565 where the μs and σs are higher-order parameters.
566

567 Parameters were estimated in a Bayesian framework. A Bernoulli likelihood was used for
568 the infection status of each *Culicoides* midge with probability of infection given by equation
569 (1). Priors for the strain-specific parameters were given by the higher-order distributions,
570 while non-informative priors were assumed for the higher-order parameters (diffuse normal
571 for the μs and diffuse gamma for the σs). The methods were implemented in OpenBUGS
572 (version 3.2.3; http://www.openbugs.info). Two chains each of 10,000 iterations were
573 generated (with the first 2,000 iterations discarded to allow for burn-in of the chains).
574 Convergence of the chains was monitored visually and using the Gelman-Rubin statistic in
575 OpenBUGS.
576

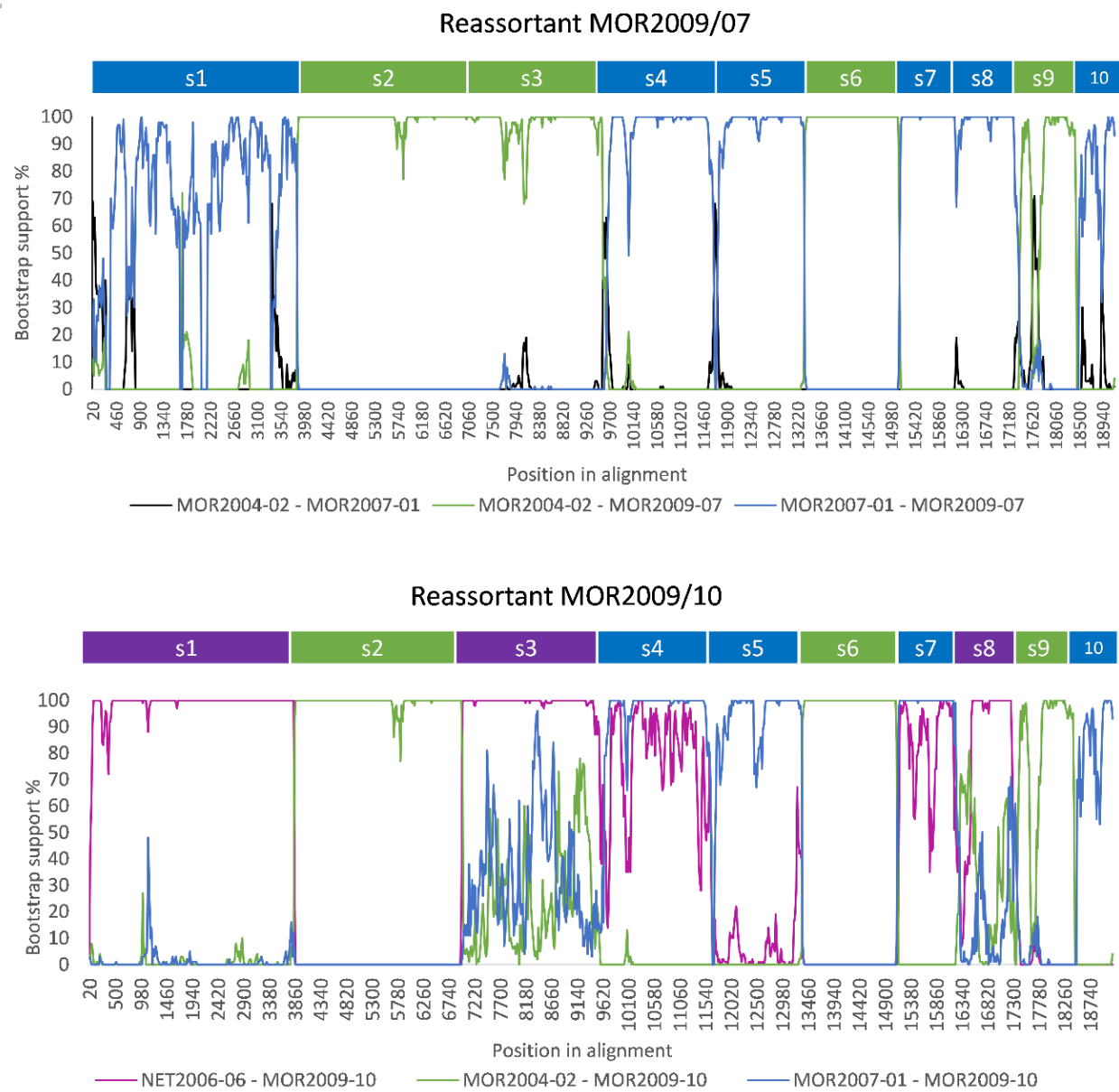577 **ACKNOWLEDGEMENTS:**

Fig 1: BOOTSCAN evidence for reassortment of BTV-4 MOR2009/07 (top) and BTV-4 MOR2009/10 (bottom).

17
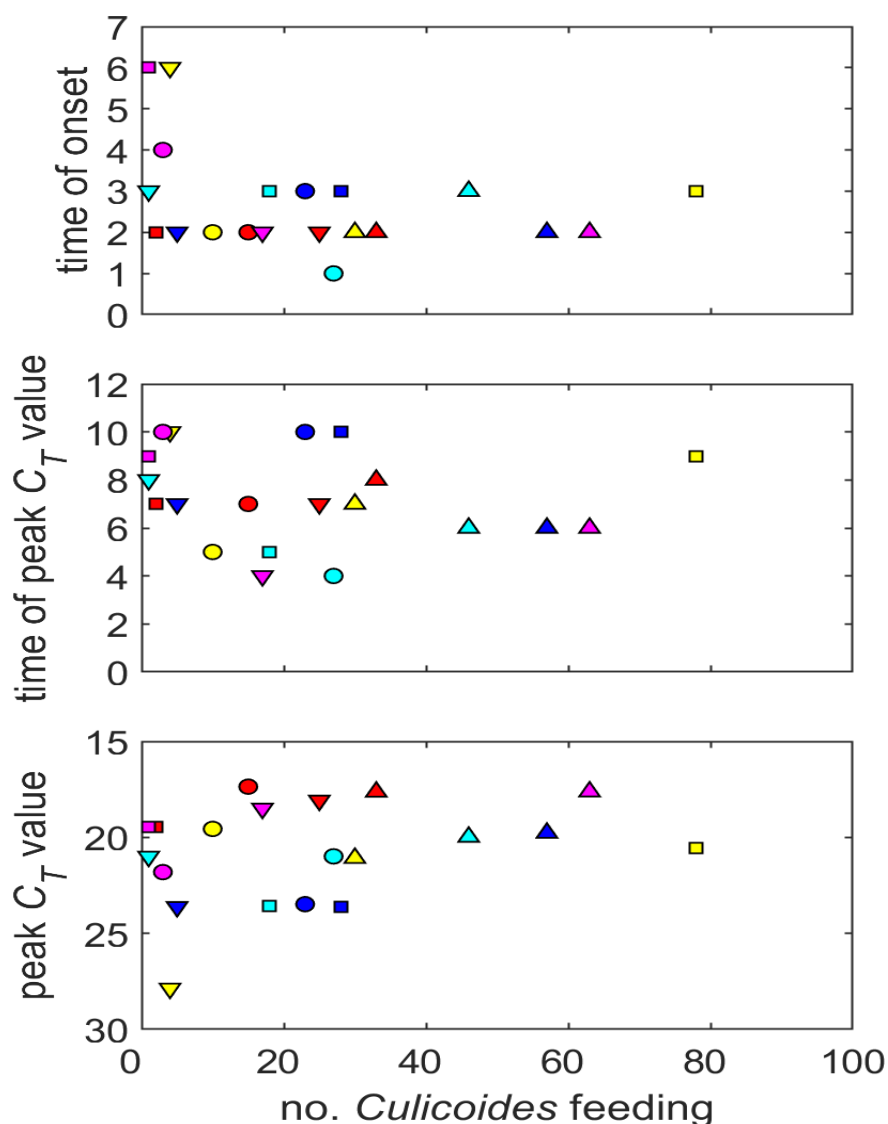
Fig 2. Time to onset of viraemia, time to peak viraemia and level of peak viraemia in sheep infected with different strains of bluetongue virus and their dependence on the number of inoculated *Culicoides* feeding to initiate the infection. Strains are: BTV-1 MOR2007/01 (red), BTV-4 MOR2004/02 (blue), BTV-8 NET2006/08 (cyan), BTV-4 MOR2009/07 (yellow) and BTV-4 MOR2009/10 (magenta). The different symbols indicate the different sheep infected with each strain.
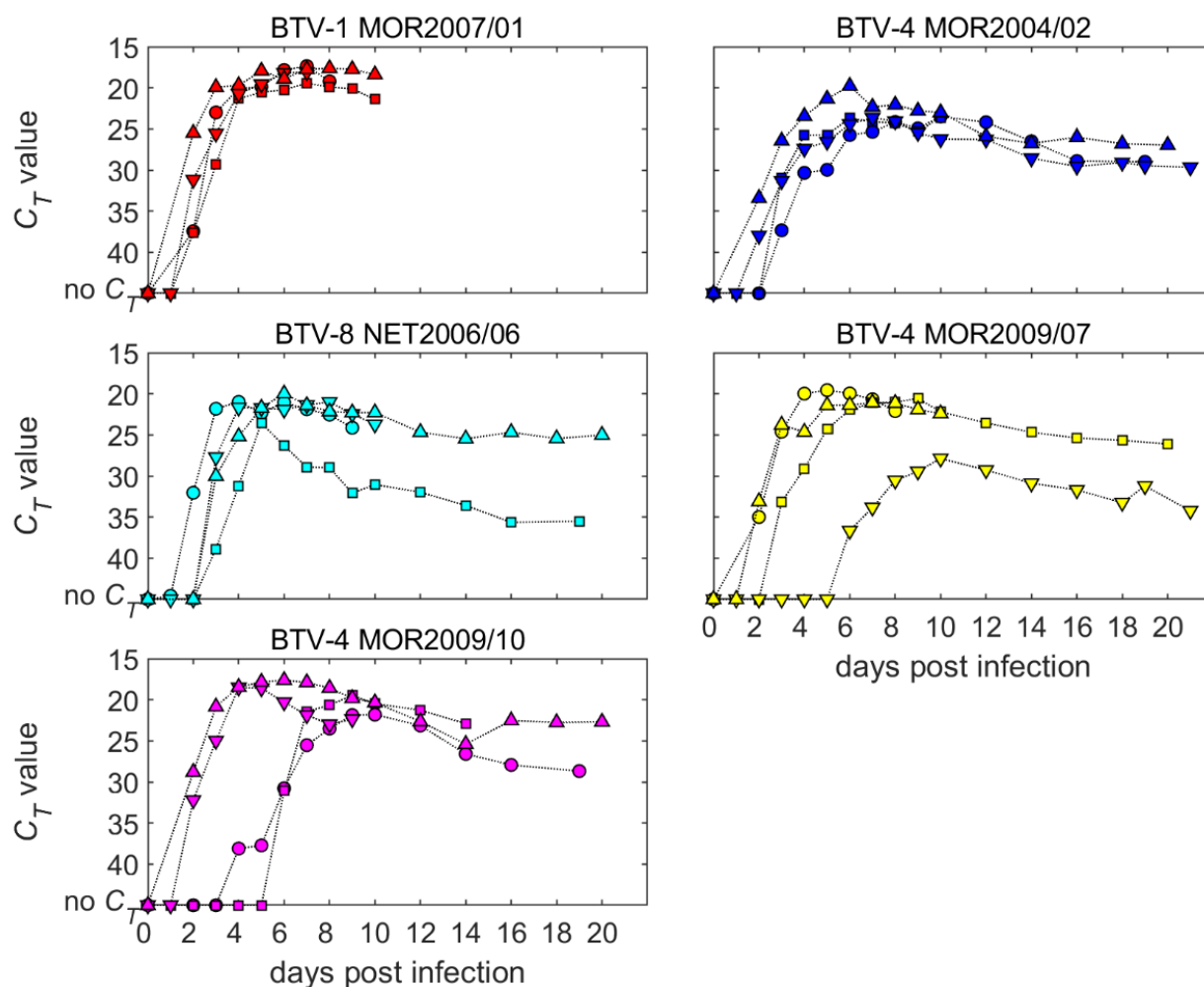
Fig 3. Viraemia in sheep infected with one of five strains of bluetongue virus. Each plot shows the changes in C_T value over time for individual sheep (indicated by symbols).

BTV-1MOR2007/01

BTV-4 MOR2004/02

BTV-8 NET2006/06

BTV-4 MOR2009/07

BTV-4 MOR2009/10

Non-infected in-contact controls

temperature score
behaviour score
red eyes
nasal discharge
diarrhoe
food intake score
red mucosal membranes
swollen face
respiratory signs
feet score average
salivation
ulcers

626   **Fig 4:** Daily accumulated average clinical score observed in sheep infected with 5 different
627   BTV strains. Clinical scores were obtained across 12 different clinical signs routinely
628   observed during BTV infection and visualised for each of the separate clinical sign for the
629   time period of 4-19 dpi (see legend). The daily clinical scores recorded for each clinical sign
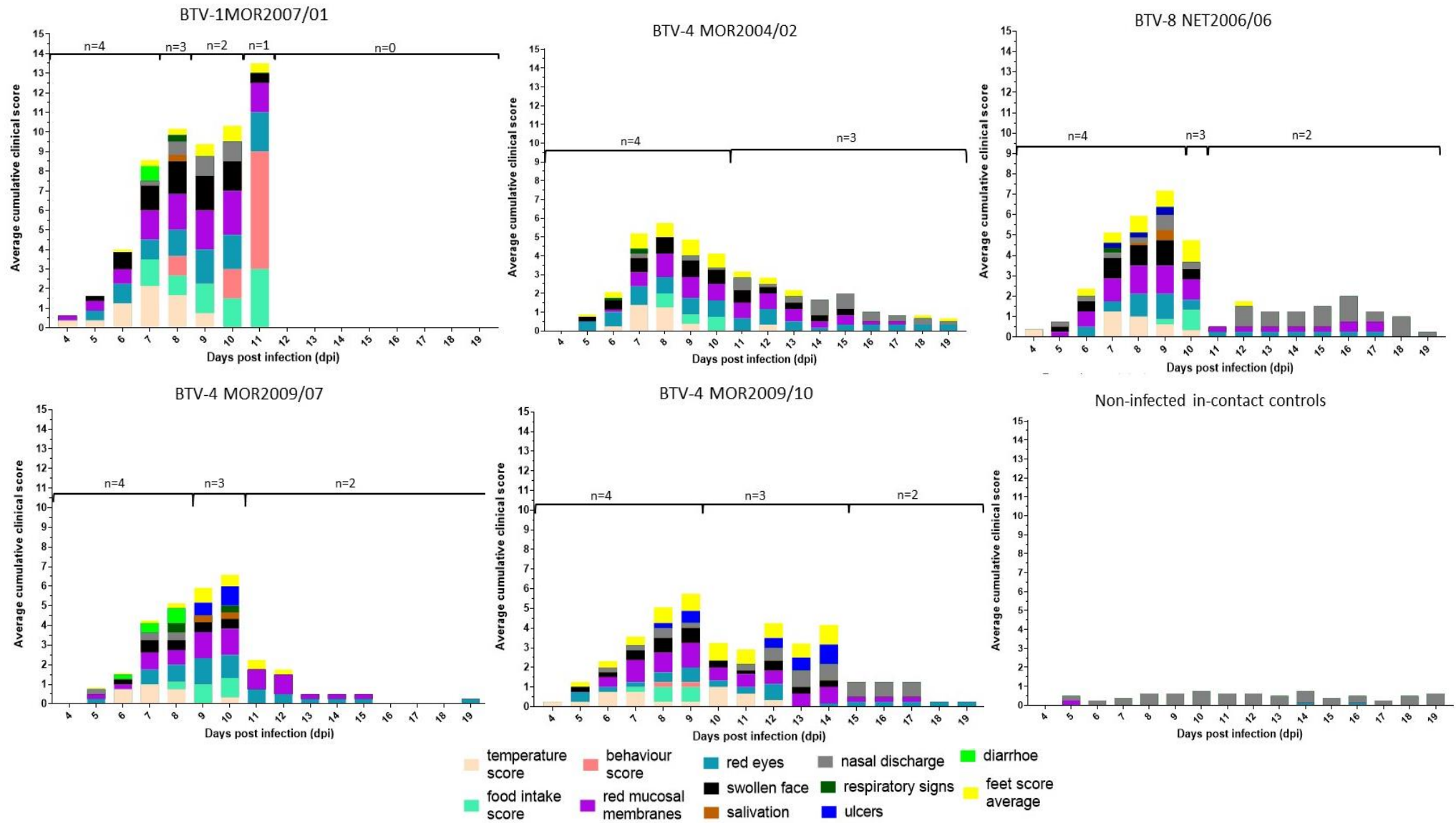630   were combined from all sheep within the group and then normalised to the sheep still
631   present on the day to account for removal of sheep from groups at different days due to
632   reaching predefined humane end points. Clinical scores for the uninfected in-contact
633   controls were also recorded throughout to highlight potential non-specific clinical
634   observations.

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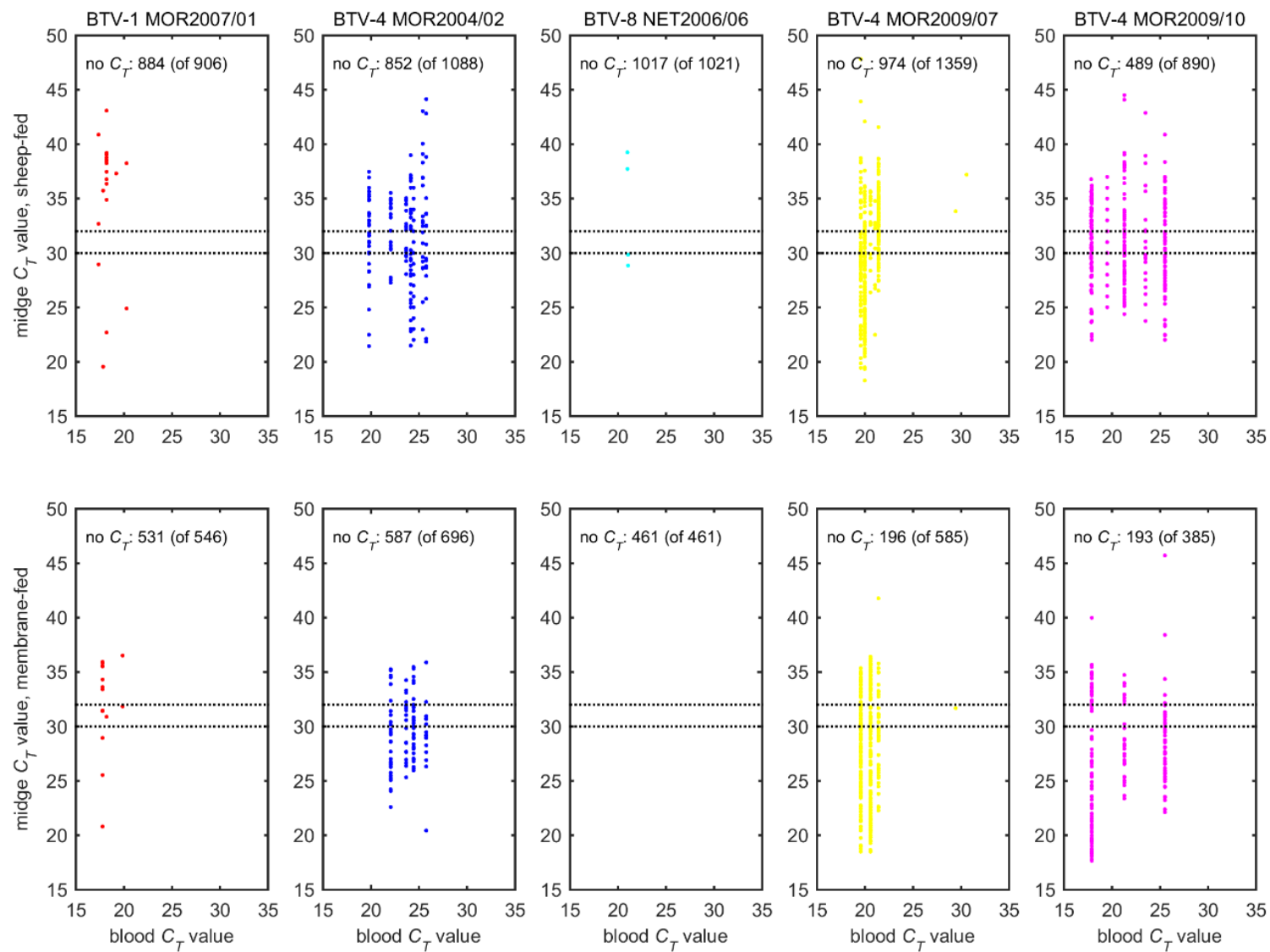645

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Fig 5. Observed C_T values for five strains of bluetongue virus in *Culicoides sonorensis* following blood feeding and incubation for eight days at 25 °C. Each plot shows the dependence of the C_T value on feeding route (sheep-fed: top row; membrane-fed: bottom row) and the C_T value of the infected blood meal. The numbers at the top of each plot indicate the number of midges with no C_T value after incubation and the total number of *C. sonorensis* tested. The dotted lines indicate C_T values which correspond to a possible ($C_T<32$) and probable ($C_T<30$) fully transmissible infection in a previous study of BTV infection in *Culicoides* (Veronesi *et al*. 2013).
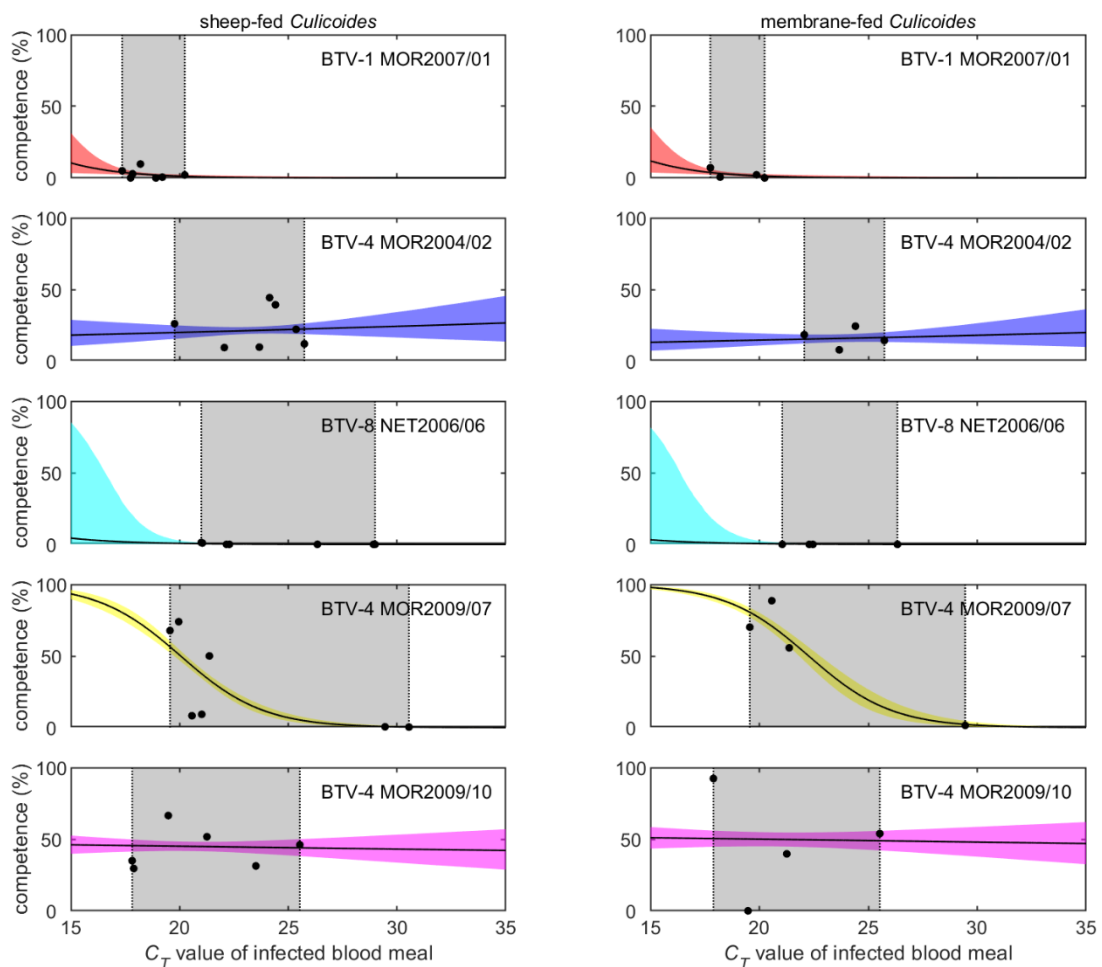
Fig 6. Vector competence of *Culicoides sonorensis* for five strains of bluetongue virus and its dependence on feeding route and blood meal C_T value. Each plot shows the posterior median (black line) and 95% credible interval (shading) for the vector competence. The black circles are the observed proportions of infected midges at each C_T value and the grey shaded area represents the range of C_T values for the infected blood meals.

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676   **Table S1:** Passage history of virus strains used during studies. All viruses were subjected to a
677   single passage in KC-*C. sonorensis* prior to measurement of titre.

Virus strain	Reassortant status	Passage history of virus strain used for infection of *C. sonorensis*	Viral titre quantified by immunofluorescence on KC cells
BTV-1 MOR2007/01	Parental	KC-*C. sonorensis* (2)	$10^{7.25}$ TCID50/ml
BTV-4 MOR2004/02	Parental	Embryonated Chicken Egg (1); Baby Hamster Kidney (4); KC- *C.* sonorensis (1)	$10^{5.75}$ TCID50/ml
BTV-8 NET2006/06	Parental	KC-*C. sonorensis* (4)	$10^{6.75}$ TCID50/ml
BTV-4 MOR2009/07	Reassortant	KC-*C. sonorensis* (2)	$10^{6.5}$ TCID50/ml
BTV-4 MOR2009/10	Reassortant	KC-*C. sonorensis* (2)	$10^{6.25}$ TCID50/ml

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695 **Table S2.** Nucleotide identity (white) and amino acid similarity (grey) expressed as %

696 between bluetongue virus (BTV) strains across all ten genome segments.

Seg-1	NET2006-06	MOR2007-01	MOR2004-02	MOR2009-07	MOR2009-10
BTV-8 NET2006-06		99.3	99.0	99.1	99.7
BTV-1 MOR2007-01	94.5		99.6	99.7	99.3
BTV-4 MOR2004-02	94.7	98.8		99.4	99.0
BTV-4 MOR2009-07	94.5	99.8	98.7		99.2
BTV-4 MOR2009-10	99.9	94.5	94.6	94.5	

Seg-2	NET2006-06	MOR2007-01	MOR2004-02	MOR2009-07	MOR2009-10
BTV-8 NET2006-06		52.5	40.8	40.8	40.8
BTV-1 MOR2007-01	55.1		40.4	40.4	40.4
BTV-4 MOR2004-02	48.6	48.7		99.4	99.2
BTV-4 MOR2009-07	48.6	48.5	99.6		99.7
BTV-4 MOR2009-10	48.5	48.5	99.4	99.8	

Seg-3	NET2006-06	MOR2007-01	MOR2004-02	MOR2009-07	MOR2009-10
BTV-8 NET2006-06		99.6	99.7	99.8	100.0
BTV-1 MOR2007-01	95.4		99.6	99.7	99.6
BTV-4 MOR2004-02	95.0	95.0		99.8	99.7
BTV-4 MOR2009-07	94.6	94.6	99.2		99.8
BTV-4 MOR2009-10	99.6	95.1	94.8	94.7	

Seg-4	NET2006-06	MOR2007-01	MOR2004-02	MOR2009-07	MOR2009-10
BTV-8 NET2006-06		99.2	98.4	99.2	99.2
BTV-1 MOR2007-01	98.2		98.9	100.0	100.0
BTV-4 MOR2004-02	95.7	95.9		98.9	98.9
BTV-4 MOR2009-07	97.9	99.8	95.9		100.0
BTV-4 MOR2009-10	98.0	99.9	95.9	99.9	

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Seg-5	NET2006-06	MOR2007-01	MOR2004-02	MOR2009-07	MOR2009-10
BTV-8 NET2006-06		99.6	99.4	99.6	99.6
BTV-1 MOR2007-01	94.4		99.4	100.0	100.0
BTV-4 MOR2004-02	94.7	96.6		99.4	99.4
BTV-4 MOR2009-07	94.3	99.8	96.7		100.0
BTV-4 MOR2009-10	94.0	99.6	96.4	99.6	

Seg-6	NET2006-06	MOR2007-01	MOR2004-02	MOR2009-07	MOR2009-10
BTV-8 NET2006-06		84.9	78.5	78.5	78.5
BTV-1 MOR2007-01	73.4		77.9	77.9	77.9
BTV-4 MOR2004-02	69.8	69.2		99.6	99.6
BTV-4 MOR2009-07	70.0	69.2	99.5		100.0
BTV-4 MOR2009-10	69.9	69.2	99.4	99.8	

Seg-7	NET2006-06	MOR2007-01	MOR2004-02	MOR2009-07	MOR2009-10
BTV-8 NET2006-06		100.0	99.7	100.0	100.0
BTV-1 MOR2007-01	97.0		99.7	100.0	100.0
BTV-4 MOR2004-02	94.2	94.0		99.7	99.7
BTV-4 MOR2009-07	97.1	99.9	94.1		100.0
BTV-4 MOR2009-10	97.1	99.9	94.1	100.0	

Seg-8	NET2006-06	MOR2007-01	MOR2004-02	MOR2009-07	MOR2009-10
BTV-8 NET2006-06		97.7	98.3	97.7	99.4
BTV-1 MOR2007-01	95.6		99.4	100.0	97.7
BTV-4 MOR2004-02	96.0	95.9		99.4	98.3
BTV-4 MOR2009-07	95.5	99.8	95.8		97.7
BTV-4 MOR2009-10	99.2	95.2	95.6	95.2	

Seg-9	NET2006-06	MOR2007-01	MOR2004-02	MOR2009-07	MOR2009-10
BTV-8 NET2006-06		96.3	95.7	95.7	95.4
BTV-1 MOR2007-01	96.3		96.9	97.5	97.2
BTV-4 MOR2004-02	96.2	97.2		98.7	98.4
BTV-4 MOR2009-07	96.2	97.0	99.1		99.6
BTV-4 MOR2009-10	96.1	96.9	99.1	99.9	

Seg-10	NET2006-06	MOR2007-01	MOR2004-02	MOR2009-07	MOR2009-10
BTV-8 NET2006-06		95.1	94.7	95.1	95.1
BTV-1 MOR2007-01	83.3		99.5	100.0	100.0
BTV-4 MOR2004-02	83.2	98.3		99.5	99.5
BTV-4 MOR2009-07	83.6	99.2	97.7		100.0

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BTV-4 MOR2009-10	83.5	99.4	97.9	99.8	

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Table S3: Inoculation and feeding success of *Culicoides sonorensis* infected with five strains of BTV across four cohorts of sheep infection studies.

Virus	Experimental Cohort	Number Inoculated	Number Surviving Incubation	Number Blood Fed (% of survived)
BTV-1 MOR2007/01 (Parental)	1	137	34	2 (5.9)
	2	141	98	25 (25.5)
	3	100	27	15 (55.6)
	4	155	47	33 (70.2)
BTV-4 MOR2004/02 (Parental)	1	129	31	5 (16.1)
	2	148	73	28 (38.4)
	3	120	48	23 (47.9)
	4	167	109	57 (52.3)
BTV-8 NET2006/06 (Parental)	1	115	23	1 (4.3)
	2	141	84	27 (32.1)
	3	105	28	18 (64.3)
	4	175	80	46 (57.5)
BTV-4 MOR2009/07 (Reassortant)	1	168	41	4 (9.8)
	2	141	57	30 (52.6)
	3	109	42	10 (23.8)
	4	167	112	78 (69.6)
BTV-4 MOR2009/10 (Reassortant)	1	160	14	1 (7.1)
	2	131	63	17 (27.0)
	3	94	9	3 (33.3)
	4	159	101	63 (62.4)

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703 **Table S4:** Clinical scoring used to assess severity of disease signs in sheep infected with BTV

704 parental and reassortant strains.

Clinical Sign	Observation	Increment of Score Assigned (Range)
1. **Redness of eyes**	Severity	0.5 (0.5 - mild to 3 – Severe)
2. **Redness of mucosal membrane**	Severity	0.5 (0.5 - mild to 3 – Severe)
3. **Facial Oedema**	Swelling	0.5 (0.5 - mild to 3 – Severe)
4. **Salivation**	Salivation	0.5 (0.5 - mild to 3 – Severe)
5. **Nasal Discharge**	Discharge	0.5 (0.5 - mild to 3 – Severe)

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6. Cough	Frequency and Severity	0.5 (0.5 - mild to 3 – Severe)
7. Ulcers (oral and/or nasal)	Severity	0.5 (0.5 - mild to 3 – Severe)
8. Diarrhoea	Severity	0.5 (0.5 - mild to 3 – Severe)
9. Body Temperature	°C	Increase of ≥1°C from resting = 1; ≥1.5°C = 1.5 and ≥2°C = 2; Temperature > 41°C = 3
10. Behaviour	General observation	Apathy and slowness = 1; Reluctance to rise and separation from flock = 2; Reluctance to rise with stimulus = 3; Refusal to rise with stimulus = 3 and humane endpoint.
11. Food Intake	Consumption scored across 2 daily meals and then averaged/day	Reduced food intake = 1; avoiding concentrate but eating hay = 2; no food intake = 3.
12. Feet	Determined separately for each foot and then averaged for an individual	0.5 (0.5-3 considering warmth, reddening, lesions and lameness)

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712 **Table S5:** Summary of clinical severity of all five parental and reassortant BTV strains in
713 sheep.

Group	Total clinical score	Days alive all sheep 4-19 dpi (max 64 days if all survived)	Normalised severity score (total score/ days alive)	Clinical observation notes
BTV-1MOR2007/01 (Parental)	142.625	24	5.94	Highly acute disease presentation, euthanasia of all sheep 7-11 dpi.

BTV-4 MOR2004/02 (Parental)	137	55	2.49	Acute clinical presentation only in one sheep, however high accumulation of scores across chronic clinical signs (red eyes and mucosal membranes but also swollen face).
BTV-8 NET2006/06 (Parental)	122.75	45	2.73	Clearly separated into two acutely affected sheep and 2 mild but chronically affected sheep.
BTV-4 MOR2009/07 (Reassortant)	97.5	44	2.22	Clearly separated into two acutely affected sheep and 2 mild but chronically affected sheep. Lower accumulated feet score than other strains.
BTV-4 MOR2009/10 (Reassortant)	134.625	49	2.75	Clearly separated into two acutely affected sheep and 2 mild but chronically affected sheep. One of the sheep developed peak clinical disease late with feet lesions and burned muzzle (ulcers) (euthanasia 14dpi)
Uninfected controls	31	64	0.49	Two sheep with nasal discharge for >10 days each

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716 **Table S6.** Infection rates of *C. sonorensis* fed either directly on viraemic sheep or indirectly
717 on sheep blood using a membrane-based device.

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	BTV-1 MOR 2007/01 (parental)	**BTV-4 MOR 2004/02 (parental)**	**BTV-8 NET 2006/06 (parental)**	**BTV-4 MOR 2009/07 (reassortant)**	**BTV-4 MOR 2009/10 (reassortant)**

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	Sheep		Sheep		Sheep		Sheep		Sheep	
Total *Culicoides* surviving incubation period	906	546	1088	696	1021	461	1359	585	890	385
Culicoides containing detectable BTV RNA (%)	22 (2.4)	15 (2.7)	236 (21.7)	109 (15.7)	4 (0.4)	0 (0)	385 (28.3)	389 (66.5)	401 (45.1)	192 (49.9)
Total *Culicoides* Ct<30 (%)	4 (0.4)	3 (0.5)	98 (9.0)	61 (8.8)	2 (0.2)	0 (0)	182 (13.4)	240 (41.0)	165 (18.5)	131 (34.0)

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Table S7. Effect of blood meal C_T value and feeding route on viral infection of *C. sonorensis* for five strains of bluetongue virus.

Strain	blood meal C_T value†			feeding route‡		
	estimate	95% credible limit		estimate	95% credible limit	
		lower	upper		lower	upper
BTV-1 MOR2007/01	**0.64**	0.43	0.88	1.15	0.63	2.10
BTV-4 MOR2004/02	1.02	0.96	1.10	**0.68**	0.53	0.88
BTV-8 NET2006/06	0.69	0.33	1.01	0.78	0.12	2.31
BTV-4 MOR2009/07	**0.59**	0.54	0.64	**3.19**	2.52	4.08
BTV-4 MOR2009/10	0.99	0.95	1.03	1.21	0.96	1.54

† odds ratio for change of one unit in blood meal C_T; those shown in bold differ significantly (*P*<0.05) from one

‡ odds ratio for membrane feeding compared with feeding on sheep blood; those shown in bold differ significantly (*P*<0.05) from one
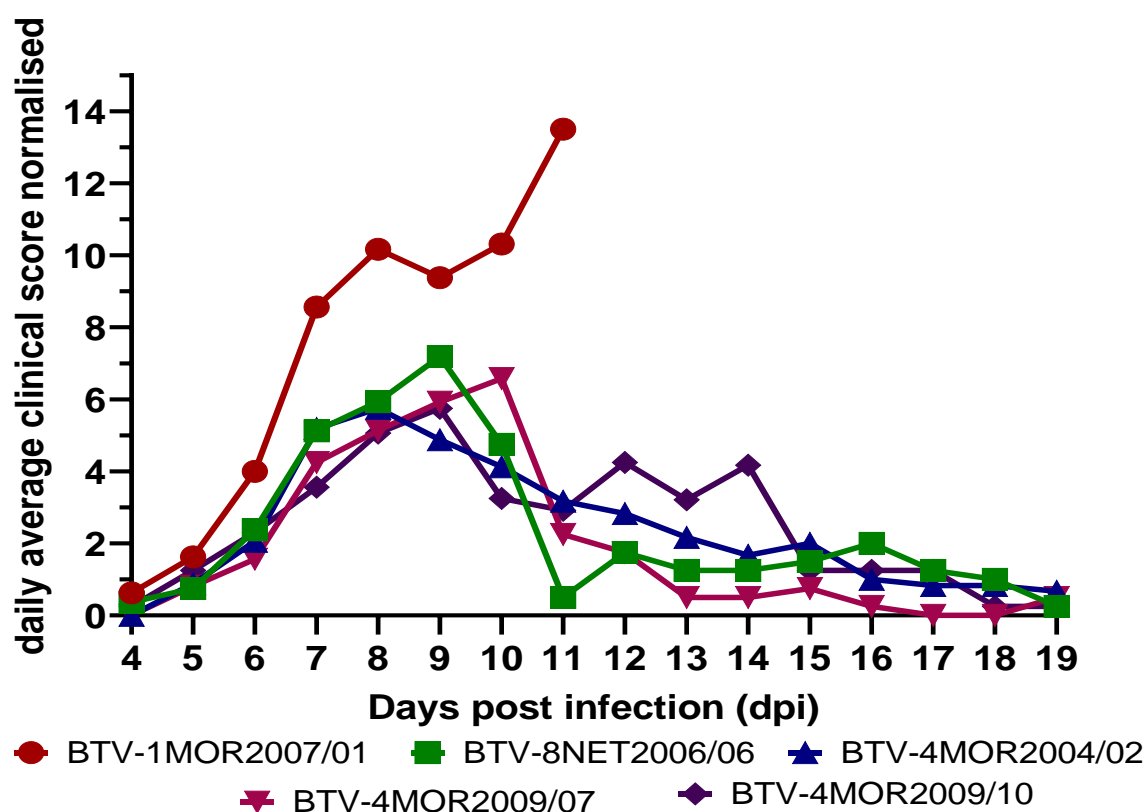
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Figure S1. Maximum likelihood tree was constructed for the VP2 coding regions of BTV using IQ-Tree software version 1.3.11.1 (Nguyen, L.T., et al) and the reliability of each tree was estimated by ultrafast bootstrap (Minh, B.Q) analysis of 1000 replicates. The GTR + I + G4 model of evolution was selected according to the Bayesian information criterion score calculated using the IQ-Tree software. Phylogenetic trees were visualised and rooted on the midpoint using the Figtree v1.4.4 software. BTV-1 lineage 2006-2009 is shown in red, BTV-4 lineage 2003-2010 in blue and BTV-8 lineage 2006-2008 in green.

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Figure S2: Clinical severity of 5 different BTV strains over time. The daily average normalised clinical score combined for all clinical signs visualised for the clinical period between 4-19 d.p.i. for each of the 5 different BTV strains.
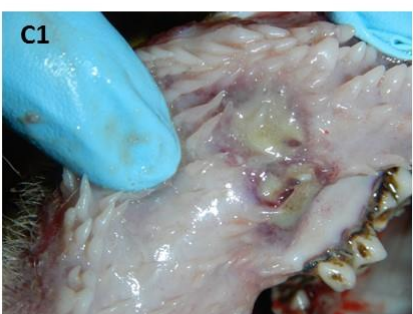
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BTV-1
MOR2007/01

BTV-8
NET2006/06

BTV-4
MOR2004/02

BTV-4
MOR2009/07

BTV-4
MOR2009/10

780 **Figure S3: Typical pathological changes in acutely affected sheep infected with 5 different strains**
781 **of BTV** Pathological signs observed in sheep euthanized for reaching humane endpoints between 7-
782 14 days post infection. The cohort of the sheep is given in each panel. BTV-1 MOR2007/01 caused
783 wide ranging haemorrhages in the oral and nasal mucosa, but also skeletal muscles, as well as
784 significant subcutaneous facial and systemic oedema (4/4 sheep). BTV-8 NET2006/06 caused
785 moderate haemorrhagic lesions to the oral and nasal mucosa and tongue (2/4 sheep). BTV4
786 MOR2004/02 only caused mild haemorrhagic lesions of the oral mucosa (1/4 sheep – both pictures
787 of the same sheep). Both BTV-4 reassortant strains (BTV4 MOR 2009/07 and BTV-4 MOR 2009/10)
788 caused oral and nasal haemorrhagic lesions exceeding those of the BTV-4 and BTV-8 parental strains,
789 but less severe than the BTV-1 parental strain (2/4 sheep for each strain). These two viral strains
790 caused significant oral or nasal ulcerations in the affected sheep that were more severe than those
791 seen for any parental BTV strain.

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