

1 **Experimental infection of European red deer (*Cervus elaphus*) with bluetongue**
2 **virus serotypes 1 and 8**

3

4 Jorge Ramón López-Olvera^{1*}, Caterina Falconi^{2,5}, Paloma Fernández-Pacheco³, Jovita
5 Fernández-Pinero³, Miguel Ángel Sánchez³, Agustín Palma³, Irene Herruzo³, Joaquín
6 Vicente², Miguel Ángel Jiménez-Clavero³, Marisa Arias³, José Manuel Sánchez-
7 Vizcaíno⁴, Christian Gortázar²

8 1 Servei d'Ecopatologia de Fauna Salvatge (SEFaS), Universitat Autònoma de
9 Barcelona (UAB), Bellaterra, Barcelona, Spain

10 2 Instituto de Investigación en Recursos Cinegéticos (IREC; CSIC-UCLM-JCCM),
11 Ciudad Real, Spain

12 3 Centro de Investigación en Sanidad Animal (CISA-INIA), Valdeolmos, Madrid, Spain

13 4 Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense
14 de Madrid (UCM), Madrid, Spain

15 5 Via E. De Magistris 9, 09123, Cagliari, Italy

16 * Corresponding author:

17 Servei d'Ecopatologia de Fauna Salvatge (SEFaS), Universitat Autònoma de
18 Barcelona (UAB), Bellaterra, E-08193, Barcelona, Spain

19 E-mail address: Jordi.Lopez.Olvera@uab.es

20 Telephone: +34935868190 Fax: +34935812006

21 **Keywords:** Bluetongue; red deer; BTV RNA; antibodies; wildlife; reservoir

22 **Abstract**

23 Bluetongue (BT) is a climate change-related emerging infectious disease in Europe.
24 Outbreaks of serotypes 1, 2, 4, 6, 8, 9, 11, and 16 are challenging Central and Western
25 Europe since 1998. Measures to control or eradicate bluetongue virus (BTV) from
26 Europe have been implemented, including movement restrictions and vaccination of
27 domestic BTV-susceptible ruminants. However, these measures are difficult to apply in
28 wild free-ranging hosts of the virus, like red deer (*Cervus elaphus*), which could play a
29 role in the still unclear epidemiology of BT in Europe. We show for the first time that
30 BTV RNA can be detected in European red deer blood for long periods, comparable to
31 those of domestic ruminants, after experimental infection with BTV-1 and BTV-8. BTV
32 RNA was detected in experimentally-infected red deer blood up to the end of the study
33 (98-112 dpi). BTV-specific antibodies were found in serum both by enzyme-linked
34 immunosorbent assay (ELISA) and virus neutralization (VNT) from 8-12 dpi to the end
35 of the study, peaking at 17-28 dpi. Our results indicate that red deer can be infected with
36 BTV and maintain BTV RNA for long periods, remaining essentially asymptomatic.
37 Thus, unvaccinated red deer populations have the potential to be a BT reservoir in
38 Europe, and could threaten the success of the European BTV control strategy.
39 Therefore, wild and farmed red deer should be taken into account for BTV surveillance,
40 and movement restrictions and vaccination schemes applied to domestic animals should
41 be adapted to include farmed or translocated red deer.

42

43 1. Introduction

44

45 Formerly considered an exotic viral disease of wild and domestic ruminants,
46 several outbreaks of bluetongue (BT) virus serotypes 1, 2, 4, 6, 8, 9, 11, and 16 have
47 challenged Europe since 1998. BT is an emerging infectious disease related to climate
48 change (Purse et al., 2005; Breard et al., 2007; Enserink, 2008; Rodríguez-Sánchez et
49 al., 2008; Eschbaumer et al., 2009; European Commission, 2009).

50 Measures to control bluetongue virus (BTV) in Europe include movement
51 restrictions and vaccination (European Commission, 2009), and surveillance systems for
52 BT are being established (Hadorn et al., 2009). As of May 2009, the largest restriction
53 zones in Europe correspond to BTV serotypes 1 (BTV-1), which is expanding
54 northwards since its first introduction in Southern Spain in 2007, and 8 (BTV-8),
55 spreading throughout Europe since it appeared in The Netherlands in 2006 (Purse et al.,
56 2005; Rodríguez-Sánchez et al., 2008). The target of the vaccination campaign is to
57 achieve at least 80% coverage of domestic ruminants using killed vaccines, although
58 doubts have arisen about its effectiveness (Enserink, 2008; Rodríguez-Sánchez et al.,
59 2008).

60 Red deer (*Cervus elaphus*) population density in Europe ranges from 2 to 30
61 individuals per square kilometre (up to 70 for food supplemented populations)
62 (Acevedo et al., 2008; Lovari et al., 2009), which could account for a significant
63 percentage of the BTV-infection susceptible ruminant population in certain regions.

64 Wild ruminants are included in the European Council Directive 2000/75/EC of
65 20 November 2000, laying down specific provisions for the control and eradication of
66 bluetongue, but vaccination and movement restrictions can only be applied in farmed or
67 managed ruminants, being almost impossible in wild free-ranging hosts of the virus.

68 High prevalence of serum antibodies against BTV has been reported in several species
69 of wild ungulates, including red deer (Linden et al. 2008; Ruiz-Fons et al. 2008; García
70 et al. 2009), suggesting widespread contact of wild ruminants with BTV. Moreover,
71 BTV RNA has been recently detected in farmed red deer in Spain (serotypes 1 and 4)
72 (Rodríguez-Sánchez et al. 2010) but BTV infection seems not to result in a significant
73 mortality in red deer (Linden et al. 2008), although sporadic fatal disease with BTV
74 isolation has been reported in mouflon (*Ovis aries*) (Fernández-Pacheco et al. 2008). BT
75 is considered endemic in wild ruminants in parts of Africa and North America
76 (Stallknecht et al. 1996; Gerdes, 2004), but up to now little is known about the role deer
77 could play in the epidemiology of BT in Europe. The aim of this study is to determine
78 the dynamics of BTV serotypes 1 and 8 infection in red deer, thus assessing the
79 potential of this species as a wild reservoir for BT.

80

81 **2. Materials and methods**

82

83 Eleven seven month-old red deer females were transported into the insect-proof
84 biosecurity level 3 (BSL3) facilities of the Centro de Investigación en Sanidad Animal
85 (CISA) in Valdeolmos (Madrid) on January 19th 2009. The deer were kept in three
86 different boxes (four in boxes A and B and three in box C). According to the routine
87 BSL3 procedures, each box was isolated from the others, sampling material was
88 exclusively used in the same box and the operators changed clothes before and after
89 working in a box, having a shower to exit from each box. One week after arriving to the
90 CISA, four of the deer (deer 1 to deer 3 in box A and deer 4 in box C) were inoculated
91 intravenously (iv) with $2,5 \times 10^6$ TCID₅₀ of BTV-1 strain Algeria/2006. This strain was
92 received from the Institute for Animal Health in Pirbright, and underwent five cell

93 culture passages prior to inoculation. Other four deer (deer 5 to deer 7 in box B and deer
94 8 in box C) were inoculated iv with $2,5 \times 10^6$ TCID₅₀ BTV-8 isolate 202326
95 (Belgium/2006). This strain was received from the Istituto Zooprofilattico Sperimentale
96 Della Lombardia e dell'Emilia Romagna (IZSLER), and was inoculated after four
97 culture passages. Finally, the three remaining deer (deer 9 in box A, deer 10 in box B,
98 and deer 11 in box C) received iv an equivalent volume of cell culture medium, acting
99 as controls. The deer were monitored daily from 0 days post-infection (dpi) to 12 dpi
100 and on 14, 17, 21, 24, 28, 31, 38, 50, 60, 66, 71, and 78 dpi. Monitoring included
101 exploration for clinical signs of bluetongue (rectal temperature, facial oedema,
102 erythema, coronitis, stomatitis, conjunctivitis), as well as collection of blood samples
103 with anticoagulant for real time RT-PCR analysis and without anticoagulant for serum.
104 Skin biopsies were taken at 14 dpi from the eight deer at boxes A and B (three BTV-1
105 inoculated, three BTV-8 inoculated and two controls). The deer were euthanized on 98
106 (four deer at box B), 105 (four deer at box A), and 112 dpi (three deer at box C).
107 Viral BTV RNA in blood was assessed using a modification of an already described
108 semi-quantitative real-time RT-PCR (Toussaint et al., 2007). Serum antibodies were
109 analyzed by commercial ELISA (Pourquier Bluetongue competitive ELISA, Institut
110 Pourquier, Montpellier, France), and by standard virus neutralization test (VNT) using
111 the inoculation virus as antigen, similarly to the methodology previously reported
112 (Hamers et al., 2009). Virus isolation was attempted in Vero and BHK cells only at
113 peak genome detection (12 dpi) and at late stages of the infection (78 dpi) one BTV-1
114 (deer 4) and one BTV-8 (deer 7) inoculated deer.

115 This study was approved by the INIA Ethics Committees on Animal
116 Experimentation and Biosafety. Handling procedures and sampling frequency were
117 designed to reduce stress and health risks for subjects, according to European (86/609)

118 and Spanish laws (R. D. 223/1988, R. D. 1021/2005), and current guidelines for ethical
119 use of animals in research (ASAB, 2006).

120

121 **3. Results**

122

123 BTV RNA was detected in all deer inoculated with BTV-1 (deer 1 to 4) and
124 three out of the four deer inoculated with BTV-8 (deer 5 to 7) from one dpi to the end of
125 the study, with a peak around twelve dpi for both serotypes, and a slow decline
126 thereafter. Figure 1 shows dynamics of BTV RNA detected in blood of the infected red
127 deer, as assessed by real-time RT-PCR. Detection of BTV RNA was low and transient
128 for BTV-8-infected deer 8 (box C), disappearing after 14 dpi. No virus RNA was
129 detected in none of the control deer until 38 dpi. However, on 38 dpi BTV RNA was
130 detected in control deer 11 (box C), followed by seroconversion between 38 and 50 dpi.
131 The virus infecting this deer was characterized as BTV-1 both by VNT and BTV-
132 1/BTV-8 multiplex real-time RT-PCR (Fernández-Pinero et al., in prep.). From all the
133 four samples where BTV isolation was tried, only BTV-1 was recovered at 12 dpi from
134 deer 4 (inoculated with BTV-1) in Vero cells. Only mild transient unspecific clinical
135 signs, which could be compatible with BT, were observed in the infected deer, and no
136 statistically significant difference, peak or trend in rectal temperature was evidenced.

137 Serum antibodies against BTV were detected in all inoculated deer both by VNT
138 and ELISA tests. ELISA revealed BTV specific antibodies by 10 dpi in the BTV-1
139 group, and between 9 and 12 dpi in the BTV-8 group, antibodies being present
140 throughout the whole study period for both serotypes. In deer inoculated with BTV-1,
141 the neutralizing antibody response was first detected at 8-11 dpi, with peak titres of
142 1/1280 around 17-21 dpi. In deer inoculated with BTV-8 neutralizing antibodies

143 appeared at 8 dpi, with peak titres of 1/640 to 1/1280 between 17 and 28 dpi. Controls 9
144 and 10 showed no specific antibodies throughout the whole experiment. However,
145 control deer 11, the one where BTV RNA was first detected at 38 dpi, seroconverted by
146 45 dpi, showing antibodies against BTV-1 until the end of the study (Figure 2).

147 BTV RNA was detected in the skin biopsies obtained at 14 dpi in five out of the
148 six BTV-inoculated deer (three inoculated with BTV-1 and two with BTV-8) analyzed.
149 The result was doubtful for the remaining BTV-8-inoculated deer, and negative for the
150 two control deer sampled.

151

152 **4. Discussion**

153

154 Our results confirm that Iberian red deer get asymptotically infected with
155 BTV serotypes 1 and 8, BTV RNA being reliably detected for long periods, comparable
156 in intensity and duration to that of domestic ungulates (Luedke, 1969; MacLachlan et al.
157 1990; Puentes et al., 2008). RT-PCR has been validated as a detection technique for
158 BTV, at least as sensitive as viral titration on Vero cells (Hamers et al. 2009). BTV
159 viraemia with mild or no clinical signs following experimental infection has been
160 reported in North American elk (*Cervus elaphus canadensis* and *Cervus elaphus*
161 *nelsoni*) (Murray and Trainer, 1990; Ellis et al., 1993). BTV was isolated as long as 105
162 dpi from the blood of experimentally infected elk after 95 days of negative results
163 followed by cortisone injection (Murray and Trainer 1970). However, duration and
164 intensity of both the virus dynamics and the immune response remains to be fully
165 described. To the best of our knowledge, this is the first report to address this issue in
166 European red deer. The kinetics of the antibody response of our experimentally-infected
167 red deer during the study period was similar to those of experimentally infected

168 domestic ruminants and North American elk (Murray and Trainer, 1970), showing the
169 validity of ELISA and serum VNT to monitor contact with BTV in red deer. The
170 clinical picture observed among BTV-1 and BTV-8 infected deer was similar to the
171 mild subclinical effects of BTV observed in cattle (MacLachlan et al., 1990) and elk
172 (Murray and Trainer, 1970; Ellis et al., 1993), rather than to the more severe clinical
173 pictures often described in sheep (MacLachlan et al., 2008) and white-tailed deer
174 (Vosdingh et al., 1968).

175 Transplacental, oral, and wound contact have been suggested as mechanisms for
176 BTV transmission in absence of the *Culicoides* vector, both in domestic and wild
177 ungulates (Vosdingh et al. 1968; Thomas and Trainer, 1970; Menzies et al., 2008;
178 Backx et al., 2009). Iatrogenic transmission can be discarded for the spontaneously-
179 infected control deer 11 of our study, since material for each deer was prepared
180 individually before entering the enclosure, so oral (the deer bit each other due to
181 hierarchic fights in the small area of the enclosure) or wound transmission would be the
182 most likely explanation. Transmission in absence of the vector is therefore possible in
183 close contact conditions, and although its epidemiological importance is unknown, it
184 could be a concern regarding the overwintering of BTV (Wilson et al., 2008).

185 Which is the role of red deer in the epidemiology of BTV in Europe? Red deer is
186 an abundant wild ruminant in many parts of the northern hemisphere, occurring in BTV
187 affected regions of central and southern Europe (Lovari et al., 2009). Other wild
188 ruminant species are considered maintenance hosts for BTV in Africa and North
189 America, the virus being endemic in their populations (Stallknecht et al., 1996; Gerdes,
190 2004). Antibodies against BTV and BTV RNA have been identified in free-ranging
191 naturally infected red deer in Europe (Linden et al., 2008; Ruiz-Fons et al., 2008; García
192 et al., 2009). Moreover, RNA of BTV-4 was found in red deer blood samples in summer

193 2007, eight months after the last detection in sympatric domestic ruminants (Rodríguez-
194 Sánchez et al., 2010). Finally, our results indicate that red deer can maintain BTV RNA
195 in blood for long periods, and therefore red deer have the potential to play a role in BT
196 epidemiology. However, the lack of BTV isolation at 78 dpi suggests that only BTV
197 RNA but no viable virus stays detectable long after the peak viraemia. Moreover, it
198 remains unanswered whether the *Culicoides* vector can get infected from red deer.
199 Nevertheless, the detection of BTV in skin samples of our experimentally-infected red
200 deer at 14 dpi seems to point that it could be infectious to midges, at least during the
201 peak of BTV RNA detection.

202

203 **6. Conclusion**

204

205 Red deer have the potential to be a reservoir for BT. A natural experiment to test
206 this hypothesis is currently running: livestock has largely been vaccinated (European
207 Commission, 2009), but annual re-vaccination is unlikely to be maintained for long.
208 Vaccine induced immunity against BTV can last for over one year, but it may be lost
209 after a certain time if no annual boost occurs (Hamers et al., 2009). Also, positive
210 results of the vaccination campaigns rely on a minimum coverage of 80% of the
211 susceptible population (Ferrari et al., 2005), and susceptible unvaccinated red deer could
212 affect this minimum required goal. Hence, if repeated BTV outbreaks occur in regions
213 with high wild ruminant densities and no clear link with livestock movements or
214 vaccination failures, the deer reservoir hypothesis will be confirmed. If, in contrast, no
215 BTV circulation takes place and livestock vaccination alone is successful in eradicating
216 bluetongue, the hypothesis will be rejected.

217

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219

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230

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352

353 **Figure captions**

354

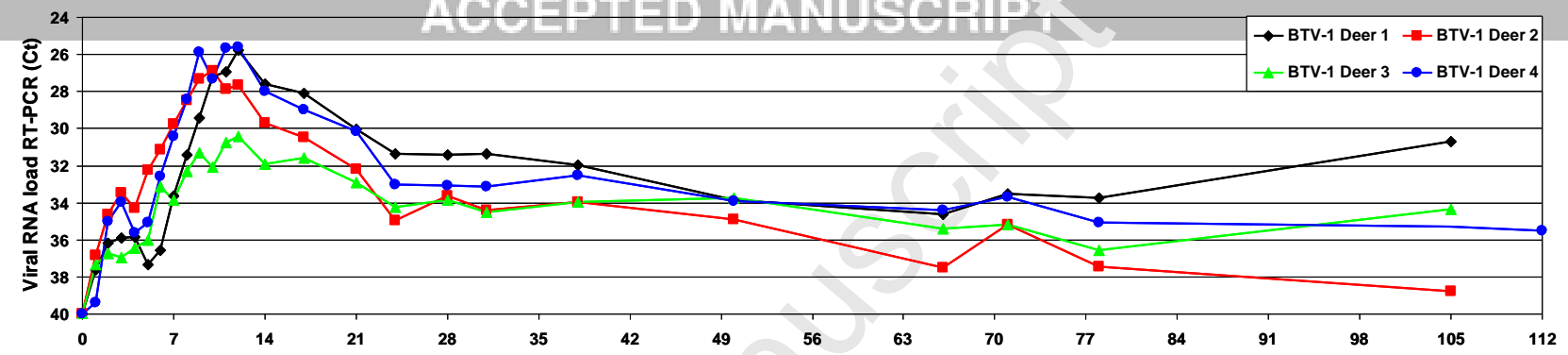
355 **Figure 1.-** Viral load (Ct=Cycle at which specific amplification starts to be detectable in
356 the real-time RT-PCR technique) determined by generic BTV RRT-PCR on whole
357 blood samples from 0 dpi to the end of the experiment (98-112 dpi). Serotype was
358 confirmed by serotype-specific RRT-PCRs of the serotype inoculated in randomly
359 selected samples for each deer. Upper panel: BTV1; Central panel: BTV8; Lower panel:
360 control.

361

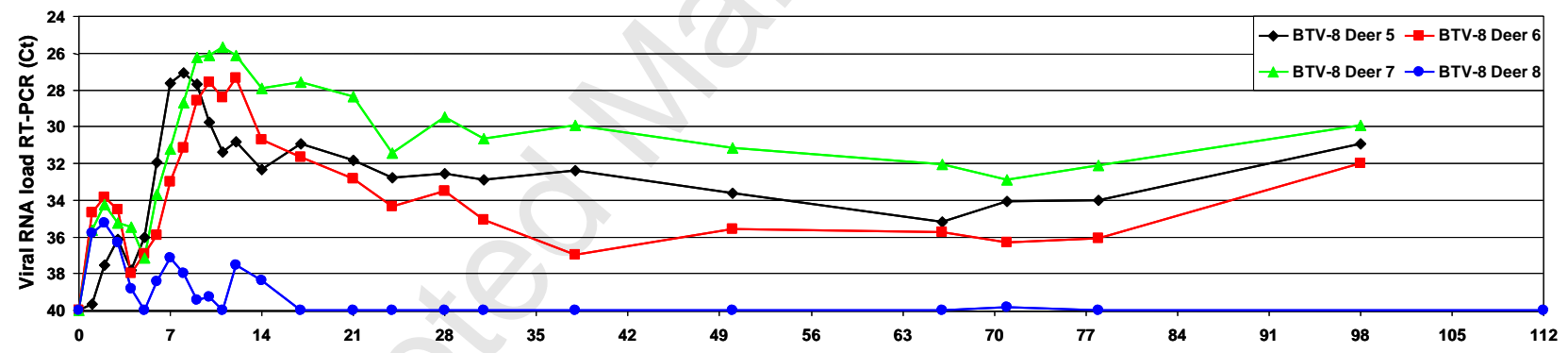
362 **Figure 2.-** Kinetics of the BTV-neutralizing antibody response measured by VNT in
363 BTV-inoculated and control red deer. Upper panel: sera from BTV-1 inoculated deer 1-

364 4 and control deer 9 and 11 tested against BTV-1. Lower panel: sera from BTV-8
365 inoculated deer 5-8 and control deer 10 and 11, tested against BTV-8.

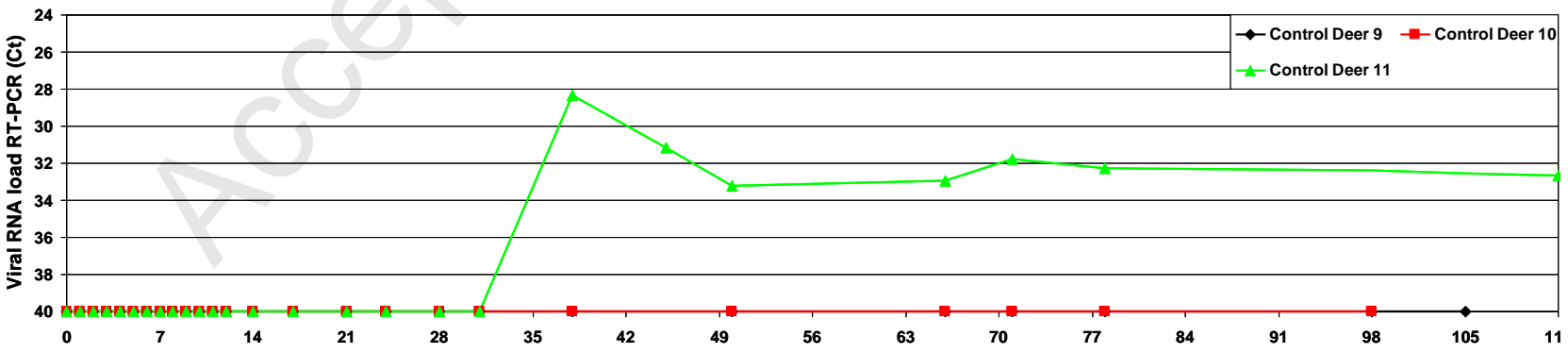
Accepted Manuscript



BTV-8



CONTROL



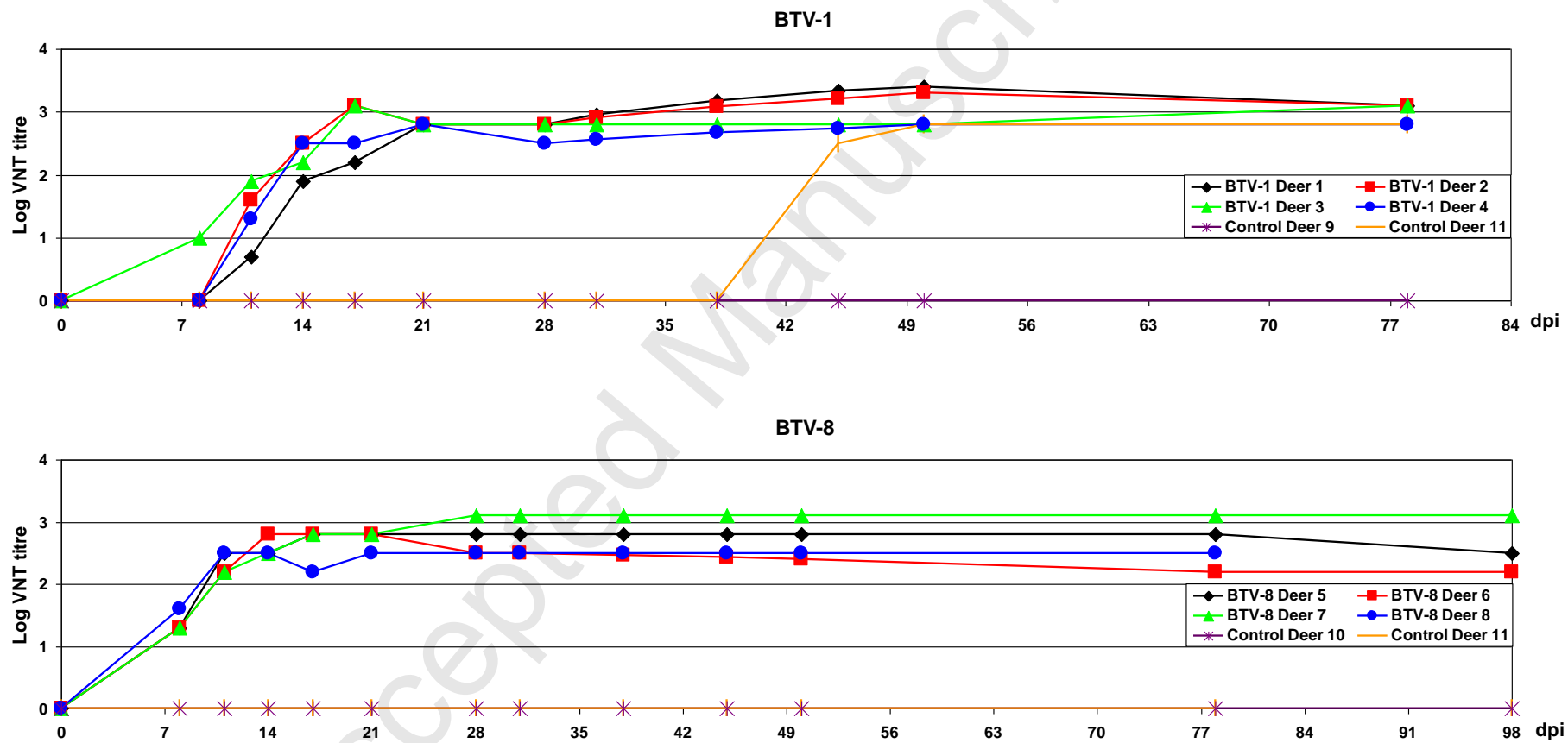


FIGURE 2