Experimental infection with chamois border disease virus causes long-lasting viraemia and disease in Pyrenean chamois (Rupicapra pyrenaica)

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Since 2001, severe outbreaks of disease associated with border disease virus (BDV) infection have been reported in Pyrenean chamois. The disease is characterized by variable degrees of cachexia, alopecia and neurological manifestations prior to death. The aim of this study was to investigate this disease under experimental conditions. To assess viral virulence, humoral immune response, dissemination and probable routes of transmission, seven chamois (five seronegative and two seropositive for BDV) were inoculated with a BDV isolated from a naturally infected chamois. A group of three chamois were maintained as uninfected controls. The five seronegative chamois became viraemic from day 2 post-inoculation (p.i.) until their death (three animals) or the end of the experiment (on day 34 p.i.) and developed neutralizing antibodies from day 18 p.i. until the end of the study. Continuous shedding of the virus was detected by RT-PCR in oral, nasal and rectal swabs in viraemic chamois from day 5 p.i. Despite none of the viraemic chamois showing obvious neurological signs, all of them had a non-suppurative meningoencephalitis as seen in naturally infected chamois. The two inoculated BDV-seropositive chamois did not become viraemic. This study confirms that BDV is the primary agent of the disease that has been affecting chamois populations in recent years in the Pyrenees and that previously acquired humoral immunity is protective.

INTRODUCTION

Border disease (BD) is a congenital infectious disease caused by border disease virus (BDV, genus Pestivirus, family Flaviviridae), an enveloped, positive-sense, ssRNA virus that mainly affects small ruminants. In 2001 and 2002 an outbreak of a disease associated with a genotype-4 BDV (hereafter, ch-BDV) infection was reported in a Pyrenean chamois (Rupicapra pyrenaica) population in the central Pyrenees (Catalonia, north-eastern Spain) (Arnal et al., 2004; Hurtado et al., 2004; Marco et al., 2007). The disease spread to the eastern Pyrenees, causing further severe outbreaks with even higher mortality rates that led to a collapse in chamois populations (Marco et al., 2009). Ten years after the first outbreak, the affected populations are recovering, although more slowly than expected, and the disease remains a major health concern in the Pyrenean chamois.

The clinical presentation of BD in sheep is characterized by the presence of barren ewes, abortions, stillbirths and the birth of weak and persistently infected (PI) lambs. BD in healthy postnatally infected sheep is mild or unapparent and animals develop a short viraemia that ends when neutralizing antibodies appear in serum (Nettleton et al., 1998). However, an outbreak of disease with high mortality associated with acute BDV infection in healthy sheep has been reported in the Aveyron region (France). Furthermore, a mucosal disease-like syndrome has been described occasionally in PI sheep (Chappuis et al., 1984; Monies et al., 2004; Hilbe et al., 2009).
Traditionally, the genus *Pestivirus* includes four species: *Bovine viral diarrhea virus* type 1 (BVDV-1) and type 2 (BVDV-2) in cattle, BVDV in small ruminants and *Classical swine fever virus* (CSFV) in swine (Becher *et al.*, 1997). Interspecies transmission of BVDV and BDV between a number of domestic animal species has been reported in recent years and has led to the detection of antibodies against pestivirus in many other domestic and wild *Artiodactyla* species (Vilcek & Nettleton, 2006). Despite the fact that pestiviruses are widespread and present in many populations of free-living ruminants (Løken, 1995) BDV isolation has only occasionally been reported (Becher *et al.*, 1997) in Pyrenean chamois.

Clinical and post-mortem studies of infected chamois reveal a previously unreported presentation for a BDV infection, consisting of cachexia, alopecia with skin hyperpigmentation and several degrees of neurological alterations such as depression, weakness and difficulty in moving, which are associated with a non-suppurative meningoencephalitis (Marco *et al.*, 2007). Although several studies of affected chamois have linked the observed disease with this virus (Arnal *et al.*, 2004; Hurtado *et al.*, 2004; Marco *et al.*, 2007), current knowledge of the disease in chamois is still limited. Unanswered questions about the pathogenesis of the disease and its infection dynamics in chamois are essential for understanding the progress of this virus in these populations, as well as its spread between wild populations and domestic livestock in the Pyrenees. In this sense, interspecies ch-BDV infection would be a diagnostic challenge for the control of other pestiviruses in livestock (Cabezon *et al.*, 2010a, b) and other animal species. In addition, information about the behaviour of this virus in infected chamois, which is associated with fatal outbreaks and severe ecological and economic losses, is important for the conservation and management of this species. The three objectives of the present work were: to demonstrate whether under experimental conditions ch-BDV causes this disease and the lesions described in naturally infected chamois; to describe the viral dynamics and the humoral immune response; and to assess whether previously acquired humoral immunity against pestiviruses protects Pyrenean chamois from ch-BDV infection and disease occurrence.

**RESULTS**

No overt clinical signs that could be unequivocally attributed to the infection were observed during the adaptation or post-challenge periods. However, chamois 2 and 4 died suddenly on days 18 and 20 post-inoculation (p.i.), respectively, with haemorrhagic diarrhoea with pasty dark faeces in the perineal region and tail. Also, chamois 5 had mild coughing and was found dead on day 24 p.i.

During the experiment mean body temperatures in viraemic chamois 1 to 5 were higher than in non-viraemic chamois (*F*=3.93, *edf*=2.6, *P*=0.015, 10% of explained deviance), especially from day 8 p.i. onwards, when the mean body temperature of the viraemic chamois increased by 0.77 degrees with respect to their counterparts (mean=39.79 °C, *SEM*=0.18, min.=38.65, max.=41.84 for viraemic versus mean=39.02 °C, *SEM*=0.17, min.=37.86, max.=41.12 for non-viraemic chamois) (Fig. 1). All chamois lost between 13 and 25% of their body weight during the adaptation period to captivity. From day 0 of the experiment to the day of death or necropsy, the viraemic chamois continued to lose, on average, an additional 4% of body weight, while non-viraemic chamois recovered 15% of their initial weight (*t*=6.96, *P*<0.001) (Fig. 2).

BDV was detected by RT-PCR in the sera of chamois 1 to 5 from day 2 p.i. until they died or were euthanized. BDV was isolated and titered from sera samples from all these chamois from day 2 p.i. until the last day of sampling, showing an increase in the virus titres until day 11 p.i., with a maximum titre of 10^{6.8} TCID_{50} ml^{-1} (Table 1). From day 18 p.i. until the death/euthanasia of the animals, virus titre decreased in one animal (chamois 1), was not detected in another (chamois 3) and remained unchanged in the other three (chamois 2, 4 and 5) viraemic chamois. The two antibody-positive challenged animals (chamois 6 and 7) and all control animals (chamois 8 to 10) tested negative for the presence of BDV in sera samples throughout the sampling by RT-PCR and virus isolation.

BDV RNA was detected by RT-PCR in oral, nasal and rectal swabs in chamois 1 to 5 from day 2 p.i. until the end of the study. Animals 6 and 7 were negative with the exception of nasal swabs on day 25 p.i. for chamois 6 and
day 34 p.i. for chamois 6 and 7 (Table 2). Swab samples tested by RT-PCR were negative for all control chamois.

All tissue and urine samples collected at necropsy from viraemic animals (chamois 1 to 5) tested positive for BDV by RT-PCR. On the other hand, chamois 6, 7 (challenged animals), 8, 9 and 10 (control animals) tested negative.

Two of the viraemic chamois (2 and 3) had low neutralizing antibody titres against the homologous BDV CADI-6 on day 11 p.i. On day 18 p.i., all viraemic chamois (1 to 5) had seroconverted and remained antibody-positive until the end of the study (Table 1). Challenged antibody-positive chamois 6 and 7 had neutralizing antibodies against homologous BDV throughout the study. No increase in neutralizing antibodies against the homologous BDV was observed. The three control animals tested negative, by a virus neutralization test (VNT), throughout the study.

**DISCUSSION**

The virological and clinico-pathological data obtained from the inoculated chamois in the present study were unexpected when compared with reported pestivirus infections in domestic and wild ruminants. Postnatal BDV infection in immunocompetent animals is normally subclinical; the viraemia is transient and difficult to detect, and usually only seen between days 4 and 11 p.i. (Nettleton et al., 1998). Experimental infections in pigs and lambs with the same ch-BDV have followed this pattern (Cabezo´n et al., 2010a, b). However, during the experimental infection of chamois we observed a long-lasting viraemia. The virus appeared in serum on day 2 p.i. and persisted until the animals died or were euthanized on day 34 p.i.

At necropsy, chamois 2 and 4 had haemorrhagic diathesis affecting mainly the gastrointestinal tract with fluid to thick pasty haemorrhagic contents from the duodenum to the rectum. In order to rule out a haemorrhagic enterotoxaemia, an ELISA for the detection of α-, β- and ε-toxins and a structural protein of *Clostridium perfringens* (Bio-K 095; Bio-X Diagnostics) was performed with sera samples from these two animals; both chamois tested negative. Chamois 3 died on day 24 p.i. with a severe bacterial fibrinous bronchopneumonia. In chamois 1 and 5, only a mild lymphadenomegaly was observed at necropsy on day 34 p.i. No significant gross lesions were observed in the rest of chamois.

Microscopic lesions were only seen in viraemic chamois. The main and most consistent lesions were detected in the brain and lymphoid tissues. All five viraemic chamois had non-suppurative lymphohistiocytic meningoencephalitis and gliosis (Fig. 3). Changes in lymphoid tissue in both lymph nodes and the spleen were characterized by moderate lymphoid depletion with a loss of lymphoid follicles and decreased lymphoid density (Fig. 3). In the animals that died with haemorrhagic diathesis (chamois 2 and 4), multifocal extravasation of red blood cells, especially in the serosa, were observed throughout the intestinal tract. Focal areas of transmural haemorrhages were detected in the large intestine of animal 4.

**Table 1. Virus Neutralization Test (VNT) and virus titration in pestivirus-free sheep fetal thymoid (SFT-R) cells of sera from viraemic chamois**

For the VNT, sera were tested against homologous virus and titres expressed as the reciprocal of the highest dilution that neutralized 100 TCID₅₀ in all cultures. Titres of virus are expressed as log₁₀ TCID₅₀ ml⁻¹ of sera. –, Negative; NA, Not available.

<table>
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<tr>
<th>Chamois</th>
<th>2 days p.i.</th>
<th>5 days p.i.</th>
<th>8 days p.i.</th>
<th>11 days p.i.</th>
<th>18 days p.i.</th>
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<td>4.6</td>
<td>–</td>
<td>6.8</td>
<td>160</td>
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<tr>
<td>RP 2</td>
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<td>4.6</td>
<td>–</td>
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<td>RP 3</td>
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<td>3.9</td>
<td>–</td>
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<td>20</td>
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<td>80</td>
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<td>RP 4</td>
<td>–</td>
<td>4.6</td>
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<td>RP 5</td>
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The high titres of viable replicating infectious virus detected in sera and in the continuous shed of virus via nasal, oral, faecal and urinary routes agrees with the findings previously reported for naturally infected Pyrenean chamois (Cabezón et al., 2010c). This high virus excretion in naturally and experimentally infected chamois might facilitate a high horizontal virus transmission rate that would explain the rapid spread of the infection during the different outbreaks throughout the central and eastern Pyrenees (Marco et al., 2007, 2009). The existence of this type of transmission is supported by the fact that chamois 6 and 7 (seropositive-challenged chamois that did not become viraemic, but which were in contact with the viraemic chamois) tested positive by RT-PCR using nasal-swab samples at days 18 and 34 p.i. It is worth remarking that the absence of viraemia in these two antibody-positive challenged chamois would seem to confirm that previously acquired humoral immunity protects against ch-BDV infection.

Viral RNA was present in all the organs of the viraemic chamois, as has been reported in naturally infected animals (Cabezón et al., 2010c). A transient and mild viraemia has been described in sheep experimentally acutely infected with the same ch-BDV, and in these cases the presence of viral RNA was only detected on day 7 p.i. in mesenteric, mediastinal and prescapular lymph nodes, tonsils and brain (Cabezón et al., 2010b). Therefore, the great capacity of this virus for dissemination and replication in almost all organs of acutely infected chamois is unprecedented. Long-lasting viraemia, the continuous shedding of the virus via oral, nasal and rectal routes, and generalized viral tissue distribution observed in the chamois of this study have only previously been described in PI animals (Vilcek et al., 1994; Nettleton et al., 1998). However, while PI animals are infected in utero, the chamois in the present study were postnatally infected.

Antibody response in the experimentally infected chamois was similar to that of acute BDV infection (Nettleton et al., 1998) and to experimental infection with ch-BDV in sheep and pigs (Cabezón et al., 2010a, b). However, only chamois 2 achieved viral clearance in sera. This animal neutralized the virus in blood by day 18 p.i., although it later died as a result of severe haemorrhagic diathesis, and detection of BDV RNA was confirmed in all its tissues at the time of death. The explanation for the long viraemia co-existing with high titres of neutralizing antibodies in the other four viraemic chamois has not been elucidated. Co-existing viraemia and antibodies have only been described in PI animals when they are infected with another pestivirus strain (Nettleton et al., 1998). It is unlikely that stress affected the outcome of the infection. During the adaptation period and experimental infection, the evaluation of the behaviour in control and infected chamois concluded that stress was successfully eliminated by the use of the long-acting tranquilizer enanthate perphenazine (I. Marco and O. Cabezón, unpublished results).

Clinical signs and severe disease following acute BDV infection in immunocompetent animals are extremely rare.
In fact, there is only one report of an outbreak of severe disease in the literature, which was characterized by a haemorrhagic syndrome and leukopenic enterocolitis with high mortality in sheep and growing lambs (Chappuis et al., 1984). It was associated with a new BDV strain (Aveyron) that produced profound leukopenia and death in 50% of 3–5-month-old lambs. However, further experimental infections in lambs with this highly pathogenic strain failed to reproduce the disease (Thabti et al., 2002). Severe neurological signs associated with a non-suppurative meningoencephalitis and chronic wasting preceding death have been described in naturally infected chamois (Marco et al., 2007). In our experimental infection none of the viraemic chamois showed any neurological signs of disease, although it is likely that such signs would have been difficult to assess, since the animals were under the effects of the perphenazine and in captivity. Nevertheless, the lesions observed in the brain were similar to those described in naturally infected chamois (Marco et al., 2007), suggesting that these signs would have occurred if they had not been tranquilized and/or the experiment had lasted longer. Non-suppurative meningoencephalitis and focal and diffuse gliosis were similar in both naturally and experimentally infected chamois, but spongiosis, which is consistently seen in naturally infected animals, was seen only mildly in one of the studied chamois. This result may indicate that this lesion could be the result of a longer, more persistent infection, a hypothesis that needs to be explored further. The neurotropism of ruminant pestiviruses in congenital infections is well-documented (Bielefeldt-Ohmann et al., 2008; Maxie & Youssef, 2007) but has only been suspected in few postnatal infections with BDV or BVDV (Blas-Machado et al., 2004; Monies et al., 2004). This study confirms the neurotropism and neuropathology of ch-BDV in postnatally infected chamois.

Severe haemorrhagic diathesis was seen in two of the viraemic chamois, and thrombocytopenia was suspected to be the most likely cause. However, this condition has never

**Fig. 3.** (a) Brain-hippocampus of control chamois. (b) Brain-hippocampus showing non-suppurative lymphohistiocytic meningoencephalitis and gliosis in a viraemic chamois (day 34 p.i.). (c) Lymph node of control chamois. (d) Lymph node from a viraemic chamois showing loss of lymphoid follicles and lymphoid density (day 34 p.i.). Bars, (a, b) 100 μm; (c, d) 500 μm.
been observed in naturally infected chamois. As mentioned previously, BDV has been associated with a haemorrhagic syndrome in acutely infected sheep (Chappuis et al., 1984), although the infection mechanism was not discovered. Haemorrhagic syndromes have also been reported in infections with other pestiviruses such as BVDV-2 and CSFV that have been associated with thrombocytopenia, which could be multifactorial and a consequence of platelet activation and aggregation, as well as defective production of virus excretion may explain the high transmission rate and spread of the disease during the reported outbreaks.

In conclusion, the present study confirms that ch-BDV is the aetiological agent of the disease that has been affecting Pyrenean chamois in the Pyrenees since 2001, and which has led to a significant reduction in the chamois population there. The long-lasting viraemia and high-load virus excretion may explain the high transmission rate and spread of the disease during the reported outbreaks.

**METHODS**

**Animals.** Ten female Pyrenean chamois between 1 and 8 years of age were captured in drive nets (López-Olvera et al., 2009) in the Fraser-Setcases National Hunting Reserve (north-east Spain, 42°22’N, 2°09’E). By means of a RT-PCR-based assay all animals were confirmed to test negative for the presence of pestivirus in sera for at least 10 days before the day of the challenge and the day of the challenge itself. Chamois 1, 2, 3, 4, 5, 8, 9 and 10 were antibody confirmed to test negative for the presence of pestivirus in sera for at least 10 days before the day of the challenge and the day of the challenge itself. Chamois 1, 2, 3, 4, 5, 8, 9, and 10 were antibody positive, while chamois 6 and 7 were negative against BDV according to a VNT.

**Inoculum.** A non-cytopathic ch-BDV isolated from a diseased chamois found in the Pyrenees in 2005 was used as inoculum. The ch-BDV was cultured in single and double passages in the SFT-R cell line (provided by the Friedrich-Loeffler Institute, Island of Riems, Germany). The virus titre was determined by end-point titration in the SFT-R cell line, obtaining a measurement of $1 \times 10^7$ TCID$_{50}$ ml$^{-1}$ of virus. The sequence of the 5' UTR of the virus used in this experimental infection was deposited in GenBank under the name CADI-6 with the accession number AM905923. The 5'-UTR fragment sequence was 241 bp long. The virus isolated from the chamois was located in the same BDV genotype (BDV-4) as described for previous outbreaks in chamois populations (Marco et al., 2009).

**Experimental design.** After 2 weeks of adaptation to captive conditions the animals were housed in two isolated boxes (challenged and control chamois) in level-3 biosafety facilities in the experimental station of the Centre de Recerca en Sanitat Animal (CReSA, Universitat Autònoma de Barcelona, Spain). In order to mitigate the adverse effects of stress in captivity, 1.5 mg enanthate perphenazine kg$^{-1}$ was intramuscularly administered every week. In addition, all the chamois were treated with a single dose of 2.5 mg tulathromycin kg$^{-1}$ (Draxxin; Pfizer Animal Health) and a single oral dose of 2.5 mg toltrazuril kg$^{-1}$ of 5% (Baycox; Bayer Animal Health) to prevent opportunistic bacterial and parasitic infections. The chamois were separated into two groups on the basis of their serological status regarding BDV antibodies. Animals 1 to 7 were challenged with $1 \times 10^7$ TCID$_{50}$ of ch-BDV CADI-6 a.m.905923. The whole virus dose was thawed immediately before inoculation and diluted in a total volume of 2 ml of Eagle’s minimal essential medium (EMEM; produced in-house). The inoculum was administered by a combination of nasal catheter (0.5 ml in each nostril) and orally (1 ml). Animals 8 to 10 (control group) were inoculated with 2 ml of sterile EMEM via the same routes. The animals were observed daily for clinical signs. Rectal temperatures were monitored three times a week. Animals were weighed on day 0 p.i. and during necropsy, which was scheduled to be performed on day 34 p.i. Animal care activities and study procedures were conducted in accordance with the guidelines of Good Experimental Practices, with the approval of the Ethical and Animal Welfare Committee of the Universitat Autònoma de Barcelona.

**Sample collection.** To monitor viraemia and antibody response, blood samples were obtained from the jugular veins of the animals on days 0, 2, 5, 8, 11, 18, 25 and 34 p.i. Blood samples were placed in commercial serum-separator tubes and were centrifuged at 1200 g for 15 min to obtain serum. Sera were stored at $-80^\circ$C until processed. Nasal, oral and rectal swabs collected on the same days as the blood samples, along with urine swabs taken at necropsy, were mixed with 2 ml of sterile PBS (pH 7.2) 6% supplemented with antibiotics (10 000 U penicillin ml$^{-1}$, 10 000 μg streptomycin ml$^{-1}$ and 10 000 U nystatin ml$^{-1}$). Samples were mixed vigorously and stored at $-80^\circ$C until analysed. At necropsy, 1.0 g of tissue from the brain, tonsils, four lymph nodes (superficial cervical, superficial inguinal, mediastinal and mesenteric), spleen, digestive tract (oesophagus, reticulum, rumen, omasum, abomasum, duodenum, jejunum, ileum and colon), pancreas, liver, respiratory tract (nasal turbinates, trachea and lung), thyroid and adrenal glands, kidney, ovaries and uterus, heart, skin (ear and eyelid) and bone marrow from the proximal femur were sampled and homogenized in 9 ml EMEM 6% supplemented with antibiotics (10 000 U penicillin ml$^{-1}$, 10 000 U streptomycin ml$^{-1}$ and 10 000 U nystatin ml$^{-1}$), and stored at $-80^\circ$C.

**RT-PCR.** Total viral RNA was extracted directly from 150 μl of sera, swabs, urine and all tissue samples by using a Nucleospin Viral RNA Isolation kit (Macherey-Nagel) as per the manufacturer’s instructions. Two microlitres of the purified RNA were used for RT-PCR with a Qiagen One-Step RT-PCR kit (Qiagen). Reverse transcription was carried out at 50°C for 30 min. Thereafter, the RNA/DNA hybrid was denatured at 95°C for 15 min and the DNA amplified for 36 cycles (60 s at 94°C, 60 s at 56°C and 60 s at 72°C), with a final extension time of 7 min at 72°C. Thirty picomoles of primers 324 (5’-ATGCCCTWTAGGACAGCACA-3’; W= adenosine or thymine) and 326 (5’-TCAACTCCATGTCGACGAGC-3’) were used for the amplification reaction (Vilcek et al., 1994). These primers were designed from the ch-BDV genome: nt 101–121 and 386–366, respectively (Vilcek et al., 2010).

**Virus isolation.** In order to confirm that the RNA detected by RT-PCR was because of the presence of viable replicating infectious ch-BDV CADI-6 and then to quantify it, sera from viraemic animals were subjected to virus isolation and to an end-point titration on monolayers of SFT-R cells. The titration was performed by making tenfold dilutions of 96-well microtitre-plate cultures, and replication was monitored by using the immunoperoxidase monolayer assay (IPMA) (OIE, 2011) with a polyclonal pestivirus antibody (produced in-house). Virus titration was performed according to the method of Reed & Muench (1938). Furthermore, swab samples from challenged chamois (animals 1 to 7) from all collection days were cultured in SFT-R cells to confirm the presence of viable replicating virus.

**VNT.** Sera from all animals and all collection days were tested for the presence of antibodies against the homologous BDV strain CADI-6 with a VNT, as described in OIE (2011). Briefly, serum samples were diluted 1:10 with sterile EMEM, heat-inactivated (56°C for 30 min) and tenfold diluted in 96-well plates (50 μl per well). After adding a volume of 50 μl containing 100 TCID$_{50}$ of the homologous BDV, the plates were incubated at 37°C for an hour. Finally, 2.8 × 10$^5$ Madin–Darby bovine kidney cells (100 μl) were added to each well. Replication was monitored by the IPMA method as described above. Titres were expressed as the reciprocal of the highest dilution that neutralized
100 TCID₅₀ in all cultures, calculated according to the method of Reed & Muench (1938).

Pathological examination. Necropsies and tissue sampling were performed according to standard protocols. On day 34 p.i., the remaining chamois were euthanized with a lethal barbiturate injection. At necropsy, tissue samples (the same samples as described above) collected for the histopathological examination were fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 4µm sections and stained with haematoxylin and eosin according to standard procedures.

Statistical analysis. To explore whether body temperature and white blood cell count (WBC) were influenced by pestivirus infection, a set of mixed models in which both dependent variables were explained by the interaction between the treatment group (‘viraemic’ versus ‘non-viraemic’ as fixed effect) and the time in days (as fixed nominal factor) were fitted. Since the chamois were sampled throughout the study period, we considered each individual as a random factor to control pseudo-replication (Zuur et al., 2009). The relationship between WBC and both date of sampling and treatment group was nonlinear (skewness=1.38, kurtosis=4.55, Shapiro–Wilk normality test=0.86, P=0.01, residual pattern from a linear mixed model) and therefore an additive modelling based on the cubic regression splines method (mgcv package, version 1.6-1, see Wood, 2008) was used. Finally, the consequences of pestivirus infection on body weight were explored by comparing the weight variation [i.e. (weight at day 0–weight at necropsy) × 100/weight at day 0] between viraemic and non-viraemic chamois. Since variation in the weights of individuals was normally distributed (Shapiro-Wilk normality test=0.96, 0=0.86) comparisons were made by using an unpaired Student’s t-test. All statistical analyses were performed by using R version 2.12.0. (R Development Core Team, 2010).

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