Epidemiological aspects of Border Disease Virus infection in Pyrenean chamois (Rupicapra p. pyrenaica): Influence of the viral strain, non-artiodactyl hosts and sheep transhumance

Andreu Colom Cadena

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Els doctors Ignasi Marco Sánchez, Professor Titular d'Universitat de l'Àrea de coneixement de Medicina i Cirurgia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona i Óscar Cabezón Ponsoda, Investigador Ordinari del Servei d'Ecopatologia de Fauna Salvatge i Centre de Recerca en Sanitat Animal (CRESA-IRTA),

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List of abbreviations

APPV: Atypical Porcine Pestivirus

BD: Border Disease

BDV: Border Disease Virus

BVDV: Bovine Viral Diarrhoea Virus

CI: Confidence Interval

cp: Cytopathic

CSFV: Classical Swine Fever Virus

Ct: Threshold cycle

CT: Congenital Tremor

dpi: Days post-inoculation

EDTA: Ethylenediaminetetraacetic acid

EMEM: Eagle's Minimum Essential Medium

Fam.: Family (taxa)

FBS: Foetal Bovine Serum

ICTV: International Committee on Taxonomy of Viruses

i.e.: In example

IPMA: Immunoperoxidase monolayer assay

MD: Mucosal disease

MDBK: Madin-Darby bovine kidney

MHC: Major histocompatibility complex

ncp: non-cytopahtic

NHR: National Hunting Reserve

nt: Nucleotide

OIE: World Organization for Animal Heatlh

ORF: Open Reading Frame

PBS: Phosphate Buffered Saline

p.i.: Post inoculation

PI: Persistent infected

qPCR: Quantitative polimerase chain reaction (Real time-PCR)

R.p.pyrenaica: *Rupicapra pyrenaica pyrenaica*

RHA: Restricted Hunting Area

RNA: Ribonucleic Acid

RT-PCT: Reverse transcription polymerase chain reaction

sd: Standard deviation

sp.: Species

SP: Structural Proteins

$TCID_{50}$: Tissue Culture Infective Dose

VNT: Virus Neutralization Test

UTR: Untranslated Region

WBC: White Blood Cells

Abstract

Pestiviruses (Family *Flaviviridae*) cause diseases with important economic and health impact on livestock. One of these pestivirus species, the Border Disease Virus (BDV) is also of importance in wildlife conservation since 2001 when it was associated with high mortality outbreaks in Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*). After 16 years of research, different epidemiological scenarios of BDV infection in chamois populations have been described. The main objective of the present thesis was to analyse the role of three factors that may explain this epidemiological diversity: viral strains, sympatric wild non-artiodactyl hosts and sheep transhumance.

Study I was designed to confirm the presence of BDV strains of a high and low virulence in free-ranging chamois populations from Pyrenees and to understand the implications of these findings to the diverse epidemiological scenarios. An experimental infection of Pyrenean chamois with a high-virulence (Cadí-6) and low-virulence (Freser-5) BDV strains was performed. Pregnant and non-pregnant animals with and without antibodies were included in each group. Cadí-6 BDV strain was confirmed to be of high virulence for adults and foetuses. Three chamois died before the end of the experiment with haemorrhagic diathesis. All but one antibody negative animals presented long viraemia, high nasal and rectal viral shedding and wide viral distribution in tissues. Foetuses infected with Cadí-6 died before the end of the experiment presenting high viral RNA loads. The antibody negative chamois infected with Freser-5 BDV strain presented less viral distribution and RNA load in tissues than Cadí-6 group, and cleared the virus from the serum. However, foetuses died before the end of the experiment and RNA virus was detected in sera and tissues although with lower RNA load than the Cadí-6 group. Chamois from both groups presented lesions in brain but the ones infected with the low-virulence Freser-5 BDV strain were mild and most likely transient. In both groups, in pregnant females with antibodies and all but one of their foetuses did not present viraemia or viral RNA in tissues. These results demonstrate that viral strains

diversity is a significant factor in the heterogeneity of epidemiological scenarios in Pyrenean chamois populations.

Since free-ranging common rabbit (*Oryctolagus cuniculus*) and Bennett's wallaby (*Macropus rufogriseus*) were found susceptible to pestivirus infections, chamois sympatric non-artiodactyl species became of interest as potential hosts in pestivirus epidemiology. Study II focused on the susceptibility to pestivirus infection of two of these species: European hare (*Lepus europaeus*) and Alpine marmot (*Marmota marmota*). None of the marmots presented pestivirus and antibodies in the analyzed sera samples. Although no pestivirus was detected, 36.2% of hares had neutralizing antibodies. Thus, the European hare is the third wild non-artiodactyl with documented susceptibility to pestivirus infection.

The anthropogenic influence in the diversity of epidemiological scenarios in chamois BDV infection was assessed in Study III and focused on transhumant sheep flocks. Five sheep flocks grazing in two alpine areas in the Pyrenees with two different BDV epidemiological scenarios in chamois populations were studied. Sheep were sampled before and after transhumance. Only one farm presented persistent BDV circulation in the flock. In that farm, joining feed lots in alpine meadows was demonstrated as the main factor for viral transmission. Moreover, the titration of neutralizing antibodies in that farm showed that most of the infections may be the result of contact with BDV strains of domestic origin. The only BDV sequenced (5'UTR region) in this farm was found genetically close related to previous BDV strains from chamois origin. This fact, together with the evidence that in another studied farm sheep antibodies seems to be originated by a chamois-like BDV strain, indicate that occasional transmission between sheep and chamois occurs.

Resum

Els *Pestivirus* (Família *Flaviviridae*) causen malalties al bestiar domèstic amb un important impacte econòmic i sanitari. Una de les espècies de pestivirus, el Border Disease Virus (BDV) és també important per a la conservació de la fauna salvatge des del 2001, moment en que es va associar amb brots d'alta mortalitat en l'isard pirinenc (*Rupicapra pyrenaica pyrenaica*). Després de 16 anys d'investigació, s'han descrit diferents escenaris epidemiològics d'infeccions per BDV a les poblacions d'isard. El principal objectiu de la present tesis ha sigut analitzar el rol de tres factor que podrien explicar aquesta diversitat epidemiològica: les soques víriques, els hostes salvatges no artiodàctils, i la transhumància ovina.

L'Estudi I va ser dissenyat per confirmar la presència de soques de BDV d'alta i baixa virulència en poblacions d'isards dels Pirineus per tal d'entendre les seves implicacions en la diversitat d'escenaris epidemiològics. Es va realitzar una infecció experimental en isard pirinenc amb una soca de BDV d'alta virulència (Cadí-6) i una de baixa virulència (Freser-5). A cada grup es van incloure animals gestants i no gestants. Al concloure la infecció experimental es va confirmar que la soca Cadí-6 era d'alta virulència per adults i fetus. Tres animals van morir abans de la fi de l'experiment amb una diàtesis hemorràgica. Tots els animals sense anticossos, excepte un, van presentar una virèmia llarga, una alta excreció vírica per via nasal i rectal, i una àmplia distribució de virus en els teixits. Els fetus infectats amb Cadí-6 van morir abans de finalitzar l'experiment presentant altes càrregues d'ARN víric. Els isards sense anticossos infectats amb la soca Freser-5 van eliminar el virus del sèrum i van presentar menys distribució de virus i càrrega d'ARN víric en teixits que el grup Cadí-6. Tot i això, els fetus van morir abans de finalitzar l'experiment i es va detectar ARN víric a sèrum i teixits, encara que amb menys quantitat que al grup Cadí-6. Els isards dels dos grups van presentar lesions a l'encèfal, però als infectats amb la soca de baixa virulència Freser-5 les lesions van ser lleus i probablement transitòries. En els dos grups, les femelles gestants i amb anticossos, i tots els fetus excepte un, no van presentar virèmia ni ARN víric en teixits. Aquests resultats

demostren que la diversitat de soques víriques és un factor significatiu per a la heterogeneïtat d'escenaris epidemiològics presents en les poblacions salvatges d'isard.

Des de que poblacions salvatges de conill comú (*Oryctolagus cuniculus*) i ualabí de Bennet (*Macropus rufogriseus*) van ser descrites com a susceptibles a infeccions per pestivirus, les espècies no artiodàctils simpàtriques de l'isard van esdevenir hostes potencials en l'epidemiologia del pestivirus. L'Estudi II es va centrar en la susceptibilitat a les infeccions per pestivirus de dues d'aquestes espècies: la llebre europea (*Lepus europaeus*) i la marmota alpina (*Marmota marmota*). Cap de les mostres de sèrum de marmota va presentar pestivirus ni anticossos. Encara que no es va detectar pestivirus, el 36.2% de les llebres tenia anticossos neutralitzants. Així doncs, la llebre europea és la tercera espècie salvatge no artiodàctil documentada com a susceptible a infeccions per pestivirus.

La influència antropogènica sobre la diversitat d'escenaris epidemiològics en les infeccions per BDV en isard va ser avaluada en l'Estudi III, centrant-se en ovelles transhumants. Es van estudiar cinc ramats d'ovelles que pasturen a dues zones del Pirineu amb dos escenaris epidemiològics de BDV en isard. Les ovelles es van mostrejar abans i després de la transhumància. Només una granja va presentar circulació constant de BDV en el ramat. En aquesta granja, la unió de lots a les praderes alpines va ser el principal factor de transmissió de virus. A més, a la mateixa granja la titulació d'anticossos neutralitzants va mostrar que la majoria de les infeccions havien estat causades probablement per soques de BDV d'origen domèstic. La única seqüència de BDV (regió 5'UTR), en aquesta mateixa granja, va resultar ser genèticament pròxima a soques de BDV d' isard. Aquest fet, juntament amb l'evidència de que en una altra granja estudiada els anticossos d'ovelles semblen haver estat originats per una soca de BDV d'isard, indica que la transmissió entre ovella i isard es dóna de forma ocasional.

Resumen

Los *Pestivirus* (Familia *Flaviviridae*) son causantes de enfermedades en el ganado con un importante impacto económico y sanitario. Una de las especies de pestivirus, el Border Disease Virus (BDV) es también importante para la conservación de la fauna salvaje desde 2001, momento en que se asoció con brotes de alta mortalidad en rebeco pirenaico (*Rupicapra pyrenaica pyrenaica*). Después de 16 años de investigación, se han descrito diferentes escenarios epidemiológicos de infecciones por BDV en poblaciones de rebeco. El principal objetivo de la presente tesis ha sido analizar el rol de tres factores que podrían explicar dicha diversidad epidemiológica: las cepas víricas, los huéspedes salvajes no artiodáctilos y la trashumancia ovina.

El Estudio I fue diseñado para confirmar la presencia de cepas de BDV de alta y baja virulencia en poblaciones de rebeco de los Pirineos para entender las implicaciones en la diversidad de escenarios epidemiológicos. Se realizó una infección experimental en rebeco pirenaico con una cepa de BDV de alta virulencia (Cadí-6) y una de baja virulencia (Freser-5). En cada grupo se incluyeron animales gestantes y no gestantes. Al finalizar la infección experimental se demostró que la cepa Cadí-6 era de alta virulencia para adultos y fetos. Tres animales murieron antes de acabar el experimento con una diátesis hemorrágica. Todos los animales sin anticuerpos, excepto uno, presentaron una viremia larga, una alta excreción vírica por vía nasal y rectal, y una amplia distribución del virus en los tejidos. Los fetos infectados con Cadí-6 murieron antes de finalizar el experimento presentando altas cargas de ARN viral. Los rebecos sin anticuerpos infectados con la cepa Freser-5 eliminaron el virus del suero y presentaron menor distribución de virus y carga de ARN vírico en tejidos que el grupo Cadí-6. No obstante, los fetos murieron antes de finalizar el experimento y se detectó ARN vírico en suero y tejidos, aunque en menor cantidad que en el grupo Cadí-6. Los rebecos de los dos grupos presentaron lesiones en el encéfalo, pero en los infectados con la cepa de baja virulencia Freser-5 fueron leves y posiblemente transitorias. En los dos grupos, hembras gestantes y con anticuerpos, y todos excepto uno de sus fetos, no presentaron viremia ni ARN

vírico en tejidos. Estos resultados demuestran que la diversidad de cepas víricas es un factor significativo para la heterogeneidad de escenarios epidemiológicos presentes en las poblaciones salvajes de rebeco.

Desde que las poblaciones salvajes de conejo de monte (*Oryctolagus cuniculus*) y ualabí de Bennet (*Macropus rufogriseus*) fueron descritas como susceptibles a infecciones por pestivirus, las especies no artiodáctilos simpátricas del rebeco se convirtieron en huéspedes potenciales en la epidemiología del pestivirus. El estudio II se centró en la susceptibilidad a las infecciones por pestivirus en dos de estas especies: la liebre europea (*Lepus europaeus*) y la marmota alpina (*Marmota marmota*). Ninguna de las muestras de suero de marmota presentó pestivirus ni anticuerpos. Aunque no se detectó virus, el 36.2% de las liebres tenía anticuerpos neutralizantes. Así pues, la liebre europea es la tercera especie salvaje no artiodáctilo documentada como susceptible a las infecciones por pestivirus.

La influencia antropogénica sobre la diversidad de escenarios epidemiológicos en las infecciones por BDV en rebeco fue evaluada en el Estudio III, centrándose en ovejas trashumantes. Se estudiaron cinco rebaños de ovejas que pastan en dos zonas del Pirineo con dos escenarios epidemiológicos de BDV en rebeco. Las ovejas se muestrearon antes y después de la trashumancia. Solo una granja presentó circulación constante de BDV en el rebaño. En esta granja, la unión de lotes en las praderas alpinas fue el principal factor de transmisión de virus. Además, en la misma granja la titulación de anticuerpos neutralizantes mostró que la mayoría de las infecciones habían sido causadas probablemente por cepas de BDV de origen doméstico. La única secuencia de BDV (región 5'UTR), en esta misma granja, resultó ser genéticamente próxima a cepas de BDV de rebeco. Este hecho, junto con la evidencia de que en otra granja estudiada los anticuerpos de oveja parecen estar originados por una cepa de BDV de rebeco, indica que la transmisión entre oveja y rebeco se da de manera ocasional.

Part I: General introduction, hypotheses and objectives

1. General introduction

1.1 Pestivirus

1.1.1 History, molecular biology and taxonomy

The genus *Pestivirus* belongs to the *Flaviviridae* family since the year 1991. Early reports of diseases attributed to a pestivirus in pigs were described in 1810 and 1833 in Tennesse and Ohio (Hanson, 1957), although the description of Classical Swine Fever Virus (CSFV) as the causative agent was reported in 1904 (Schwenitiz and Dorset, 1904). Some years later, Olafson et al. (1946) described a bovine diarrhoea disease of unknown aetiology. The first characterization of the causative agent was 11 years later, when a pestivirus called Bovine Viral Diarrhoea Virus (BVDV) was identified (Lee and Gillespie, 1957). In 1959 in the border between England and Wales a disease affecting sheep was described being called Border Disease (BD) (Hughes *et al.*, 1959). Soon after this first report, it was also observed in several other countries such as New Zealand and USA (Hartley and Kater, 1962) but it was not until 1977 that a Border Disease Virus (BDV) was isolated and characterized (Harkness *et al.*, 1977). Initially, those *Pestivirus* genus species were classified into the *Togaviridae* family but in 1991 they were finally placed into *Flaviviridae* family. Nowadays, the *Pestivirus* genus comprises the traditional pestivirus species (BVDV-1, BVDV-2, CSFV and BDV) and putative species (Simmonds *et al.*, 2017). However, the improvement of molecular techniques in recent years has increased the number of species proposed to be included into the genus (For more information, see Chapter 1.1.2). Within the *Flaviviridae* family, *Pestivirus* genus shares the taxonomic rank with the *Flavivirus*, *Pegivirus* and *Hepacivirus* genus, whose diverse molecular biology reflects their adaptations to different propagation strategies and hosts (Loken, 1995; Deregt, 2008; Schweizer and Peterhans, 2014; Tautz *et al.*, 2015).

The pestivirus genome is characterized by a single-stranded (ss) RNA of positive polarity with a single large open reading frame (ORF). Within an enveloped viral particle of 40-60nm there is a viral RNA genome that has a length of about 12.3 kb, which is translated into a polyprotein of 3900 amino acids. After co- and posttranslational processing facilitated by viral and host proteases, 12 mature proteins are generated. In the virion there are four structural proteins (SPs), a basic core protein C and the envelope (E) glycoproteins E^{rns}, E1 and E2. Moreover,

eight non-structural proteins are located in the genome presenting diverse functions: NS3-NS5B constitutes the replication complex; Npro represents the N-terminal autoprotease; p7 the ion channel; NS3 an NTPase/RNA helicase/protease; NS4A functions as a cofactor of the NS3 protease and is at least essential for processing the NS4B/NS5A and the NS5A/NS5B sites. Although NS4B and NS5B seem to play an essential role in virus replication, its functions have not been well characterized yet. Finally, NS5B represents a RNA-dependent RNA polymerase (Fig. 1.1). The general stability of virions has been shown to be of a half-life of approximately 7h at 37ºC and neutral pH. Despite the fact that pestiviruses are very resistant to low pH, they have a lipid envelope that makes them easily inactivated by detergent treatment (Schweizer and Peterhans, 2014; Tautz *et al.*, 2015).

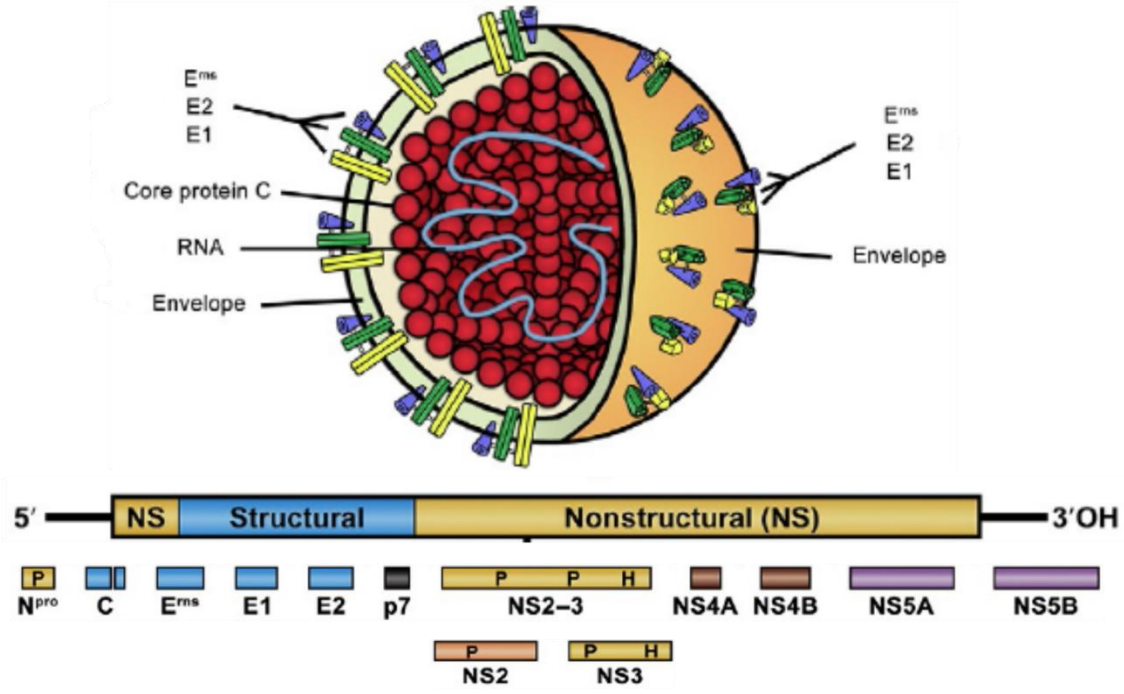

Figure 1.1 Pestiviral particle, viral genome and polyprotein product of the single open reading frame (modified from Tautz *et al.,* 2015).

Pestivirus are classified into two biotypes depending on *in vitro* cell damage: those that cause the death of infected cells (cytophatic, cp) or those that cause none or low damage in infected cells (non-cytophatic, ncp). For cytopathic pestiviruses, enhanced expression of NS3 is observed, whereas the uncleaved form, NS2-3,

predominates in noncytopathic pestiviruses. The 5' untranslated region (UTR) is not capped and there is no poly(A)-tail at the 3' end of the viral RNA. The 5' and 3' nontranslated regions are of about 400 and 200 nucleotides, respectively. This 5'UTR has been widely used to genetically group pestivirus because is a highly conserved region in the genome. Therefore, many analysis and diagnosis of a wide range of hosts focus on 5'UTR as several sequences available in databases are based on this region. Other regions are also included in taxonomy studies, such as the N^{pro} and the E2. The first is a quite conserved region of 504 nucleotides, and the second, a region of about 1116-1119 nucleotides, with high variations. E2 is of importance because it is the main target for neutralizing antibodies (Tautz *et al.*, 2015).

Taxonomic classification of pestiviruses is a constant changing picture, mainly when regarding genogroups and subtypes. Many authors have proposed standardizations and have tried to harmonize pestivirus taxonomy (Vilček *et al.*, 1994; Liu *et al.*, 2009; Giammarioli *et al.*, 2011), although the debate is still unsolved. The genome structure of pestivirus suggests a quick evolution, with about 10^{-3} substitutions per site and year (Duffy *et al.*, 2008) as corroborated by Luzzago *et al.* (2016) recently, who estimated $2.9x10^{-3}$ substitutions per site and year in BDV. Therefore, the genetic diversity of BDV is greater than other pestivirus species, since up to eight major genotypes are reported (Dubois *et al.*, 2008; Peletto *et al.*, 2016). Liu *et al.* (2009) proposed a reliable pestivirus phylogeny inferred from a molecular dataset combining the 5'UTR, N^{pro} and E2 gene regions by Maximum likelihood and Bayesian approach. Luzzago *et al.* (2016) have also recently presented an interesting classification by using a Bayesian framework allowing spatial and temporal reconstruction of the evolutionary dynamics of highly variable viruses. Continuing with this approach, pestivirus consist on a large variety of closely related sequences that determine their evolution. These are called mutant clouds or quasispecies and their distributions may change over time either spontaneously or after a selection pressure (Domingo *et al.*, 2012). Studies regarding different regions of the pestivirus genome, and selection pressure factors on quasispecies are scarce but their relation to virulence and pathogenesis of these

mutant clouds are promising (Jones *et al.*, 2002; Tokstad *et al.*, 2004; Pfeiffer and Kirkegaard, 2006; Töpfer *et al.*, 2013). The uncertainty that exists in the classification of pestivirus is being increased when new putative species appear, growing in number as molecular techniques are being improved.

1.1.2 Putative (non-traditional) pestivirus species

Apart from the traditional recognized species, CSFV, BVDV-1, BVDV-2 and BDV, new viruses have been proposed to represent additional pestivirus species (Fig. 1.2) although they have not been approved yet by the International Committee on Taxonomy of Virus (ICTV). The first temptative species, Giraffe-1, was described in Kenya after an outbreak of a disease in giraffes (Harasawa *et al.*, 2000). A virus named PG-2 from bovine cells from Kenya was proposed as the second member of this putative species (Becher *et al.*, 2014). Vilček *et al.* (2005) described a new tentative species found in a Pronghorn antelope (*Antilocapra americana*) in the USA. One year before, Schirrmeier *et al.* (2004) identified a HoBi-like virus (Atypical pestivirus or BVDV-3) in foetal bovine serum (FBS) from Brazil. Since then, many other authors around the world identified phylogenetically similar viruses in contaminated FBS. In 2007, Kirkland *et al.* (2007) reported a highly divergent pestivirus. This virus, named Bungowannah was isolated in pigs from Australia. Another temptative species was found in a sheep from Turkey in 2012 (Becher *et al.*, 2012). More recently, molecular techniques as next-generation sequencing have led to the discovery of new and highly divergent pestivirus. In rats (*Rattus norvegicus*) from New York, Firth *et al.* (2014) found a putative pestivirus species which polyprotein sequence (3993aa) shared a maximum amino acid identity of 60% with the previously known pestivirus polyproteins. Moreover, Wu *et al.* (2012) identified another highly divergent novel pestivirus in a virome of bats. A curious example of this constantly growing putative species and the limitations of taxonomy clades is the description of a novel flavivirus with homology to pestivirus in a soybean cyst nematode (Bekal *et al.*, 2014). The last virus to come into the tentative species of the genus *Pestivirus* was recently described in pigs and called Atypical Porcine Pestivirus (APPV) (Hause *et al.*, 2015). With high genetic divergence from the typical pestiviruses, the discovery of this new APPV has

become an important finding when being identified as the causative agent of Congenital Tremor (CT) type II, a disease of pigs of unknown etiology until that discovery (Postel *et al.*, 2016).

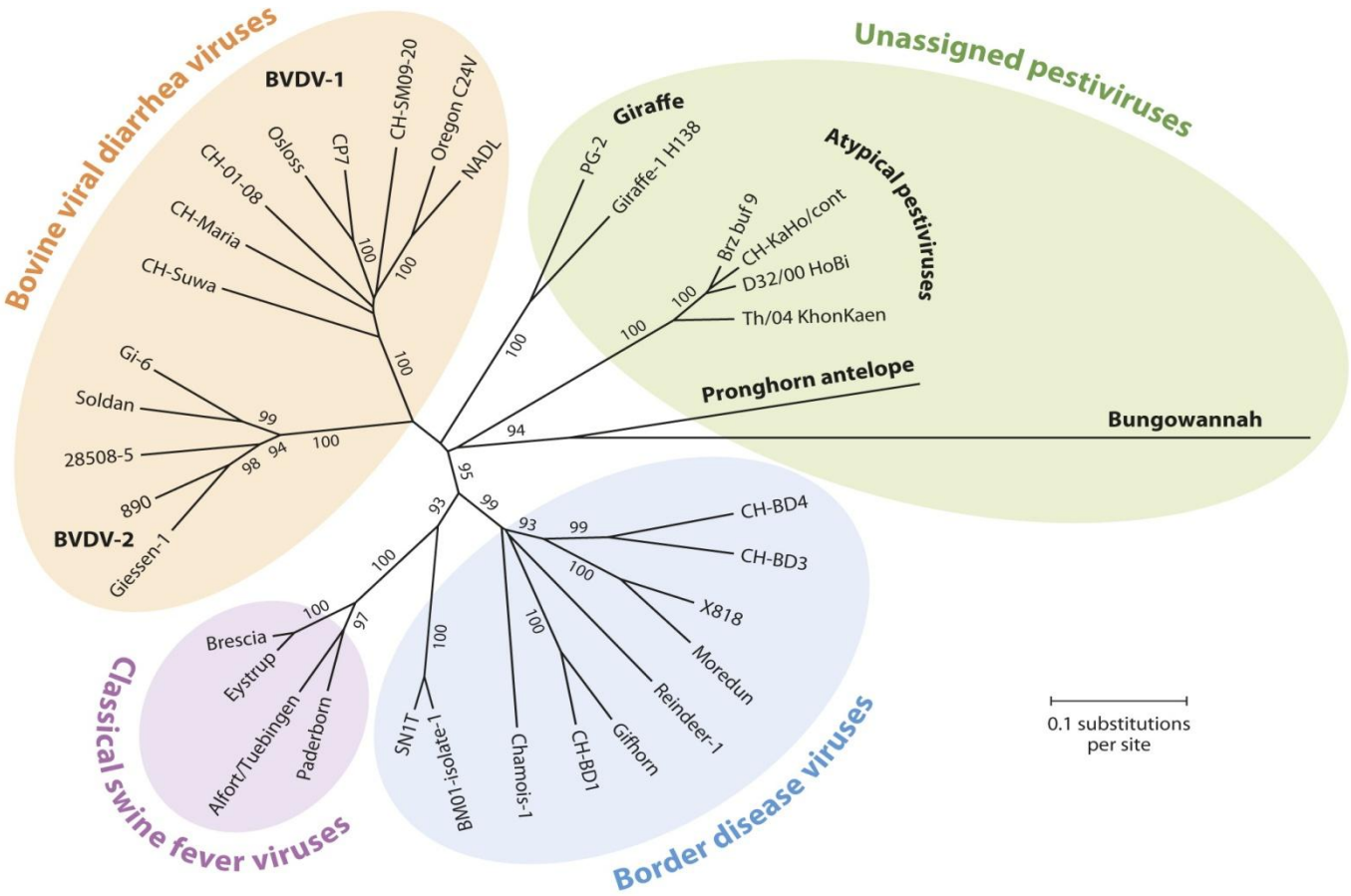

Figure 1.2 Phylogenetic tree based on the N^{pro} region (504nt) of traditional and putative (unassigned) pestiviruses. The evolutionary distance was calculated according to the Maximum Composite Likelihood Method. The number in branches represent the values in percent of 1000 bootsrap replicates and only values greater than 90% are indicated. Lines lengths are proportional to genetic distance (Schweizer and Peterhans, 2014). Rat, Bat and APPV pestiviruses are the highly divergent putative pestiviruses not represented in this figure.

1.1.3 Hosts and diseases

At the beginning of pestivirus research, the traditional species were associated to a single host but these beliefs have changed over years. CSFV infects different members of the *Suidae* Family. Apart from domestic pig (*Sus scrofa domesticus*) and the European wild boar (*Sus scrofa scrofa*), other species such as common warthogs (*Phacochoerus africanus*) and bushpigs (*Potamochoerus larvatus*) have been demonstrated

to be susceptible hosts for CSFV (Blome *et al.*, 2017). The traditionally known as ruminant pestivirus species, BVDV-1 and BVDV-2, and BDV, have been described affecting over 50 wild and domestic species from *Artiodactyla* order, including the families *Antilocapridae*, *Bovidae*, *Camelidae*, *Cervidae*, *Giraffidae*, *Suidae* and *Tragulidae* (Vilček and Nettleton, 2006; Passler and Walz, 2010; Ridpath, 2010). This wide range of potential hosts, the high mutation rate of the virus and its capability to originate persistently infected (PI) animals allow these viruses to persist in ruminant populations (Nettleton *et al.*, 1998; Dubois *et al.*, 2008). However, other not even-toed ungulates have been found to be susceptible to these traditionally known as ruminant pestiviruses. The inclusion of Bennett's wallaby (*Macropus rufogriseus*) and the common rabbit (*Oryctolagus cuniculus*) as free-ranging susceptible species to pestivirus infections may change the traditional nomenclature (Munday, 1972; Grant *et al,*. 2015) (See chapter 1.1.4).

Pestiviruses are one of the most economically-threatening pathogens in livestock. Impact on farm productivity due to death or to the associated clinical signs is enlarged by the difficulty in controlling the quick virus spread and the resulting long-lasting persistent infections. Reproductive alterations due to pestivirus infections usually represent the biggest impact on farm productivity. Implementation of eradication plans, based on PI animals removal, biosafety measures, or vaccine strategies generates a huge economical and research effort worldwide (Loken, 1995; Ridpath, 2010; Schweizer and Peterhans, 2014; Pinior *et al,*. 2016).

CSFV is a notifiable disease to the World Organization for Animal Health (OIE) because of its impact on pig and wild boar populations. This fact has led to successful eradication plans in many countries, although it is assumed to be endemic in several countries of South and Central America, parts of Eastern Europe and Asia. The situation in Africa nowadays remains unclear. The fact that wild boar is a wild reservoir of the disease and the increasing world trade in pig sector has become a threat to surveillance plans in several countries worldwide and the eradicated status of an area, a fragile reality.

Clinical signs of CSFV infections can vary depending on virulence of the virus strain and different host factors. Presentations from peracute deaths to unapparent courses have been described throughout time, although over the last decades CSFV strains decreased their virulence in many outbreaks. There is a debate whether these strains are variants of CSFV adapted for long-term perpetuation in wildlife or are a consequence of monitoring and detection biases (Lange *et al.,* 2012; Blome *et al.,* 2017). The predominating absence of symptoms in the mild forms of the disease may complicate CSFV diagnostic and increase virus spread. The acute-lethal forms of the disease could present haemorrhages on skin and in inner organs, severe thrombocytopenia, pulmonary oedema, gastrointestinal symptoms, significant leukopaenia and increased vascular leakage (Blome *et al.*, 2017). CSFV is mainly transmitted oronasally, and the forms of the disease, as pointed before, can be acute (transient or lethal), chronic or persistent. The former, is usually ruled by an infection during pregnancy although postnatal infecting forms have been recently demonstrated (Cabezón *et al.*, 2015; Muñoz-González *et al.*, 2015). Depending on stage of pregnancy when infection occurs, it could result in different foetal impact. Absorption or mummification, abortions or stillbirth are often seen on CSFV infections during pregnancy. When pigs are infected between days 50 and 70 of pregnancy, immunotolerant phenomenon can lead to a PI offspring. Those PI animals are born without neutralizing antibodies against CSFV and chronic viraemia (Liess, 1984; Blome *et al.*, 2017), although their importance on CSFV epidemiology is still on debate.

Regarding BVD and BD, both diseases have many similarities in terms of clinical presentation and infection features. The historically known as ruminant pestiviruses can infect their host either transiently or persistently. As in CSFV, many clinical presentations of the disease can be seen, although the most virulent forms are less likely to appear. Haemorrhagic diseases with severe trombocytopaenia - commonly associated with high mortality rates - have been reported in some BVDV strains and few BDV strains (Chappuis *et al.*, 1984; Walz *et al.*, 1999; Vega *et al.*, 2015). Despite the fact that this is not the main disease

presentation of these pestiviruses, their impact on livestock is significant. The acute form of the disease is usually mild and characterized by fever, leukopaenia, depression and seroconversion. Whereas these mild or asymptomatic presentations are caused in acute infections occurring postnatally, the congenital forms are the most important issues on BVDV and BDV infections. Depending on the stage of gestation when infection occurs, different scenarios can appear. Similar than in CSFV, when animals are infected during the first half of gestation, this can lead to embryonic death, mummification, abortion, stillbirth, dysmorphogenesis or the birth of an apparently normal, or weakborn animal. If the congenital infection occurs before the immune system maturation of the foetus, a PI animal can get born. PI animals play a key role in pestivirus epidemiology (Nettleton *et al.*, 1998). In PI lambs infected by BDV, a characteristic nervous sign can be found ranging from a continuous light tremor or shaking movement of the tail and head, to tonic-clonic muscle contractions that involve the whole body. Those animals are known as "hairy shakers". Ataxia, low birth weight and general weakness can be found in these PI animals.

A special presentation of disease related to BVDV infections is the Mucosal Disease (MD). This is a non-transmissible lethal form of the disease that appears only in low proportion cases in a herd. MD is characterized by high fever, anorexia, salivation, erosions and ulcers in the gastrointestinal tract and bleeding diarrhea. After many years of research on this particular presentation, it has been demonstrated that MD appears in some cases when a PI animal generated by a BVDV ncp biotype is infected horizontally by another cp biotype BVDV strain. Interestingly, both strains need to be closely antigenic related to generate MD. This fact is also supported by the finding of high genome similarity between both ncp and cp BVDV strains developing the MD (Loken, 1995; Nettleton and Willoughby, 2008; Schweizer and Peterhans, 2014; Tautz *et al.*, 2015).

Finally, the novel APPV as the causative agent of Congenital Tremor (CT) and classified as a putative species into *Pestivirus* genus represents a new disease presentation into the taxa. Congenital Tremor type AII is a disease of neonatal pigs

characterized by action-related repetitive myoclonus, with visible histological lesions in brain and spinal cord and historically attributed to an unknown virus (Arruda *et al.*, 2016). Interestingly, since APPV was described in USA in 2015 (Hause *et al.*, 2015), it has been found in pigs from Austria (Schwarz *et al.*, 2018), England (Williamson and Group, 2017), Germany (Beer *et al.*, 2016; Postel *et al.*, 2016), Netherlands (De Groof *et al.*, 2016), Spain (Muñoz-González *et al.*, 2017) and Sweden (Blomström *et al.*, 2016). Muñoz-González *et al.* (2017) showed that APPV was present at least since 1997 in pigs from North-Eastern Spain. Pigs with APPV genome and no CT have been described in many of the recent reports, especially in adults (Postel *et al.*, 2016; Muñoz-González *et al.*, 2017). Also, CT in piglets is usually a temporary condition lasting from several weeks to several months, and most piglets are clinically normal at weaning age (De Groof *et al.*, 2016). The existence of PI animals in APPV infections has also been proposed (De Groof *et al.*, 2016; Muñoz-González *et al.*, 2017; Schwarz *et al.*, 2017) and if confirmed it could play a major role in APPV epidemiology.

1.1.4 Non-artiodactyl hosts

The role of wildlife in pestivirus infection epidemiology is an issue with many variable situations and actors. Hence, the implication of free-ranging hosts is still under study. Some authors have reported potential wild reservoirs of pestivirus for livestock (Vilček and Nettleton, 2006; Passler and Walz, 2010;) while other authors described independent domestic cycles versus sylvatic cycles of infection (Marco *et al.*, 2009a). This heterogeneity of results had encouraged the analysis of pestiviruses in wild species, mainly in countries with BVDV eradication programs implemented for livestock (Casaubon *et al.*, 2012) and the interest of potential non-ungulate hosts for pestiviruses has increased.

Regarding the historically called ruminant pestiviruses, BVDV and BDV, the common rabbit (*Oryctolagus cuniculus*) was the first non-artiodactyl species identified as a potential reservoir of BVDV (Baker *et al.* 1954). Serological evidence of BVDV antibodies in 40% free-ranging rabbits from Germany has been reported (Frölich and Streich, 1998). Recently, the role of these lagomorphs as a reservoir has been

explored reporting viraemia and antibody response after an experimental infection with a BVDV (Bachofen *et al.*, 2014). Moreover, Grant *et al.* (2015) presented a serological analysis of wild rabbits in Scotland and Northern England with 1.2% of seroprevalence. The same authors demonstrated transplacental infection inoculating BVDV in pregnant rabbits.

Strikingly, a study of wildlife diseases in Tasmania confirmed the Bennett's wallaby or red-necked wallaby (*Macropus rufogriseus*) as the second wild non-artiodactyl species susceptible to pestivirus infections (Munday, 1972). By means of a virus neutralization test (VNT), the authors found antibodies against the BVDV Oregon strain in 2/44 samples analyzed from the marsupial species.

More recently, Seong *et al.* (2015) reported a BVDV experimental infection in mice (*Mus musculus*) detecting the virus and related histopathological changes in some infected animals. In the last years, the improving of molecular techniques has led to new pestiviruses identification. The most remarkable examples are the identification of new possible pestivirus species in sera of brown rat (*Rattus norvegicus*) from New York (Firth *et al.*, 2014) and in viromes of bats from China (Wu *et al.*, 2012). The reports presented above, may be essential when trying to implement an eradication program on free-ranging livestock and also when monitoring the impact of these viruses in wildlife.

1.2 Pestivirus and chamois

1.2.1 Disease appearance and evolution

Several Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) were found dead unexpectedly in 2001 at the National Hunting Reserve (NHR) of Alt Pallars-Aran, in the Central Catalonian Pyrenees (Fig. 1.3). Virological studies demonstrated that a BDV of the genogroup 4 was the causative agent of the epizootic (Arnal *et al.*, 2004; Hurtado *et al.*, 2004). This first outbreak lasted 2 years and was the first reported case of a pestivirus causing high mortalities in wild ruminant species worldwide. In spite of the difficulty in assessing morbidity and mortality in wildlife, the decrease of the population during the first outbreak was estimated to be of 42% (Marco *et al.*, 2007). Meanwhile, in the French side of the Pyrenees, a decrease in chamois population related to BDV was also reported in 2002 (Alzieu *et al.*, 2004). It was not until 2005 when another outbreak associated to BDV appeared. In this occasion the disease was located at the Cerdanya-Alt Urgell NHR, about 60Km further east from the first outbreak area. This epizootic caused the highest population mortality with a drop in the population of approximately 86% (Marco *et al.* 2009b). One month later the first cases of BDV infection were found in chamois at the neighbouring National Hunting Reserve of Cadí. Since then, mild outbreaks and sporadic cases have been reported in previously affected areas and some parts of French Pyrenees. In 2010 a severe outbreak was detected in Andorra (Fernández-Sirera *et al.*, 2012a) and in 2011 in the Benasque National Hunting Reserve, with a drop of the 30-45% of the population.

Retrospective studies carried out in stored samples from chamois revealed the presence of BDV in Pyrenean populations at least since 1990. The first virus isolated date back to 1996 at the Catalan Pyrenees (Marco *et al.*, 2011) and back to 1997 in the French side of the mountain range (Pioz *et al.*, 2007). The first strain isolated in sera of two chamois sampled in 1996 belonged to a chamois from the Freser-Setcases NHR. Interestingly, despite having constant pestivirus circulation at least since the 90's, no outbreaks have been ever registered and only one clinical case was found in 2007 (Marco *et al.*, 2015).

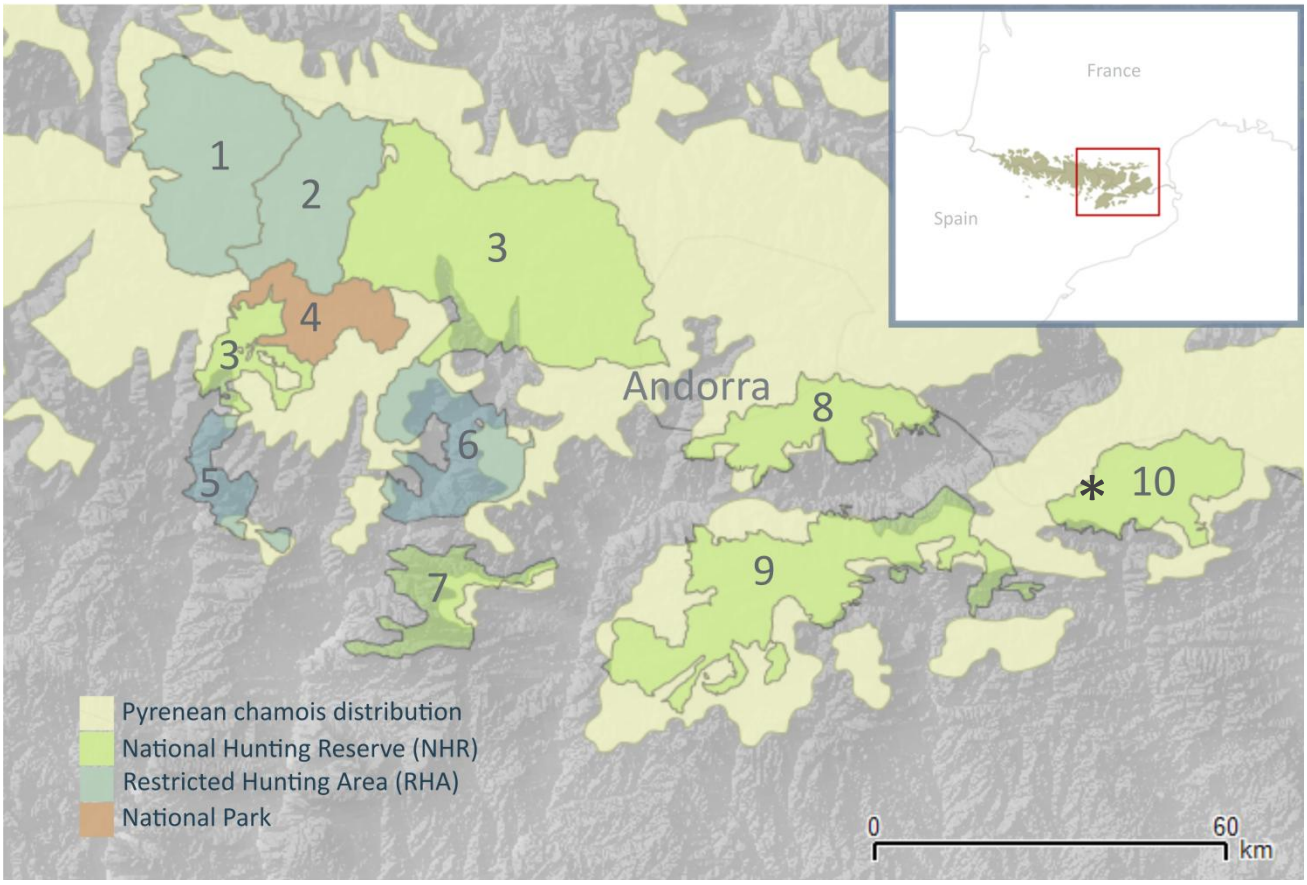

Figure 1.3 Pyrenean chamois (*Rupicapra p. pyrenaica*) distribution with numbered main protected and game areas in the Catalonian Pyrenees superimposed. **1)** Val d'Aran RHA, **2)** Naut Aran RHA, **3)** Alt Pallars NHR, **4)** Aigües Tortes i Estany de Sant Maurici National Park, **5)** Pont de Suert RHA, **6)** Sort, Soriguera, Rialp i Tornafort RHA, **7)** Boumort NHR, **8)** Cerdanya-Alt Urgell NHR, **9)** Cadí NHR, **10)** Freser-Setcases NHR. * Estimated coordinates (42.42N and 1.9E) of the hypothetical origin of Pyrenean chamois BDV strains transmitted by sheep, dated back to the early 90s (Luzzago *et al.,* 2016).

1.2.2 Epidemiology of BDV infection at the chamois population level

After 16 years since the first outbreak, different epidemiological scenarios have been described in the Pyrenees. Fernández-Sirera *et al.* (2012b) studied accurately two of these scenarios in areas with previous severe epizootics. On the one hand, some populations showed frequent circulation of BDV with low but constant decrease in population census. It is unclear if this negative impact could be attributed to adult mortality, reproductive failure or both. On the other hand, some populations presented a decrease in BDV circulation, highlighted by the reduced seropositivity in the population.

In other geographic areas, such as the above mentioned Freser-Setcases NHR, BDV is endemic with no apparent negative impact on chamois population. This area is nowadays presenting one of the highest densities of chamois in the Pyrenean mountains and currently about 300 chamois are legally hunted over an increasing population of near 3000 individuals in about 21000 Ha. In the French Pyrenees, Pioz *et al.* (2007) studied the epidemiology of BDV infections in the National Game and Wildlife Reserve of Orlu. Their epidemiological research on a valley level showed that incidence and seroprevalence is dependent on host population structure, conditioned by the number of individuals under the age of 2 and with seasonal variations. Beaunné *et al.* (2015) used a mathematical model to represent this proposed seasonal spread of infection. The authors included previously known parameters of pestivirus infections in chamois, and assumed that other features of BDV in chamois infections are the same as in BD of sheep and goat, such as the existence of PI animals.

In another mathematical approach, Serrano *et al.* (2015) demonstrated that BDV is an exceptional driver of chamois populations. They compared it to other diseases with a reported severe impact on chamois population dynamics in Europe such as sarcoptic mange and infectious keratoconjunctivitis. The model included data on reproduction, mortality and other catastrophic events affecting chamois populations.

As reviewed before, PI animals play a key role in BVDV and BDV epidemiology. However, the existence of PI animals in chamois is still unclear. The evolution of the outbreaks, with fast expansion and high mortality, seems to indicate that the virus was mainly transmitted horizontally and by oro-nasal route. Cabezón *et al.* (2010a) demonstrated that natural infected animals shed BDV through nasal, oral, rectal, and urinary excretion routes. These results were confirmed in two experimental infections of chamois inoculated oronasally (Cabezón *et al.*, 2011; Martin *et al.*, 2013) adding the vaginal excretion route on the known shedding routes of infection. Moreover, in both experimental infections viral excretion was present from day 5 post inoculation (p.i.) (Cabezón *et al.*, 2011) and from day 12 p.i. (Martin

et al., 2013) onwards. Viraemia of at least 51 days was recorded during these experimental infections, reinforcing the importance of horizontally infected and chronically viraemic chamois in virus spread.

Although horizontal transmission seems to predominate in some areas, in other regions such as Freser-Setcases NHR where the virus has been circulating for at least 20 years with no outbreaks, the transmission could be ruled by vertical infections. BDV can cross the chamois placenta barrier infecting the foetus as was demonstrated by Martin *et al.* (2013). However, although it was proposed in the mentioned study, the existence of PI animal was not clearly demonstrated. In an experimental infection of one pregnant Pyrenean chamois inoculated at day 90-100 of gestation, the newborn was RT-PCR positive at birth and at the time of death 84 days later, indicating the possibility that it was a PI chamois (Vautrain and Gibert, 2008). The epidemiological diversity found in field studies, mainly in areas with high mortality outbreaks, may indicate that BDV-4 infections in chamois could be less ruled by PI animals than pestivirus infections of livestock. However, if the existence of PI chamois is confirmed, the epidemiology of chamois pestivirus infections in other Pyrenean areas (i.e. Freser-Setcases NHR) will have more similarities to livestock pestivirus infection.

Regarding the high circulation of pestiviruses in Pyrenean chamois populations, some authors have assessed the BDV presence in sympatric wild ungulates. Roe deer, fallow deer, red deer, wild boar and mouflon samples have been analyzed by ELISA antibody tests and VNT, but only the red deer and mouflon have presented seroprevalence (Marco *et al.*, 2009a; Marco *et al.*, 2011 Fernández-Sirera *et al.*, 2012b). In these studies, only two mouflons and one red deer antibody positive have been described (Marco et al. 2009a; Marco et al. 2011). In contrast, Martin *et al.* (2011) described pestivirus antibody seroprevalence of 60% in mouflon from the French Alps. Four years after, the same authors described antibody seroprevalence of 22.2% in mouflon and 28.7% in roe deer (Martin *et al.,* 2015). Although susceptibility to BDV infection in wild ruminants has been widely demonstrated

(Vilček and Nettleton, 2006; Martin *et al.*, 2011, 2015;), in the Pyrenees, it seems that other wild ruminants than chamois act only as spillover species of BDV.

The description of the first outbreak in Pyrenean chamois encouraged the scientific community to investigate the health status of chamois populations. Retrospective, transversal and prospective studies described BDV antibody seroprevalence and sequenced several BDV, mainly in the Pyrenees. In these studies, high seroprevalence between 49-73.5% were found in Pyrenean chamois from Spain and France (Marco *et al.*, 2009a; Marco *et al.*, 2011; Fernández-Sirera *et al.*, 2012b). In the rest of Europe, antibodies against pestivirus have been found in Alpine chamois (*Rupicapra rupicapra*) in the Swiss Alps (2.1%) (Casaubon *et al.*, 2012), Italian Alps (25.5-42%) (Olde Riekerink *et al.*, 2005; Gaffuri *et al.*, 2006; Fernández-Sirera *et al.*, 2012c) French Alps (2-45.9%) (Martin *et al.*, 2011, 2015) and Austrian Alps (29.4%) (Krametter *et al.*, 2004).

As mentioned above, the sub-classifications within pestivirus species is a constantly changing issue. For BDV, 8 subgroups have been proposed to date, analyzing 5'UTR, Npro and partial C gene regions by Maximum likelihood criterion (Peletto *et al.*, 2016). All the pestivirus isolated from Pyrenean chamois clustered into the putative BDV genogroup 4 (Fig. 1.4). However, other virus belonging to other genogroups and species have been indentified in Alpine chamois in Europe. A BDV-6 genetic variant circulating in sheep was identified from a healthy animal in the French Alps. In this area, no mortality cases have been described to date but antibody seroprevalence in chamois was found to be of 38.7% (Martin *et al.*, 2015). Another singular case was the identification of a BVDV-1h in a healthy chamois from Switzerland. This strain was also supposed to be from livestock origin (Casaubon *et al.*, 2012) highlighting interspecies transmission. A special report to be included in this chapter is the recently published work by Caruso *et al.* (2016). The first description of an Alpine chamois death associated to infection with BDV from the genogroup 8 is of concern and increases the dimension of pestivirus threat to wild rupicaprins.

Recently, an interesting study of the spatial and temporal phylogeny of Pyrenean chamois isolates was carried out by Luzzago *et al.* (2016). The authors included 50 BDV-4 Pyrenean chamois strains, one BDV-6 isolate from an Alpine chamois, and 44 sequences from domestic animals, sampled between 1996 and 2011. A phylogenetic analysis of the 5'UTR was developed using a Bayesian framework allowing the spatial-temporal reconstruction of the evolutionary dynamics of viruses (Lemey *et al.*, 2009). In this research, the estimated mean evolutionary rate of the BDV 5'UTR sequence was $2.9x10^{-3}$ sub/site/year. This is similar to other RNA viruses (Duffy *et al.* 2008) and highlights that this region - commonly considered a conserved one - evolved in a short period of time. Moreover, the study proposed a geographic pattern of strain distribution and a different clade from those BDV-4a and BDV4b proposed in sheep (Valdazo-González *et al.*, 2006). This "Pyrenean chamois cluster" was also proposed to be originated from a BDV-4a strain from ovine origin. The approach realized by Luzzago *et al.* (2016) geographically placed the evolutionary origin of chamois strains at certain estimated coordinates (42.42N and 1.9E) in the early 90s (Fig. 1.3). After that foundational episode, it was assumed that the epidemic diffusion rate was about 13.1Km/year.

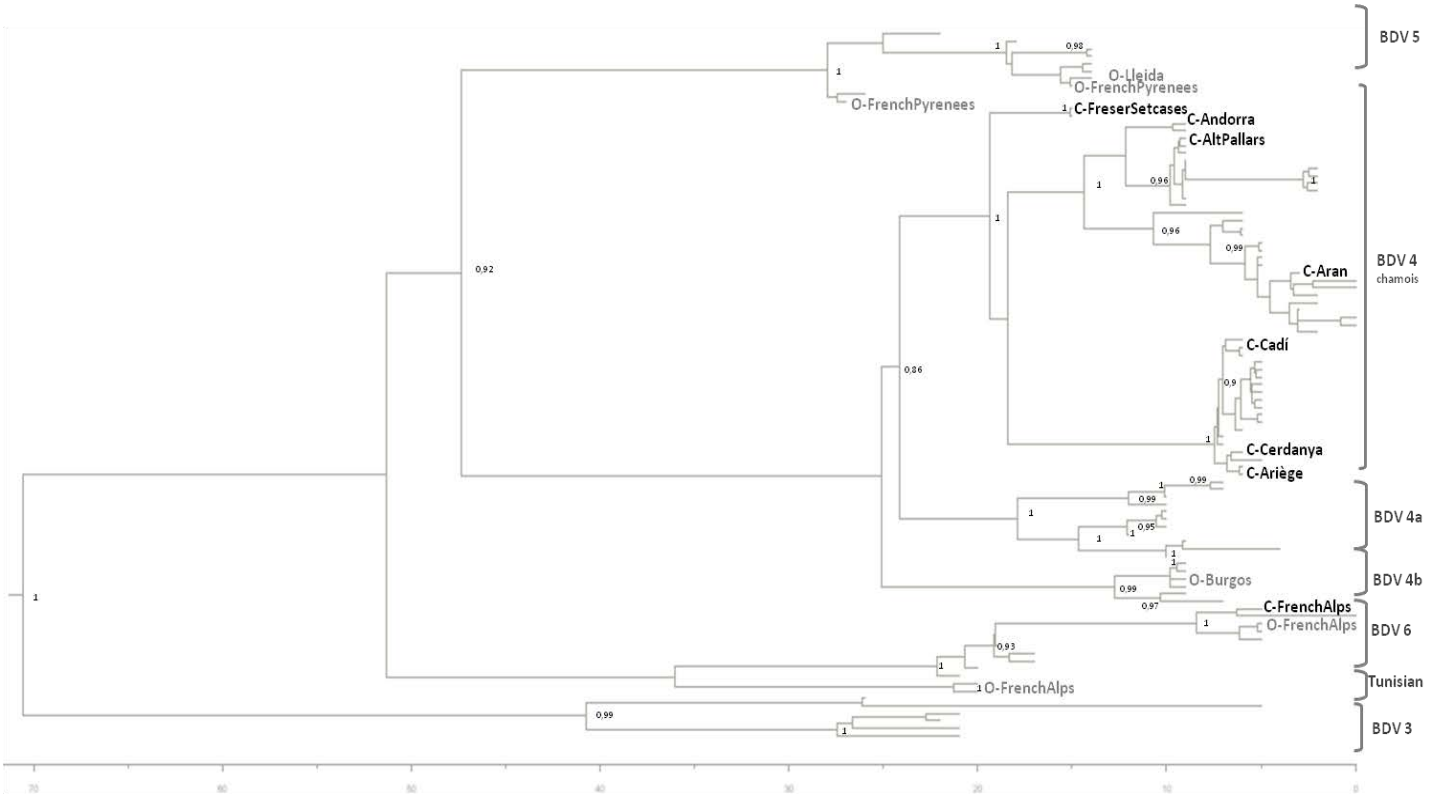

Figure 1.4. Maximum clade credibility tree of the BDV 5'UTR sequences from domestic hosts (coloured in grey) and chamois (coloured in black) with the sample location. Only the names of representative strains are included in the figure. The numbers indicate significant posterior probabilities (pp>0.8) of the corresponding nodes. The scale at the bottom of the tree represents the number of years before the last sampled sequence (2011). Modified from Luzzago *et al.* (2016).

In light of the diversity of epidemiological scenarios in chamois pestivirus infections, different factors have been pointed out when trying to explain it. Regarding wildlife infections, it is usually complicated to completely define the factors that rule their epidemiology when compared to livestock infections (Delahay *et al.,* 2009). Moreover, two factors complicate pestivirus in chamois research approaches: the fact that the pathogen is a RNA virus with high mutagenic rate and that the affected species is an ungulate inhabiting in climatic and orographic extreme areas.

The high mutagenic rate is one of the most important factors related to the studied pathogen. As said before, the genetic diversity of BDV isolates seems to agree with a geographical pattern of distribution (Luzzago *et al.,* 2016). This fact, together with

the different epidemiological scenarios described in the field, suggest different level of virulence between strains. Hence, the genetic diversity of BDV could be an important factor of epidemiological scenary variation. In other pestivirus species the genetic basis of virus virulence are an important issue with consequences in vaccine development and disease presentation management (Risatti *et al.,* 2005; Leifer *et al.,* 2013; Wang *et al.,* 2015). As reviewed before, the characterization of viruses together with the quasispecies concept may lead to a better prediction and understanding of epidemiological scenarios. For example, Bergstrom *et al.* (1999) proposed that virulence of an RNA virus could be driven down by severe bottlenecks. Moreover, Vignuzzi *et al.* (2006) studying the quasispecies diversity implications found that reduced viral diversity led to loss of neurotropism and to an attenuated pathogenic phenotype.

Factors related to host, individually or as a population, are also of importance in pestivirus epidemiology. The immune competence of a host in front of a viral infection could be regulated by several factors. Genetic diversity in the major histocompatibility complex (MHC) genes could be one of those important features. Cavallero *et al.* (2012) showed that the population of chamois from Freser-Setcases NHR has a higher diversity on MHC class II DRB1 exon 2 locus when compared to severely affected areas. Regarding humoral immune response in chamois populations, longitudinal field studies in Pyrenees had reported differences in antibody seroprevalences between areas and in the same one (Pioz *et al.,* 2007; Marco *et al.,* 2009a; Fernández-Sirera *et al.*, 2012b). The level of immunological protection can be related to the viral strain antigenicity but also to the population immune response variability. Also, other factors related to the biology of the species, such as space-use patterns could intervene in pestivirus epidemiology (Crampe *et al.,* 2007; Marco *et al.,* 2015).

Climate influences chamois population dynamics, especially in winter (Jonas *et al.,* 2008). This fact, together with the availability of food resources and the co-existence of other pathogens in free-ranging chamois populations are some of the environmental factors to be taken into account. Nevertheless, pneumonia were the

main final cause of death in mass mortality pestivirus outbreaks (Marco *et al.,* 2009b). BDV infection causes leukopaenia maintained in time as it has been demonstrated in chamois experimental infections (Cabezón *et al.,* 2011; Martin *et al.,* 2013). So, coinfections with BDV and other pathogens could be determinant for disease presentation and evolution.

Finally, human influence on pestivirus epidemiological presentations is also of concern as it has been seen in other diseases (Daszak *et al.,* 2012). Anthropogenic factors such as the hunting pressure could represent an impact on chamois populations and genetic diversity. Moreover, livestock presence on alpine meadows is a known pathogen carrier (Macpherson, 1995). Hence, the pastoral practices and the sanitary status of the domestic animals could have a major influence on pestivirus epidemiology.

1.2.3 Pathogenesis in the field and under experimental conditions

Chamois infected in the field with BDV present previously unreported clinical and post-mortem findings. The main clinical findings are neurological signs - such as depression, absence of flight reaction and loss of fear to humans - and skin disorders - such as alopecia and hyperpigmentation - although non-specific signs - such as emaciation and apathy - are also found. Pneumonia and abscesses are also commonly reported and associated with secondary infections. Main microscopic lesions are observed in the brain, where non-suppurative meningoencephalitis with gliosis is the main finding. Also, follicular atrophy and telogeneization, epidermal hyperplasia and melanosis with orthokeratotic hyperkeratosis are described (Marco *et al*., 2007, 2015).

Two experimental infections of Pyrenean chamois with have confirmed and widen the pathogenesis of BDV infections in field (Cabezón *et al*., 2011; Martin *et al*., 2013). In both experiments, chamois were inoculated with field strain isolates from diseased chamois. Three out of five and two out of three animals died before the end of the challenges, respectively, demonstrating the severity of some BDV strains infecting chamois. Although no apparent neurological signs and skin lesions

were found, non-suppurative meningoencephalitis with gliosis was described. More severe lesions were described in the telencephalon, diencephalon and mesencephalon than in metencephalon. Cabezón *et al.* (2011) also observed changes in lymphoid tissue and spleen characterized by moderate lymphoid depletion with loss of lymphoid follicles and decreased lymphoid density. Lymphoid depletion was also described by Martin *et al.* (2013) in their experimental infection. Interestingly, in both studies chamois died of haemorrhagic diathesis, with haemorrhagic diathesis with multifocal extravasations of red blood cells throghout the intestinal tract. These lesions have never been reported in naturally infected chamois.

To assess the reproductive impact of BDV-4 in chamois, Martin *et al.* (2013) infected three pregnant Pyrenean chamois with a BDV-4 strain from chamois origin. Although none of the animals arrived at labour, foetal death, viral presence in foetal and adult tissues and viraemia of at least 51 days was observed. One animal died at day 24 p.i., a second one presented a profuse diarrhoea from day 13 p.i. to death (day 51 p.i.) and the third aborted at day 46 p.i. The authors proposed that BDV was the main cause of abortion, as the virus was detected by PCR in the cotyledons of inoculated animals. Viral RNA was also detected in all organs of the foetuses and the tissues from infected adults. Regarding tissue distribution, Cabezón *et al.* (2011) found a wide distribution of BDV in free-ranging and experimentally infected chamois, as all tissue sampled and analysed by PCR and cell culture titration were positive.

Other remarkable findings in experimental infections in chamois were the higher mean body temperature and the weight loss in infected chamois when comparing to control group. Moreover, regarding haematological findings, a significant leukocyte count decrease in experimental infected chamois was found (Vautrain and Gibert, 2008; Cabezón *et al.*, 2011; Martin *et al.*, 2013), a finding that has previously been described in naturally infected chamois (Fernández-Sirera *et al.*, 2011).

In other studies, Cabezón et al. (2010b,c) showed that infection with the same strain (named Cadí-6) did not cause remarkable pathogenic alterations in sheep and pig. Although these species developed brief viremia and seroconverted within 14 days p.i., no clinical signs or histological lesions were observed.

1.3 Ovine influence in chamois pestivirus infections

1.3.1 Pestivirus infection in sheep

Previously reported data on seroprevalence of antibodies against pestivirus in sheep showed highly heterogeneous epidemiological scenarios, especially in alpine commune grazing areas. While moderate to high pestivirus seroprevalences have been described in the Spanish Pyrenees (23-69%) (Alba *et al.*, 2008; Marco *et al.*, 2009b), and Austrian, French and Italian Alps (26-90%) (Gaffuri *et al.*, 2006; Krametter-Frotscher *et al.*, 2007a; Fernández-Sirera *et al.*, 2012c; Martin *et al.*, 2015), low seroprevalence was reported in Andalusia and the Cantabrian mountains (Spain) (5.9-10.8%) (Paniagua *et al.*, 2016; Fernández-Aguilar *et al.*, 2016) and Swiss Alps (6.9%) (Braun *et al.*, 2013a). In other non-alpine regions from Spain, BDV infection has been reported to be widely spread with antibody seroprevalence ranging from 17.9% to 93% (Berriatua *et al.*, 2006; Valdazo-González *et al.*, 2008).

BDV antigen prevalence in previous studies in ovine flocks ranges from 0.3-0.7% (Valdazo-González *et al.*, 2006; 2008; Braun *et al.*, 2013a). As reviewed before, the genetic classification of pestivirus species is accepted internationally but less consensus is found in BDV group and subgroup classifications (Valdazo-González *et al.*, 2006). Many phylogenetic relations are based on the highly conserved region 5'UTR, the Npro and the E2 region. Currently a classification from up to 8 subgroups of BDV has been proposed (Giammarioli *et al.*, 2011; Peletto *et al.*, 2016). Spanish isolates from sheep have been reported as BDV-4 (Valdazo-González *et al.*, 2006) (Fig. 1.4). The putative BDV-4a cluster includes: isolates from Basque country (Berriatua *et al.*, 2006), a previously described group namely BDV-C (Hurtado *et al.*, 2003), three ovine isolates by Valdazo *et al.* (2006) and Pyrenean chamois isolates. BDV-4b is the other cluster described in BDV isolates from Spanish sheep flocks (Valdazo *et al.*, 2006, 2008). Studies in sheep flocks in the French Pyrenean were included in BDV-5 genotype (Dubois *et al.*, 2008), similar to the Aveyron strain that caused high mortality in sheep in 1984 (Chappuis *et al.*, 1984). Recently, the Esp-97 isolate from Spanish sheep belonging to a region near the Pyrenees was classified as BDV-5. This isolate was from a farm that

imported animals from Aveyron region and presented a mortality episode in 1997 (Vega *et al.*, 2015).

1.3.2 Evidence of interspecific contact

Pestiviruses mostly need direct contact to infect new hosts, although other routes of infection may be possible (Schweizer and Peterhans, 2014). Because of the wide distribution of pestivirus infections in livestock, several studies in wildlifehave focused on the interactions between domestic and wild animals. Contact rates between sheep and chamois have been reported to be unusual (Ryser-Degiorgis *et al.*, 2002; Rüttimann *et al.*, 2008; Casaubon *et al.*, 2012). The distribution and presence of attractive resources have an influence on the behaviour of animals in alpine meadows and so different scenarios can be observed. Some authors reported more frequent encounters of very short distances of less 10m (Ryser-Degiorgis *et al.,* 2002) while others described a passive tolerance between Pyrenean chamois and sheep up to >50m (Pépin and N'Da, 1992). Also, in some situations such as salt point) the rate of direct contact may be increased (Casaubon *et al.,* 2012; personal field observation) (Fig. 1.5). Other studies have assessed the contact rates between wild ungulates and livestock by using camera traps and have shown similar low rates of interspecific direct contact (Kukielka *et al.*, 2013; Payne *et al.*, 2017). In contrast, Rebollo *et al.* (1993) observed that Pyrenean chamois were segregated by the presence of sheep. In this study, chamois did not have any contact with sheep in grazing areas. Although direct contact between chamois and sheep was anecdotic, these interactions could suppose a virus introduction in a wild or domestic population.

While interespecific transmission of BVDV and BDV is well described among domestic animals (Krametter-Froetscher *et al.*, 2007b; Braun *et al.*, 2013b), viral circulation between wildlife and domestic animals is more tricky. Intraspecific maintenance in wild ruminants has been reported for BVDV in white-tailed deer (*Odocoileus virginianus*) from USA (Passler and Walz, 2010) and for BDV in Pyrenean chamois (Marco *et al.*, 2009a; Fernández-Sirera *et al.*, 2012b). Interspecific pestivirus transmission between domestic and wildlife ruminants has been demonstrated in

few studies (Bertin-Cavarait, 2006; Casaubon *et al.*, 2012; Martin *et al.*, 2015; Passler *et al.*, 2016). An example of this possible relation was proposed by Martin *et al.* (2015) when two isolates from chamois and sheep origin from the French Alps presented a 92% identity in 5'UTR. In contrast, other authors have reported absence of transmission between wild and domestic animals (Paniagua *et al.*, 2016) or have demonstrated that sheep is not important for wildlife BDV epidemiology (Fernández-Aguilar *et al.*, 2016).


A)
B)
C)
D)
E)
F)
G)
H)
I)
J)

Figure 1.5 Species identified by means of a camera trap in a salt lick. Freser-Setacases NHR (2014-2016). **A)** Pyrenean chamois (*Rupicapra p. pyrenaica*); **B)** Mouflon (*Ovis aries musimon*); **C)** Roe deer (*Capreolus capreolus*); **D)** Fox (*Vulpes vulpes*); **E)** *Turdus sp.*; **f)** Alpine marmot (*Marmota marmot*); **G)** European hare (*Lepus europaeus*); **H)** *Murinae* subfamily; **I)** Cattle (*Bos taurus*); **J)** Horse (*Equus f. caballus*).

VNT has been used as an indirect analysis to assess interspecific contacts between Pyrenean ungulates and sheep. Marco *et al.* (2009a) reported BDV infection in a mouflon most probably caused by a strain of ovine origin. In another study, Fernández-Sirera *et al.* (2012b) did not find significant differences in sheep when using BDV strains from sheep and chamois origin. Other reports from VNT studies in Pyrenean chamois did not find antigenic differences between domestic and wild BDV strains (Pioz *et al.*, 2007; Fernández-Sirera *et al.*, 2012c). The explanation of this cross-reactivity could be due to the use of a few related heterologous strains in VNT analyses. Another influential factor may be related to the interval between infection and time of sampling (Terpstra and Wensvoort, 1988).

1.3.3 Transhumance and pestivirus transmission

Pastoral practices are of importance because they increase the contact between domestic animals and between domestic and wild species. In this context, transhumance plays a main role in the epidemiology of diseases worldwide (Macpherson, 1995). Transhumance is defined as seasonal moving of livestock to regions of different climate or seasonally productive lands (Eckert and Hertzberg, 1994). It was already practiced in Pre-Roman and Roman times and historians have described abundant drove roads (Manzano and Casas, 2010). Nowadays in Europe, more than four million hectares of agricultural land are grazed by transhumant livestock (Herzog *et al.*, 2005). In Catalonia, North-Eastern Spain, transhumance has been organized and well documented since XII century, and the proximity and land source that offers the Pyrenean mountains have determined the movement of livestock (Camí *et al.*, 2012). Some studies have focused on the importance of studying transhumance in order to control several parasite infections (Eckert and Hertzberg, 1994; Macpherson, 1995; Paiz *et al.*, 1998), bluetongue virus (Nannini *et*

al., 2004), *Mycoplasma bovis* (Spergser *et al.*, 2013) and BVDV (Braun *et al.*, 1998; Bodmer *et al.*, 2008; Presi and Heim, 2010).

Eradication plans, mainly focused on BVDV, has been based on PI animals removal in many countries. These practices started in Sweden and Norway in 1993, and in Finland and Denmark in 1994. Afterward, Austria, Germany and Switzerland have performed their own programs (Rossmanith *et al.*, 2010; Stahl and Alenius, 2012). The focus on controlling transhumant animals has been developed mostly in BVDV eradication strategies without vaccination. An interesting study carried out in Switzerland, removed PI animals and studied a season of transhumance PI animals-free. Although it failed to completely prevent BVDV infection, the new infection rate was diminished (Bodmer *et al.*, 2008). Also, Presi and Heim (2010) assessed the identification and slaughter of PI cattle in a pre-pasture period as a previous step to a complete BVDV control measure in Switzerland. Although BVDV plays a major role in pestivirus and transhumant investigations (Siegwart *et al.,* 2006), the knowledge about BDV presence in sheep is also relevant when interspecies infection occurs (Braun *et al.*, 2013b) and when BDV from sheep might impede serological BVDV surveillance (Kaiser *et al.*, 2017). Pestivirus infections after transhumance have also been assessed by seroprevalence changes in flocks. Krametter-Froetscher e*t al.* (2007b) reported changes in BDV seroprevalence in sheep before and after communal alpine pasturing (from 67.6 to 83%). Also, Braun *et al.* (1998) showed results in accordance when studying BVDV seroprevalences in cattle. They found changes of seroprevalence from 63.3% previous to pastures to 80.1% after pastures in farms with PI animals.

2. Hypotheses and objectives

2.1 Hypotheses

After 16 years of the first mass mortality outbreaks associated to Border Disease Virus (BDV) infection in Pyrenean chamois, after at least 27 years of pestivirus presence in Pyrenean chamois populations and after several scientific investigations on epidemiology and virology of pestivirus in wildlife; many old questions remain unknown and new ones appear for each new uncovering.

Epidemiological studies and in-field-monitored populations have shown that pestivirus infection in wild chamois is highly diverse and has different impact in free-ranging populations. The fact that some areas were affected by high mortality epizootics while other geographically close populations remain unaffected since many years of pestivirus presence, lead the scientific community to hypothesize on factors that rule these differences. The factors pointed out in this debate are mainly based on genetic diversity of viruses, host factors such as coinfections, genetic diversity and immunological status of the populations, environmental factors and interspecies interactions. The present thesis was developed expecting to give light on some of the above mentioned factors.

RNA viruses are known to have a high rate of mutation and, therefore, virulence diversity of different strains may play an important role in the epidemiology. Moreover, these genetic variants result in a constant challenge for phylogenetic studies and may confuse the pathological implications of a viral stain when relating it to certain cluster. For this reason and the different epidemiological scenarios in pestivirus infections in Pyrenees above mentioned, we hypothesize that strain diversity is playing a major role on disease presentations, immune protection and reproductive alterations on Pyrenean chamois populations. Also, phylogeny analysis of these strains could help to understand their epidemiological situation on field.

Another important factor that may be ruling the epidemiology of pestivirus infections in wildlife is the interespecific transmission of viruses. Following this trend, many artiodactyl hosts have been identified as susceptible to pestivirus infections. However, the adaptability of these viruses to new hosts has been

highlighted when non-artiodactyl hosts have been described. Although only the rabbit and the Bennett's wallaby are known as wild non-artiodactyl susceptible species to BVDV infections this fact increases the interest on sympatric species studies in areas where pestivirus is a threat to wildlife populations. For these reasons we hypothesize that non-artiodactyl species in areas with high pestivirus circulation, such as the Pyrenean mountains, could be also susceptible to pestivirus infection.

Finally, the implication of livestock in wildlife pestivirus infections has been demonstrated in some studies and may be classified as an anthropogenic factor of variation. Livestock animals grazing in alpine areas where chamois inhabits could represent an opportunity to share virus in any direction, although intraspecies maintenance has been already shown. With the knowledge acquired by these previous studies, we hypothesize that transhumant sheep could be a source of virus to wild populations, despite playing a minor role on pestivirus maintenance in Pyrenean chamois populations. Moreover, the lack of Pyrenean pestivirus isolates from ovine origin encourages investigations on the phylogeny classification of those strains.

Graphical representation of the hypotheses

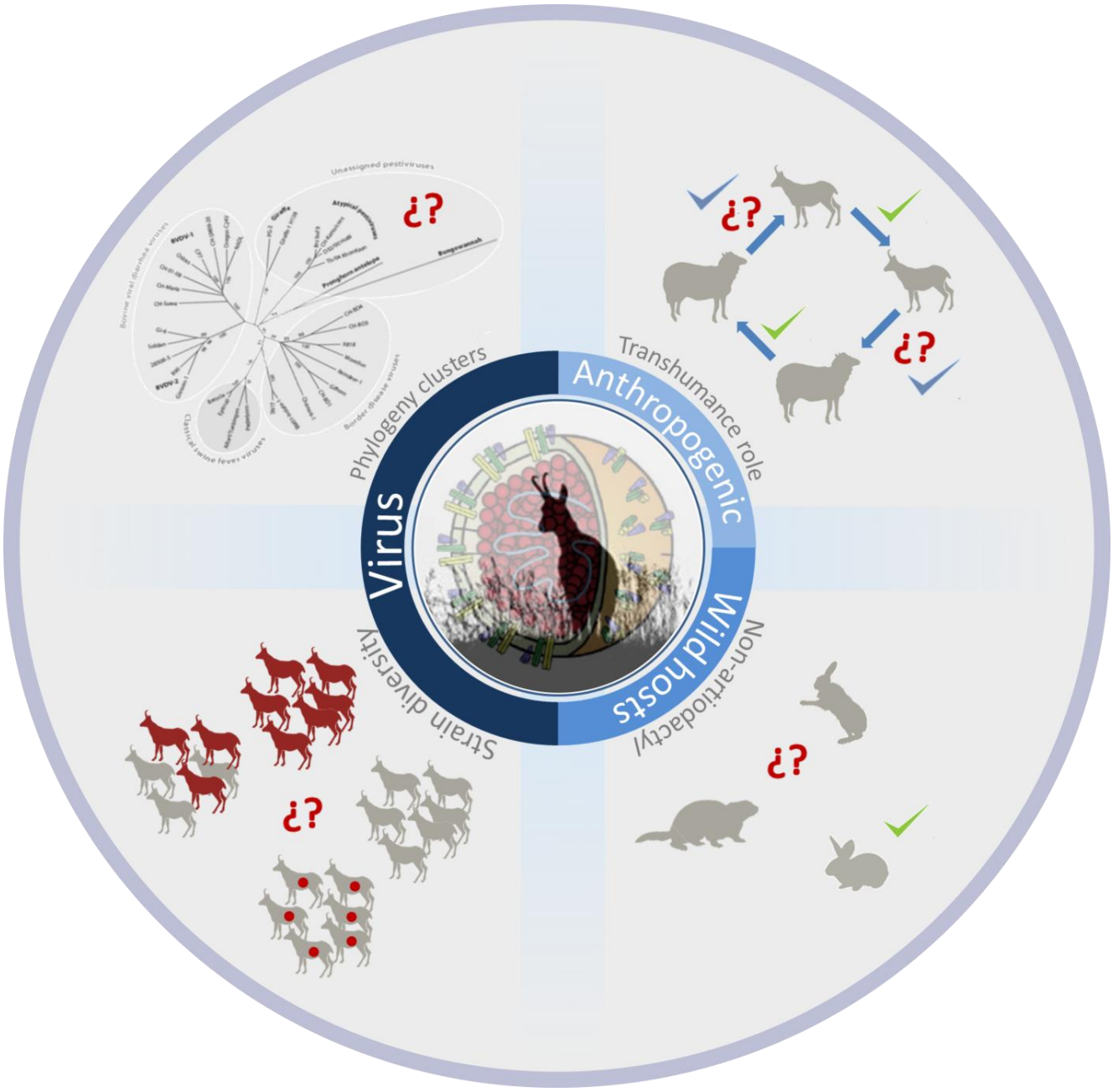

Figure 2.1. Factors studied in the present thesis as they hypothetically may affect the epidemiology of Border Disease Virus infections in Pyrenean chamois. On the left half of the circle, factors related with the virus: (up) implication of including strains into certain phylogenetic clusters and (down) strain related presentations of pestivirus infections; red chamois represents diseased animals, red dots represents foetal infections and grey chamois are healthy/immune protected chamois. On the right half of the circle: (up) transhumance could play a role in virus maintenance and increase virus presence on chamois populations, defined as an anthropogenic factor; (down) common rabbit has been demonstrated to be susceptible to BVDV infections, so sympatric non-artiodactyl species in Pyrenees, such as Alpine marmot or European hare, could be also susceptible hosts to pestivirus infections.

2.2 Objectives

1. To study experimentally the virulence and transmissibility of a high- and low-virulence BDV-4 strain in Pyrenean chamois in order to explain the different epidemiological scenarios described in the wild.

2. To describe effects on foetus of a high-virulence BDV-4 strain and a low-virulence BDV-4 strain.

3. To assess the susceptibility to pestivirus infection of the European hare (*Lepus europaeus*) and Alpine marmot (*Marmota marmota*).

4. To characterize the role of transhumant sheep in the epidemiology of pestivirus infections in Pyrenean chamois (*Rupicapra p. pyrenaica*).

5. To identify BDV strains circulating in transhumant sheep that graze seasonally in the Pyrenees.

Part II: Studies

3. Study I

Experimental infection with high- and low-virulence strains of Border Disease Virus (BDV) in Pyrenean chamois (*Rupicapra p. pyrenaica*) shed light on the epidemiological diversity of the disease.

Introduction

Border Disease Virus (BDV) is one of the four traditionally recognized species of the *Pestivirus* genus (Fam. *Flaviviridae*). Bovine Viral Diarrhea Virus (BVDV-1, BVDV-2) and CSFV are the most studied due to their economic impact on livestock industries (Tautz *et al.*, 2015). However, BDV is also important in the agricultural sector, causing economic losses mainly in sheep flocks and interfering in the detection of BVDV when eradication programs are implemented (Nettleton *et al.*, 1998; Kaiser *et al.*, 2017). Despite being the least studied pestivirus species, BDV is the only member of the *Pestivirus* genus that has caused epizootic mortalities in a wild ruminant species (Marco *et al.*, 2007).

Since 2001, Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) populations have been affected by BDV strains classified into the BDV-4 genogroup (Arnal *et al.*, 2004), causing mortalities in more than 80% of the population in some areas (Marco *et al.*, 2009b). Animals infected in the field presented previously unreported clinical and post-mortem findings for a pestivirus infection. These consisted of cachexia, alopecia with skin hyperpigmentation and several types of neurological alterations such as depression, weakness and difficulty in moving, which were associated with a non-suppurative meningoencephalitis (Marco *et al.*, 2007). In light of these field findings, Cabezón *et al.* (2011) developed an experimental infection in chamois demonstrating that a BDV-4 strain isolated from a diseased chamois causes long-lasting viraemia with pathological changes mainly characterized by non-suppurative meningoencephalitis, although no neurological signs were recorded. In another study, the same authors showed that infections with the same strain (named Cadí-6) did not cause remarkable pathogenic alterations in sheep and pig (Cabezón *et al.*, 2010a,b). Although these species developed short viraemia and seroconverted within 14 days post-inoculation (dpi), no clinical signs or histological lesions were observed. Moreover, Martin *et al.* (2013) infected three pregnant Pyrenean chamois with the same BDV strain. All of the animals died before parturition, and foetal death, viral presence in foetuses and adult tissues with viraemia for at least 51 days were found.

In both of the abovementioned experimental infections of chamois, viral shedding through nasal, rectal, oral and vaginal routes were found. Viral excretion was present from day 5 p.i. (Cabezón *et al.*, 2011) and from day 12 p.i. (Martin *et al.*, 2013) onwards, highlighting the importance of horizontally infected chamois in virus spread. Moreover the high virulence of BDV strains causing high mortality outbreaks in the field was demonstrated. Typically, a key role in pestivirus maintenance at a population level is played by persistently infected (PI) animals. Although this epidemiological figure has not been clearly demonstrated in chamois, an experimental infection of one pregnant Pyrenean chamois inoculated at day 90-100 of gestation showed an animal RT-PCR positive at birth and at the time of death 84 days later (Vautrain and Gibert, 2008).

Field studies carried out in France, Andorra and Spain have shown different epidemiological scenarios (Pioz *et al.*, 2007; Martin *et al.*, 2011; Marco *et al.*, 2015). Pestivirus infections in chamois populations mainly cause mortality outbreaks with different impacts on population censuses. After these episodes, at least two scenarios can be seen: constant BDV circulation with negative impacts on population dynamics in some areas, or a lack of virus circulation and rapid recovery of the chamois population in others (Fernández-Sirera *et al.*, 2012b). Strikingly, pestivirus circulation has been seen in an area of the southeastern Pyrenees (Freser-Setcases National Hunting Reserve) since 1996 without a significant impact on the chamois population (Marco *et al.*, 2011). Different hypotheses may explain the persistence of the pestivirus in this population, related to BDV strain variability, genetic diversity of chamois and environmental factors. Cabezón *et al.* (2011) found that chamois captured in this area are also susceptible to developing the disease when infected with a BDV-4 strain confirmed as a causative strain of high mortality rates in chamois in other areas of the Pyrenees. Of the 5' UTR sequences of BDV strains that have been isolated in the last 15 years from Pyrenean chamois, all of them have clustered into the BDV-4 genogroup with low phylogenetic divergence, although geographical patterns of distribution have been proposed (Luzzago *et al.*, 2016).

To shed light on the epidemiological diversity of pestivirus infections and to contribute to the knowledge of pathological implications of different strains from the same viral genogroup, we developed an experimental infection of Pyrenean chamois with a previously reported high-virulence strain and a supposedly low-virulence BDV. The main objectives of the study were: 1) To describe clinical, virological and pathological differences between strains; 2) To assess the impact of these strains on pregnant chamois and their foetuses; and 3) To evaluate the mechanisms of transmission and cross-protection to understand their implications on pestivirus epidemiology.

Materials and methods

Animals: Capture and management

Fifteen free-ranging Pyrenean chamois (11 females, 4 males, between 3 and 16 years old) were captured by drive net (López-Olvera *et al.*, 2009) in Freser-Setcases National Hunting Reserve (FS-NHR). This reserve covers a 20.200 hectare area of alpine ecosystem in the Pyrenees mountains, where about 300 Pyrenean chamois are legally hunted per year (northeastern Iberian Peninsula, 42º22'N, 2º09'E). Acepromazine maleate (0.1 mg/kg IM; Calmivet 5 mg/ml; Vétoquinol S.A.) was administered to all chamois to reduce stress after capture (López-Olvera *et al.*, 2007). In order to mitigate the adverse effects of stress in captivity, 1mg/Kg Zuclopenthixol acetate (Clopixol Acuphase 50mg/ml; Lundbeck Limited) was intramuscularly administered every three days. In addition, all the chamois were treated with a single intramuscular dose of 2.5 mg/Kg tulathromycin (Draxxin; Pfizer Animal Health), a single oral dose of 2.5 mg/Kg toltrazuril (Baycox 5%; Bayer Animal Health) and a single subcutaneous dose of 0.2mg/Kg ivermectine (Ivomec 1%; Merial Laboratorios S.A.), to prevent opportunistic bacterial and parasitic infections.

Before the challenge, all animals were tested for BDV presence in sera by means of RT-PCR. Antibodies against BDV were assayed by a Virus Neutralization Test (VNT) to establish their immunological status for the challenge groups. Four out of

fifteen animals (Rp 6, 7, 14 and 15) showed antibodies and were included in the study as seropositive inoculated animals. To evaluate whether or not the females were pregnant, the presence of a foetus was detected by trans-rectal echography. The image test showed that 8 out of 11 females were pregnant. Although the time of gestation could not be determined accurately, we estimated it to be between 70 and 100 days based on the natural history of Pyrenean chamois and the capture date.

Inoculum

Two non-cytopathogenic BDV-4 strains where used as inoculum. BDV Freser-5 was isolated from the spleen of a foetus belonging to a healthy hunted female chamois from Freser-Setcases National Hunting Reserve in 2014. As it was isolated from a healthy antibody-positive chamois from an area where no outbreaks had been recorded, we hypothesized that this could be a low-virulence strain. The second virus, BDV Cadí-6 (GenBank accession number AM905923), was isolated from a diseased chamois found in the Pyrenees (Cadí National Hunting Reserve) in 2005. This virus was demonstrated as highly virulent in a previous experimental infection (Cabezón *et al.*, 2011). Both BDV-4 were 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 10^6 $TCID_{50}$/ml of virus.

Study design

The animals were divided into two groups – group A (GA) and group B (GB) – and placed in two isolated boxes in a level-3 biosafety area of the Centre de Recerca en Sanitat Animal (CReSA-IRTA, Universitat Autònoma de Barcelona, Spain) facilities for 26 days. Within each group the animals were subdivided into two subgroups based on antibodies against pestivirus presence at the beginning of the experiment – subgroup 1, antibody negative (GA-1, GB-1); subgroup 2 antibody positive (GA-2, GB-2) – but not physically separated in each box (GA and GB). In group A, GA-1 was made up of 2 pregnant females (Rp 1 and 2), 2 non-pregnant females (Rp 3 and 4), and 1 male (Rp 5). The two antibody positive pregnant females (Rp 6 and 7) made up GA-2. In group B, GB-1 was made up of 2 pregnant females (Rp 8 and 9),

1 non-pregnant female (Rp 10), and 3 males (Rp 11 to 13). Two antibody positive pregnant females (RP 14 and 15) were included in GB-2 (Table 3.1). GA and GB were challenged with 10^6 $TCID_{50}$/ml of Cadí-6 and Freser-5 strains, respectively. The whole virus dose from both viruses was thawed immediately before inoculation and administered by a combination of nasal catheter (0.5 ml in each nostril) and orally (1 ml). The duration of the challenge was 26 days. Chamois displaying any or combinations of the following signs were euthanized: complete anorexia, recumbence with inability to rise, or signs of severe dehydration. 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 of Barcelona. From this point onwards, chamois from GA and GB without antibodies at the beginning of the challenge are noted in the text as GA-1/GB-1. Chamois from GA and GB that had antibodies at the beginning of the experiment are noted as GA-2/GB-2.

Sampling procedure

The animals were observed daily to evaluate clinical signs. Rectal temperature was recorded on twelve days throughout the study, and all animals were weighed on 0, 8, 15, 19, 22 dpi and when the necropsy was performed. Blood samples were obtained by venepuncture of the jugular vein on 0, 2, 4, 8, 15, 19 and 26 dpi and centrifuged at 1200 g for 15 minutes to obtain serum. Sera were stored at -80ºC until analysis. Blood from foetuses was obtained during necropsy. Nasal and rectal swabs were obtained the on same days as blood. Swabs were mixed with 1ml of sterile PBS (pH 7.2) and stored at -80ºC until analysis.

For the determination of the white blood cell (WBC) count, an aliquot of blood was placed in a commercial tube containing EDTA and was counted manually using microscopy and a Neubauer chamber. The differential leukocyte count was performed by identifying 100 leukocytes on blood smears stained with a commercial Diff-Quick-like stain (Química Clínica Aplicada, Amposta, Spain).

After necropsy, tissues for virological studies were weighed with a 0.1g precision scale, homogenized in 0.9ml EMEM and stored at -80ºC. Those samples were spleen, liver, bone marrow, kidney, Peyer patch, urine, lungs, brain and two lymph nodes (submandibular and retropharyngeal) for adult chamois, and thymus, spleen, brain, and placentome for foetuses.

Virus Neutralization Test (VNT)

Sera were tested for the presence of neutralizing antibodies against the homologous BDV strains CADI-6 (GA) and Freser-5 (GB) with VNT (OIE, 2008). Briefly, serum samples were diluted 1:10 with sterile EMEM, heat-inactivated (56 ºC for 30 min) and diluted ten-fold 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 ºC for an hour. Finally, $2.8x10^4$ Madin–Darby bovine kidney cells (100 µl) were added to each well. Replication was monitored using the immunoperoxidase monolayer assay (IPMA) (OIE, 2008) with a polyclonal pestivirus antibody (produced in-house). Titres were expressed as the reciprocal of the highest dilution that neutralized 100 $TCID_{50}$ in all cultures, calculated according to the method of Reed and Muench (1938).

Real-time reverse transcriptase-PCR

Total viral RNA was extracted directly from 200 µl of sera, swabs, urine and tissue samples using MagAttract 96 *cador* Pathogen Kit (Qiagen, Venlo, Netherlands) as per the manufacturer's instructions. A one-step reverse transcription-PCR kit was used for SYBR® Green-based real-time RT-PCR (Thermo-fisher Scientific, Waltham, Massachsetts, USA). Positive results were considered for threshold cycle values (Ct) less than 40. Differences in 3.3 Ct units were estimated to be a ten-fold increase in viral load (Nolan *et al.,* 2006). Samples in which fluorescence was undetectable were considered negative.

Primers 324 (5'-ATGCCCWTAGTAGGACTAGCA-3'; W=adenine or thymine) and 326 (5'-TCAACTCCATGTGCCATGTAC-3') were used for the amplification reaction (Vilcek *et al.*, 1994). These primers were designed from the BDV genome: nt 101–121 and 386–366, respectively (Vilcek *et al.*, 2010). Analysis of the sequence

of the 243 base pair 5'UTR fragment generated by RT-PCR was performed on positive samples from foetuses. Amplified DNA was purified and sequenced. The phylogenetic tree was made by the neighbour-joining method using an automatic root location. To test the reliability of the branches in the tree, a bootstrap analysis of 1,000 replicates was performed by creating a series of bootstrap samples.

Pathological examination

Necropsies and tissue sampling were performed according to standard protocols. The chamois were euthanized on 19 dpi (Rp 2), 22 dpi (GA-2/GB-2) and 26 dpi (GA-1/GB-1) 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 4 μm sections and stained with haematoxylin and eosin according to standard procedures.

Statistical analysis

To assess statistically significant differences in mean temperatures, weight, WBC and differential counts between GA and GB, or between GA-1/GB-1 and GA-2/GB-2 animals, a non-parametric unpaired Wilcoxon test (Mann-Whitney test) was used. Differences between Group A and B in median titres obtained by VNT were statistically assessed by the Mood's median test. The limit of significance was defined as $P \leq 0.05$. All the analyses were carried out with the statistical software R version 3.4.0 (R Development Core Team, 2016).

Results

Clinical findings and pathological examination

The main clinical observation in chamois from GA-1 was apathy. This was present in all but one animal from this subrgoup, from 20 dpi in Rp 1, 12 dpi in Rp 2, 14 dpi in Rp 4 and 15 dpi in Rp5 until death. Three out of these five animals were found dead or were euthanized before the end of the experiment. Rp 2 was euthanized on 19 dpi because of severe apathy, prostration and dyspnoea. Rp 4 and 5 were found dead at 15 dpi and 26 dpi, respectively. In GB, all animals remained active and apparently healthy with the exception of the two pregnant females (Rp 8 and 10)

who presented mild apathy between 12 dpi and 17 dpi. Chamois from subgroups GA-2 and GB-2 remained active throughout the experimental period.

All the animals presented high temperatures at 0 dpi (>39.2ºC) that progressively decreased until 5 dpi. No statistically significant differences were established between groups at any sampling time (Fig. 3.1a). Regarding weight data, all animals from the two groups lost weight from the capture day until 8 dpi (Fig. 3.1b). The largest drop in weight was 26% in chamois from GB-2 between -7 dpi and 15 dpi. During the same period the chamois from GB-1 also lost body weight (14% of body weight from -7 dpi). From 15 dpi until the end of the experiment all chamois from GB-2 increased their weight except at 26 dpi. All animals from GA-2 showed a similar trend of body weight but stabilized from 8 dpi until 22 dpi. On the last sampling day (26 dpi), chamois without antibodies at the beginning of the experiment presented a decrease in body weight of 8.8% (GA-1) and 3.5% (GB-1).

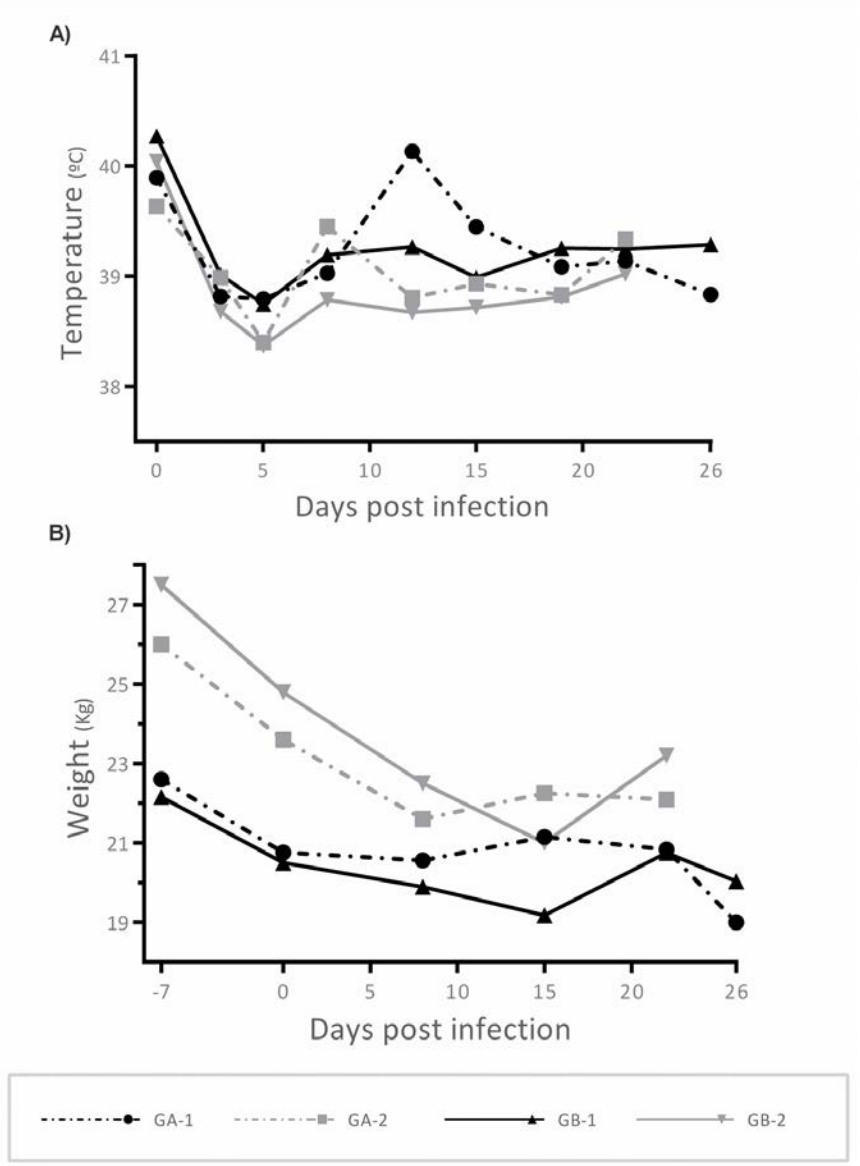

Figure 3.1 A) Mean body temperature in Celsius degrees from day of virus inoculation (0 dpi) until the end of the experiment (26 dpi). **B)** Mean weight in Kg from the day of capture in the wild until the end of experiment (26 dpi). GA: Group infected with Cadí-6 BDV strain; GB: Group infected with Freser-5 BDV strain. Subgroups according to antibody presence at the beginning of the experiment: without antibodies (numbered as 1) or with antibodies (numbered as 2).

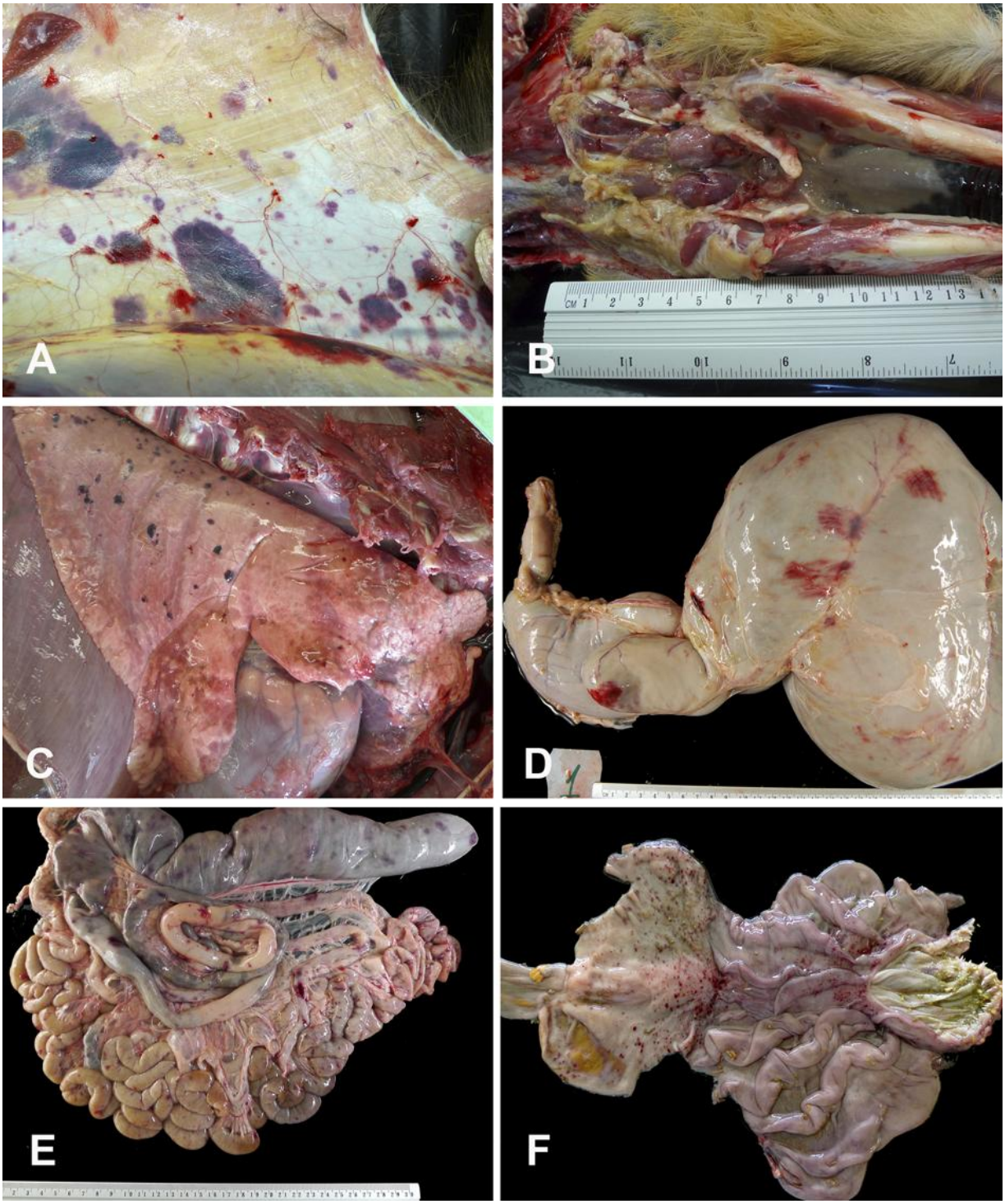

Figure 3.2 Border Disease Virus experimental infection in chamois (*Rupicapra pyrenaica*). **A)** Ecchymotic haemorrhages (ecchymoses), subcutis (Rp 2). **B)** Retropharyngeal lymphadenopathy (Rp 2). **C)** Ecchymotic haemorrhages in the lung parenchyma (Rp 4). **D)** Serosal and transmural haemorrhages in the reticulum and rumen (Rp 1). **E)** Serosal and transmural haemorrhages in the small and large intestines (Rp 2). **F)** Mucosal haemorrhages in abomasum (Rp 4).

The three animals that died before the end of the experiment had lesions consistent with haemorrhagic diathesis. Petechial to ecchymotic haemorrhages were present in the subcutaneous tissue, in the serosa wall and mucosa along the gastrointestinal tract, lungs, epicardium and endocardium, mucosa of the urinary bladder and in the pregnant females in the placentomes (Fig. 3.2 and 3.8). The only neurological sign recorded during the experiment was apathy, mainly in GA. However, lesions of different severity were seen in the brain in all animals from GA-1 and GB-1. In GA-1, three out of five animals (Rp 1, Rp 2 and Rp 5) had moderately severe non-suppurative meningoencephalitis with diffuse gliosis, glial nodules, perivascular oedema and inflammatory perivascular lymphohistiocytic infiltrates (Fig. 3.3). Rp 3 presented similar lesions in a milder form, with only few scattered glial nodules and mild perivascular infiltrates, and Rp 4 had only occasional area of microglial activation. Similar to Rp 3, all animals from GB-1 presented mild non-suppurative meningoencephalitis with few small glial nodules and occasional lymphohistiocytic perivascular infiltrates. None of the seropositive animals at the beginning of the experiment from either group (GA-2 and GB-2) presented histopathological lesions in the brain. Changes in lymph nodes and tonsils in GA-1 consisted mainly in moderate lymphoid depletion with loss of lymphoid follicles and decreased lymphoid density in interfollicular and paracortical areas except for Rp 3, where only small haemorrhages were seen. In Rp 4 and Rp 5 and to a lesser extend in Rp 1 and Rp 2 there were apoptotic bodies and numerous tingible body macrophages scattered in all lymph nodes. No lymphoid depletion or tingible body macrophages were seen in GA-2, GB-1 or GB-2.

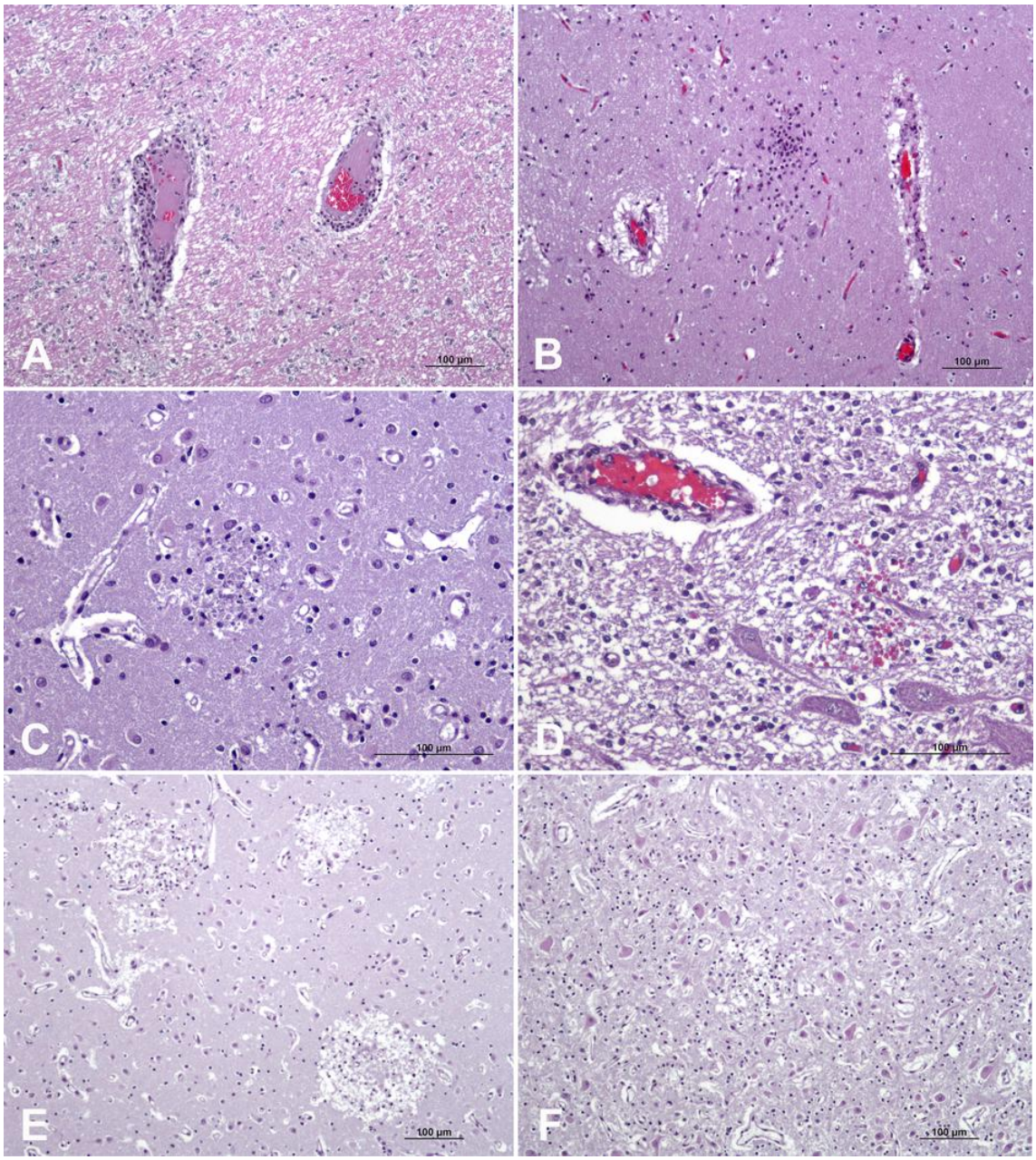

Figure 3.3 Brain lesions in female adult chamois (**A,B**) and foetuses (**C-F**) experimentally infected with Border Disease Virus. **A)** Perivascular lymphohistiocytic infiltrates, Rp 1. **B)** Focal gliosis and perivascular oedema, Rp 5. **C)** Focal necrosis and gliosis with karyorrhectic nuclei, foetus from Rp 1. **D)** Mild perivascular lymphohistiocytic infiltrate and haemorrhage, foetus from Rp 2. **E** and **F)** Multifocal necrotizing encephalitis and gliosis, foetus from Rp 10.

WBC and differential counts

White Blood Cell (WBC) counts and differential counts are represented in Figure 3.4. Mean WBC decreased in all animals until 5 dpi in group A and B. In GA-1 the WBC count continued decreasing until 22 dpi, while in GB-1 the WBC count increased. However, no statistical differences were found between them. Changes in the leukocyte differential count in GA were mainly associated with neutropaenia. The three animals that died or were euthanized before the end of the challenge in GA had very low numbers or undetectable neutrophils before death. All animals from GA-2 and GB-2 showed similar WBC and neutrophil count trends with a peak on 12 dpi significantly higher than the GA-1/GB-1 chamois. Band neutrophil counts presented high peaks on 5, 12, 22 and 26 dpi in GA-1 chamois when compared to GB-1 chamois, but statistically significant differences were found only at 12 dpi (P=0.049). Mean monocyte and lymphocyte counts did not show statistically significant changes between groups during the experiment.

Serology

Neutralizing antibody titres were detected by VNT in animals from GA-1 and GB-1 chamois from 15 dpi until the end of the experiment (Fig. 3.5). In GB-1, antibody titres increased until 26 dpi reaching median titres of 1/1280 (range 1/640-1/2560). These titres were not statistically different from those of GA-1. Chamois from GA-2/GB-2 presented neutralizing antibodies from the beginning until the end of the experiment. GB-2 had a remarkable peak at 15 dpi with titres of 2560 decreasing to titres of 320 five days later.

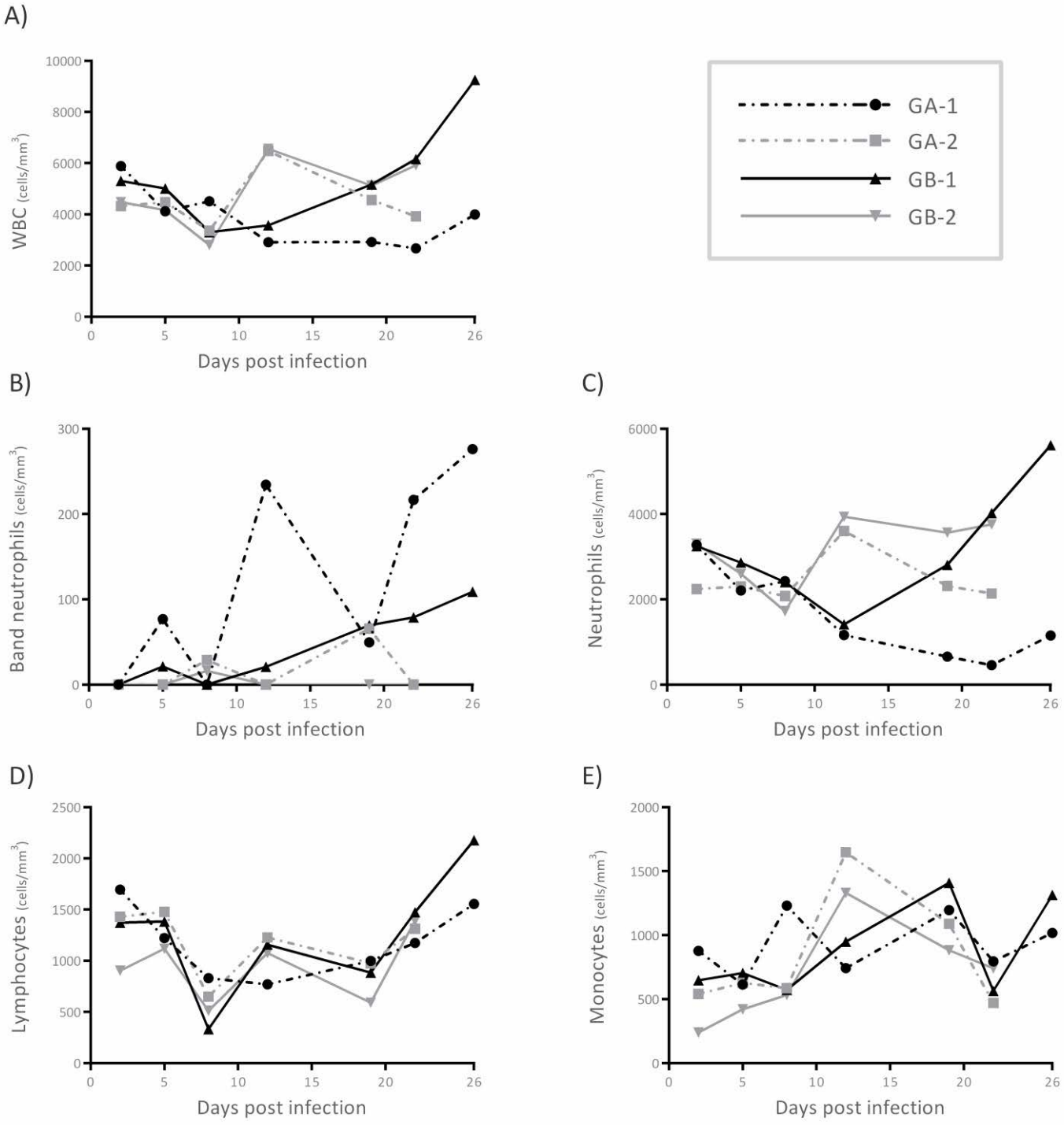

Figure 3.4 Mean values for total and differential white blood cell counts for each challenge group during the experimental infection. **A)** White blood cell count; **B)** Band neutrophils; **C)** Mature Neutrophils; **D)** Lymphocytes; **E)** Monocytes. GA: Group infected with Cadí-6 BDV strain; GB: Group infected with Freser-5 BDV strain. Subgroups according to antibody presence at the beginning of the experiment: without antibodies (numbered as 1) or with antibodies (numbered as 2).

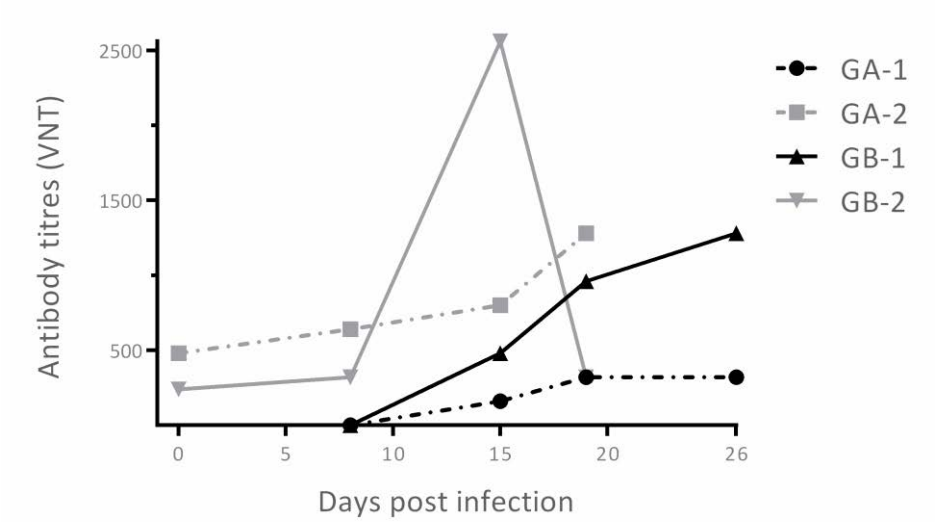

Figure 3.5 Mean neutralizing antibody titres obtained by the Virus Neutralization Test (VNT), represented by the challenge group throughout experimental infection. GA: Group infected with Cadí-6 BDV strain; GB: Group infected with Freser-5 BDV strain. Subgroups according to antibody presence at the beginning of the experiment: without antibodies (numbered as 1) or with antibodies (numbered as 2).

Viral RNA on sera and tissues

A higher mean RNA load (less Ct mean) was found in sera samples of GA-1 from 4 dpi onwards, maintaining a difference between 4.3 and 8.9 Ct – equivalent to a 10 to 100-fold increase in viral load – from animals of GB-1 (Fig. 3.6). Viral RNA was detected in sera from GA-1 in two out of five animals at 4 dpi. At 8 dpi, all chamois in this group presented virus in sera (Ct mean=26.6, sd=0.99). The higher mean RNA load was found on 15 dpi (Ct mean=24.5, sd=2.0). Interestingly, Rp 3 from this group only presented viral RNA at 8 dpi (Ct=26.63) and 26 dpi (Ct=34.37). In GB-1, three out of six chamois presented viraemia on 8 dpi (Ct mean=30.9, sd=1.5), five out of six animals on 15 dpi (Ct mean=30.6, sd=1.0) and one out of six chamois at 19 dpi (Ct=32.74). At the end of the challenge, all the chamois from GB-1 have cleared the BDV in sera as no viral RNA was detected in sera or tissues. Chamois from GA-2/GB-2 did not present viral RNA in sera during experiment.

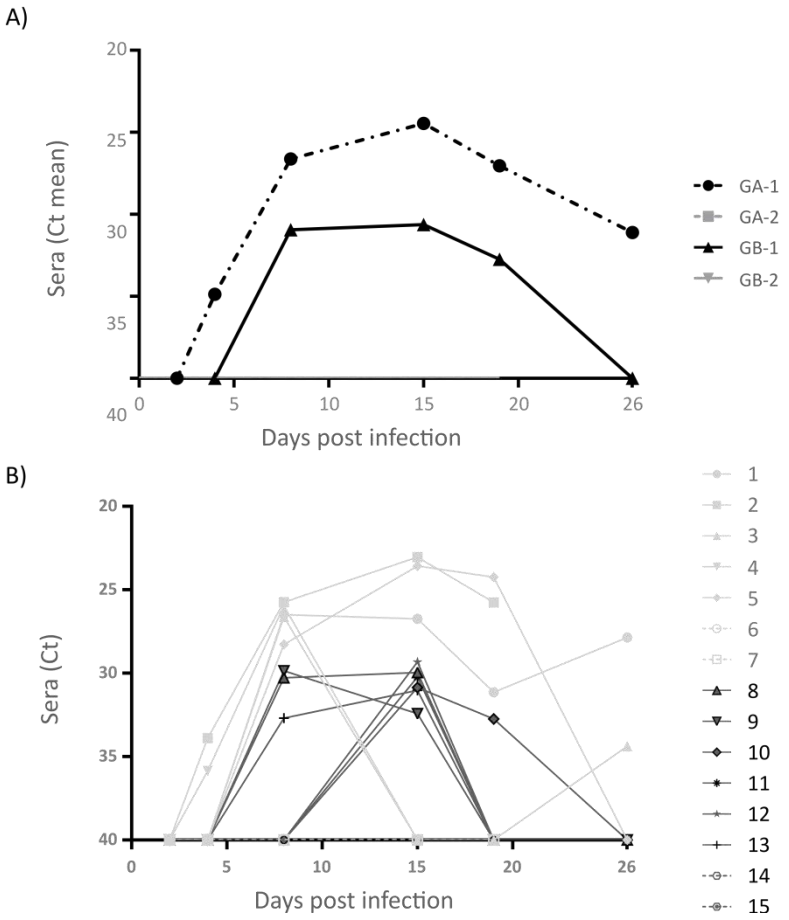

Figure 3.6 BDV-4 RNA load obtained by Real-time RT-PCR in sera samples. Results are presented in threshold cycle (Ct). **A)** Mean Ct values of positive samples; **B)** individual Ct values in each chamois and time of sampling. The limit of detection was established on Ct value ≥ 40. GA: Group infected with Cadí-6 BDV strain; GB: Group infected with Freser-5 BDV strain. Subgroups according to antibody presence at the beginning of the experiment: without antibodies (numbered as 1) or with antibodies (numbered as 2).

In group A, virus was found widely distributed in tissue samples in chamois without antibodies at the beginning of the experiment (GA-1) (Table 3.1). The higher RNA load was found in the brain (Ct mean=21.6, sd=0.35), lymph nodes (LNretrof. Ct mean=22.37, sd=2.8 and LNsubm. Ct mean=22.76, sd=3.9) and lung (Ct mean=22.1, sd=2.4). Interestingly, Rp 3 (GA-1) only presented viral RNA in the submandibular lymph node, tonsil and spleen, with a lower RNA load than the other GA-1 animals. In GB-1, viral RNA was found in lower quantities and less distributed than in GA-1. BDV RNA was detected mainly in lymph nodes (LNretrof. Ct mean=30.87, sd=3.6 and LNsubm. Ct mean=30.65, sd=3.3). Differences (≥8 Ct mean) were found in all tissues between GA-1 and GB-1 chamois, equivalent to more than a 100-fold increase in viral load. Viral RNA was also found in some animals from GB-1 in the tonsil, liver and lungs. No GA-2 and GB-2 animals presented BDV RNA in tissues.

Viral shedding

The presence of the BDV genome was detected in nasal swabs in three out of five GA-1 animals at 8 dpi (Fig. 3.7a). From 12 dpi onwards, all animals from GA-1 (challenged with the high-virulence BDV CADI-6 strain) presented viral excretion in nasal fluids, with the exception of chamois Rp 3, which only presented viral RNA on 12 dpi with high Ct (32.684). In GB-1, three animals presented viral RNA in nasal swabs at 12 dpi and two animals on 15 dpi. Differences from 3 Ct to more than 10 Ct were found between GA-1 and GB-1 chamois, equivalent to an increase in viral load between 10 and 1000-fold. GA-1 presented the highest RNA load at 15 dpi (Ct mean=21.26, sd=2.4), 19 dpi (Ct mean=21.67, sd=1.4) and 26 dpi (Ct mean= 21.87, sd=0.9). Regarding GA-2/GB-2 chamois, only animal Rp 7 presented low viral excretion (Ct=33.36) by the nasal route at 12 dpi.

RT-qPCR detected less viral shedding in rectal swabs than in nasal swabs (Fig. 3.7b). One out of five GA-1 animals presented viral RNA (Ct=32.34) at 8 dpi, all animals from this group at 12 dpi (Ct mean=30.17, sd=3.3), three GA-1 chamois at 15 dpi (Ct mean=31.28, sd=1.1) and in one animal at 19 dpi (Ct=25.34). Again, in the chamois Rp 3 rectal swabs, viral RNA was detected only at 12 dpi (Ct=33.79). Neither the GA-2, GB-1 or GB-2 chamois presented the BDV genome in any of the rectal swab samples.

Regarding BDV presence in the urine collected at necropsy, four out of five GA-1 presented positive RT-qPCR results (Ct mean=24.59, sd=3.6). GA-2, GB-1, and GB-2 animals did not present viral RNA in urine samples (Table 3.1).

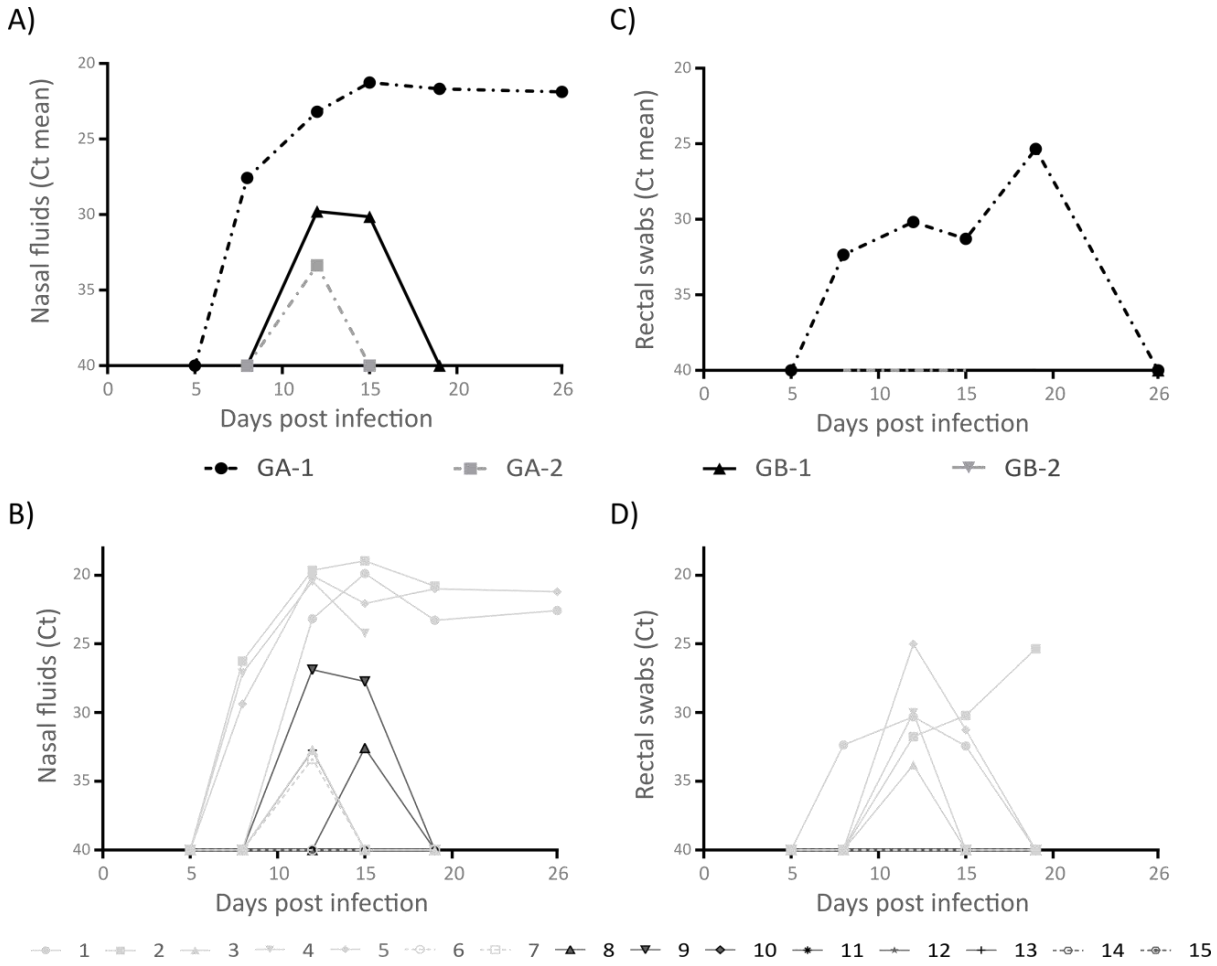

Figure 3.7 BDV-4 RNA load obtained by Real-time RT-PCR in nasal and rectal swabs. Results are presented in threshold cycle (Ct). **A)** Mean Ct values of positive samples in nasal fluids; **B)** individual Ct values in each chamois and time of sampling in nasal fluids; **C)** Mean Ct values of positive samples in rectal swabs; **D)** individual Ct values in each chamois and time of sampling in rectal swabs. The limit of detection was established at Ct value ≥ 40. GA: Group infected with Cadí-6 BDV strain; GB: Group infected with Freser-5 BDV strain. Subgroups according to antibody presence at the beginning of the experiment: without antibodies (numbered as 1) or with antibodies (numbered as 2).

Group	ID	Sex	Pregnancy	Real-time RT-PCR (Ct)										
				Subm. LN	Retrofa. LN	Tonsil	Peyer patch	Spleen	Liver	Lungs	Kidney	Bone marrow	Brain	Urine
Group A-1	Rp 1	F	Yes	19.64	19.60	19.00	21.80	27.27	24.88	20.57	27.31	20.56	21.25	22.66
	Rp 2	F	Yes	22.08	22.83	ns	u	u	u	20.90	u	22.88	21.59	20.48
	Rp 3	M		29.32	u	30.35	u	29.90	u	u	u	u	u	u
	Rp 4	F	No	22.89	26.04	ns	u	u	31.95	25.61	u	28.30	u	27.16
	Rp 5	F	No	19.85	21.00	22.21	24.64	u	32.24	21.16	u	23.18	21.94	28.08
Group A-2	Rp 6	F	Yes	u	u	ns	u	u	u	u	u	u	u	u
	Rp 7	F	Yes	u	u	ns	u	u	u	u	u	u	u	u
Group B-1	Rp 8	F	Yes	u	u	31.01	u	u	u	u	u	u	u	u
	Rp 9	M		27.42	25.60	27.18	34.51	u	38.12	35.58	u	u	u	u
	Rp 10	F	Yes	u	u	34.42	u	u	u	u	u	u	u	u
	Rp 11	M		u	32.18	u	u	u	u	38.20	u	u	u	u
	Rp 12	F	No	33.67	33.22	u	u	u	u	u	u	u	u	u
	Rp 13	M		31.57	31.59	u	u	u	u	u	u	u	u	u
Group B-2	Rp 14	F	Yes	u	u	ns	u	u	u	u	u	u	u	u
	Rp 15	F	Yes	u	u	ns	u	u	u	u	u	u	u	u

Table 3.1 Challenge groups, individual chamois information (Group, ID, sex and reproductive status) and Real-time reverse transcriptase-PCR results in tissue samples. Real-time RT-PCR Results are presented in threshold cycle (Ct).The limit of detection was established at Ct value ≥ 40. u=undetected, no viral RNA was found; ns=not sampled

Effects on pregnancy and foetus

Clinical findings in pregnant females of group A (GA) were characterized by apathy as with the other chamois in the same group. GB-1 pregnant females were the only animals in this group that presented mild and temporary apathy. Moreover, Rp 10 aborted on 25 dpi. GA-2 and GB-2 pregnant females were apparently active and healthy throughout the experiment.

The post-mortem examination showed that the two foetuses from GA-1 died during the challenge. Severe placentitis was seen in both, with abundant clear haemorrhagic amniotic fluid, oedematous placenta and haemorrhagic or necrotic caruncles. Foetuses had subcutaneous gelatinous fluid and fluid-filled cavities. Foetuses from GB-1 also died during the experiment. Rp 8 had necrotic caruncles and placenta and a mummified foetus of about 7-8 cm. Rp 10 aborted on 25 dpi. In this case, a malformation of the head was evident with marked shortening of the maxilla and the mandible. Subcutaneous gelatinous fluid in the foetus and necrotizing placentitis were also noted (Fig. 3.8). In all cases of foetal death, the brain was soft and difficult to evaluate but no obvious malformation was seen.

Regarding the development of foetus, hair distribution, Crown-Rump Length (CRL), and weight, seem to indicate that GA-1 animals were in an earlier phase of development when compared with the aborted foetus from GB-1 (Table 3.2). Foetal ages based on CRL (Sivachelvan *et al.* 1996) were estimated at 70-100 days as suggested above.

Histopathological examination of brains from GA-1 and GB-1 foetuses showed similar lesions. There was a moderate to severe multifocal necrosis with mild gliosis and occasional and mild lymphohistiocitic perivascular infiltrates (Fig. 3.3). The foetus from Rp 2 also had multifocal haemorrhages in both grey and white matter and the foetus from Rp 1 had mild multifocal deposits of basophilic granular extracellular material (calcium deposits). The mummified foetus was not examined. The foetuses from GA-2/GB-2 animals did not present histopathological lesions. Histopathological lesions in the placentomes were seen in all GA-1/GB-1 pregnant

chamois (Fig. 3.9). The lesions ranged from oedema of the chorioallantoid membrane and multifocal cryptal dilation (Rp 2) to epithelial cryptal fibrinohaemorrhagic necrosis (Rp 1) to diffuse necrosis of the placentome (Rp 10) with multifocal mineralization (Rp 8).

Results of real-time RT-PCR of tissue samples from pregnant females are summarized in Table 3.1. Two chamois only presented viral RNA in the tonsils and in low quantity (Rp 8, Ct=31.01; Rp10 Ct=34.42). Foetal tissues were also assessed for viral presence (Table 3.2). Interestingly, foetuses from GA-1 presented the highest RNA load in the experiment. Real-time RT PCR values in sera from these foetuses were of Ct=14.92 and Ct=14.49 and in placenta Ct=16.74 and Ct=17.75. Also, these foetuses presented viral RNA in the brain and thymus with a lower RNA load. In one foetus from group B, viral RNA was widely distributed (placenta, brain and thymus) but with a difference of 10 Ct (equivalent to more than a 1000-fold decrease in viral load) from foetuses of GA-1. BDV RNA was not detected in either sera from this animal or in the mummified foetus from Rp 8 (only presented viral RNA in placenta; Ct=35.24). Surprisingly, a foetus from GA-2 presented viral RNA in the sera (Ct=35.24) and brain (Ct=36.19). The analysis of the 5'UTR region revealed that all foetuses except one were infected with the homologous virus inoculated in each group. The heterologous virus was detected in a foetus from a GA-2 that showed a higher phylogenetic relationship with the Freser-5 virus. This result indicates that the animal was infected before capture. All sera samples from foetuses were negative by VNT.

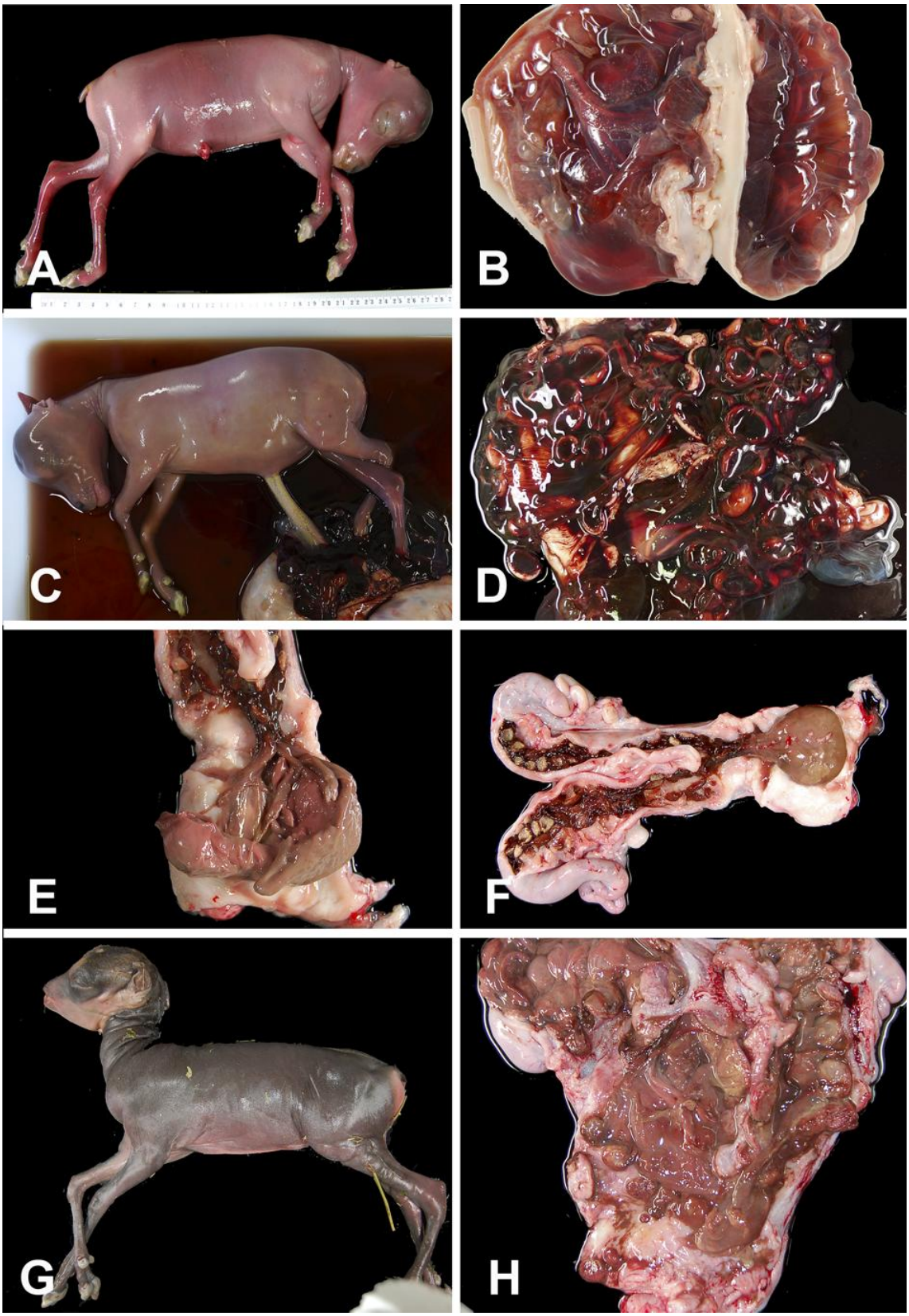

Figure 3.8 Failure of pregnancy in female adult chamois experimentally infected with Border Disease Virus-4. (**A, B**) Foetal death and placental oedema, Rp1. (**C, D**) Foetal death, haemorrhagic amniotic fluid, oedematous and haemorrhagic placentomes, Rp2. (**E, F**) Mummified foetus and necrotic caruncles and cotyledons, Rp8. (**G, H**) Foetal death and malformation – brachygnathia superior and inferior, and necrotic caruncles and cotyledons.

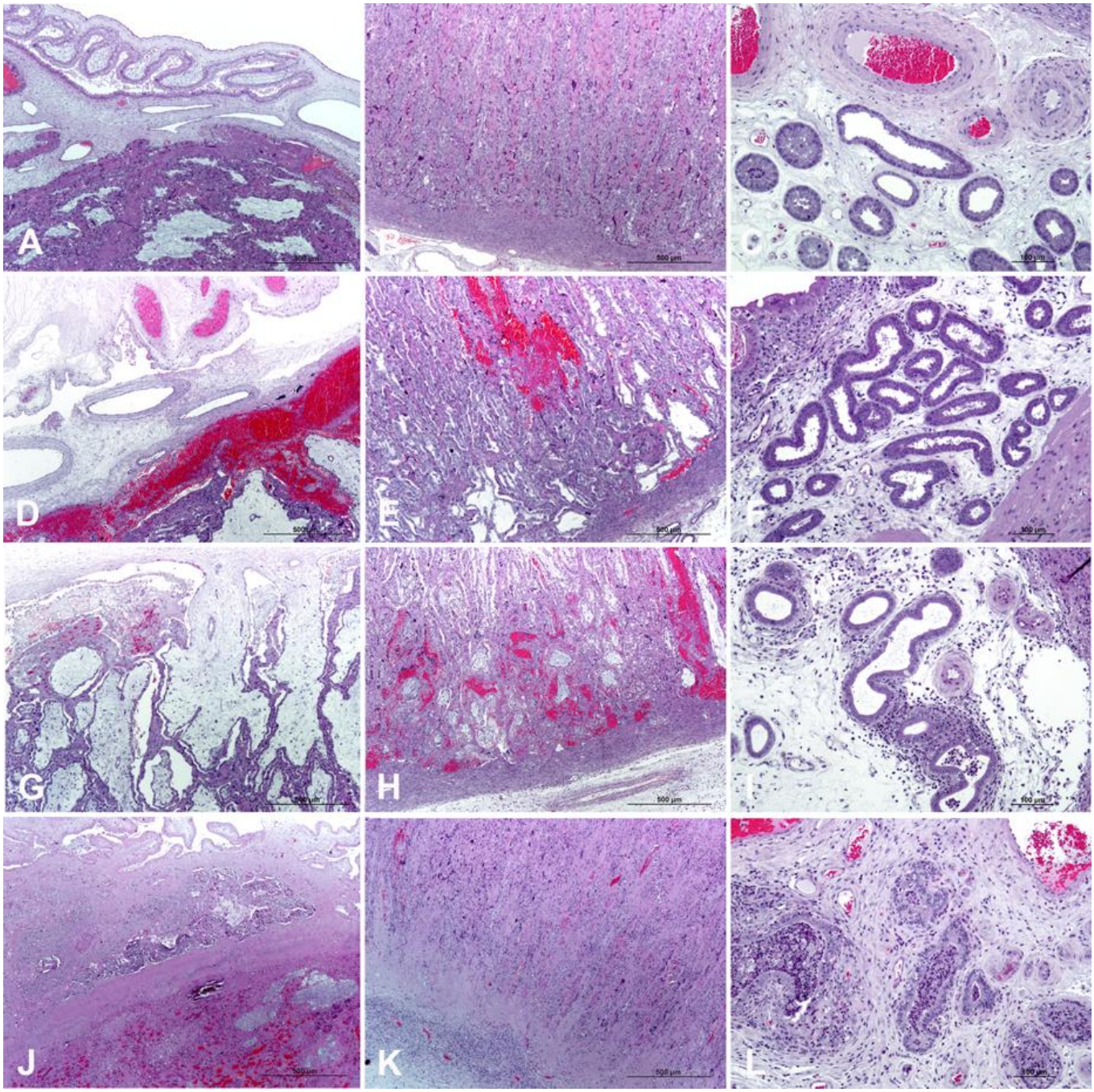

Figure 3.9 Findings in placentomes and endometrium in female adult chamois experimentally infected with Border Disease Virus-4. (**A, B, C**) Rp 15, no lesions in chorioallantoid membrane, base of the caruncle, and endometrium. (**D, F, G**) Rp 2, necrosis of chorioallantoid membrane and haemorrhages, focal haemorrhagic necrosis at the base of caruncles, and mild lymphoplasmocytic endometritis. (**G, H, I)** Rp 1, cotyledonary oedema and haemorrhage, multifocal necrosis and haemorrhages at the base of caruncles, moderate chronic suppurative endometritis. (**J, K, L**) full thickness necrosis and haemorrhages of the placentomes with mineralization and severe chronic suppurative endometritis.

Group	ID	Sex	Viability	CRL (cm)	Weight	Hair distribution	Hoof formation	VNT titres	Real-time RT-PCR (Ct)				
									Sera	Placenta	Brain	Thymus	Spleen
Group A-1	Foetus Rp 1	F	Dead	23	515	Lips, chin	Initial	0	14.92	16.74	24.57	19.45	25.84
	Foetus Rp 2	F	Dead	24	609	Lips, chin	Initial	0	14.49	17.75	23.01	29.13	u
Group A-2	Foetus Rp 6	M	Ok	30	882	Whole body	Complete	0	35.24	u	36.19	u	u
	Foetus Rp 7	F	Ok	27	597	Head	Complete	0	ns	u	u	u	u
Group B-1	Foetus Rp 8	ND	Mummified	ND	ND	ND	ND	0	ns	25.40	u	ns	u
	Foetus Rp 10	M	Aborted	30	877	Whole body	Complete	0	u	27.49	34.53	31.92	u
Group B-2	Foetus Rp 14	F	Ok	26.5	587	Whole body	Complete	0	u	u	u	u	u
	Foetus Rp 15	F	Ok	27.5	772	Head	Complete	0	ns	u	u	u	u

Table 3.2 Foetus information (Groups, ID, sex, viability, CRL (Crown-rump length), weight, hair distribution and hoof formation); Neutralizing antibody titres obtained by Virus Neutralization Test (VNT) and real-time RT-PCR results in tissue samples. Real-time RT-PCR Results are presented in threshold cycle (Ct).The limit of detection was established at Ct value ≥ 40. u=undetected, no viral RNA was found; ns=not sampled

Discussion

Clinical and pathological changes

Clinical and necropsy findings in group A (GA) were in accordance with those described previously (Cabezón *et al.*, 2011) and confirm the high level of virulence of the BDV strain Cadí-6 in Pyrenean chamois.

The high body temperature observed in all animals at the beginning of the experiment may be related to the stress from capture, handling and adaptation to captivity. GA-1 – chamois without antibodies against pestivirus at the beginning of the challenge – had an increased body temperature between 5 dpi and 12 dpi as observed before in an experimental infection with BDV in sheep (García-Pérez *et al.*, 2009). In contrast, Cabezón *et al.* (2011) reported that the higher mean body temperature in the infected group remained high when compared to the control group.

The loss of body weight in all chamois was also observed by Cabezón *et al.* (2011) in challenged and control groups, highlighting the compromise that represents captivity of wild species. However, in contrast to the above-mentioned experiment, differences between GA-1/GB-1 and GA-2/GB-2 animals were not found. Interestingly, similar trends in body weight were observed between all animals in the same box (GA and GB). This fact could be associated with differences in hierarchy and food waste in groups more than the effects of the BDV strain. In group B, the animals remained more active throughout the challenge, so more social interactions were established.

The neurological signs and the alopecia and skin hyperpigmentation described in naturally-infected chamois (Marco *et al.*, 2007) were not reproduced in the present nor in previous experimental infections. However, non-suppurative meningoencephalitis with diffuse gliosis was observed in the brain of chamois from GA-1. Interestingly, Rp 4 only had mild lesions in the brain when it died (15 dpi), which suggests that brain lesions may occur later than two weeks after infection. The lack of neurological signs in experimentally-infected chamois may be related to challenge duration and to the effect of the tranquilizers used. Alopecia and skin

hyperpigmentation in naturally-infected chamois may be due to a greater chronicity of the disease with follicular atrophy (Marco *et al.*, 2007). Hair alterations are also described in lambs infected during pregnancy with BDV demonstrating the viral tropism towards those cells (Nettleton and Willoughby, 2008).

The most remarkable pathological finding between field and experimental infections in Pyrenean chamois is haemorrhagic diathesis. In the present study, the three chamois that died before the end of the experiment presented haemorrhagic diathesis affecting mainly the serosa of the gastrointestinal tract, as described before (Cabezón *et al.*, 2011; Martin *et al.*, 2013). This haemorrhagic diathesis has been reported in other pestiviruses such as BVDV-2 and CSFV associated with thrombocytopenia (Walz *et al.*, 1999, 2001; Bautista *et al.*, 2002). Regarding BDV, lesions of haemorrhagic enteritis and fibrinous pneumonia have been described (Nettleton and Willoughby, 2008), but there are few reports in the literature that describe severe disease associated with BDV infection: the Aveyron disease in 1984 (Chappuis *et al.*, 1984), the CSFV vaccine probably contaminated with a BVDV or a BDV in 1988 in the Netherlands (Wensvoort and Terpstra, 1988) and the mortality outbreak in Spanish sheep in 1997 (Vega *et al.*, 2015). Contrary to what was observed in GA-1, clinical signs and pathological findings in GB-1 were restricted to abortion and most likely transient encephalitis and confirm the hypothetically lower virulence of strain Freser-5. This is in accordance with the mild BDV infection commonly described in horizontally-infected sheep (Nettleton *et al.*, 1998; Cabezón *et al.*, 2010b).

The decrease in the WBC and neutropaenia agreed with previously reported haematological changes in BDV infection in chamois and in other pestivirus infections (Vautrain and Gibert, 2008; Cabezón *et al.*, 2011; Martin *et al.*, 2013). However, in naturally infected chamois, lymphopaenia was the main haematological alteration (Fernández-Sirera *et al.*, 2011). Two GA-1 chamois showed no neutrophils at one sampling time, most likely associated with severe immunosuppression. This may facilitate secondary infections, frequently described in naturally-infected chamois (Marco *et al.*, 2015). Neutropaenia has been associated with suppression of granulopoiesis or decreased proliferative capacity of the bone marrow progenitor cells in highly virulent BVDV-2 (Keller *et al.*, 2006) and to apoptosis of the bone marrow neutrophil-lineage cells in CSFV infection (Summerfield *et al.*, 2001). Left-shift neutropaenia with an increase in

band neutrophils was observed in GA-1. Although this is not an typical finding in pestivirus infections, mild left-shift has been previously described (Byers *et al.*, 2009; Pypers *et al.*, 2011).

Humoral response and virology

Seroconversion was first observed in both groups at 15 dpi, which agrees with previous studies that detected it between 12 dpi and 18 dpi (Cabezón *et al.,* 2011, Martin *et al.,* 2013). The humoral response of infected chamois of GB-1 was able to clear the virus in blood at 26 dpi. The longest viraemia in these chamois was of 7 days, corresponding to the results seen previously in subclinical BDV infections in postnatal sheep and pig (Nettleton *et al.*, 1998; Thabti *et al.*, 2002; García-Pérez *et al.*, 2009; Cabezón *et al.*, 2010a,b). The humoral response in GA-1 chamois did not clear the virus in the blood. The longest viraemia was 18 days, stopped by the end of the experiment. This long-lasting viraemia has also been described in previous experimental infections in chamois, reaching 51 days in one animal (Martin *et al.,* 2013). The exceptionally long viraemia of BD in chamois when compared to other species of ruminants is of importance in BDV epidemiology as discussed below.

The chamois that had antibodies at the beginning of the challenge included in group A (GA-2) and B (GB-2) demonstrated the cross-protection between the different BDV strains. Since these chamois and the BDV strain Freser-5 used in this study belong to the same area, we hypothesize that this low-virulence strain may protect the chamois population in this area against the entrance of a more virulent strain. This also could explain the peak of antibody titres observed in GB-2 chamois that may have had an effect similar to a boost by a second infection.

The evolution of the viraemia in both infected groups agrees with viral RNA detection and quantification in tissues. On the one hand, chamois infected by the high-virulence BDV CADI-6 strain (GA) presented a wide distribution of virus in the tissues, as observed before by Cabezón *et al.* (2011) and Martin *et al.* (2013), with the highest levels in the brain, lymph nodes and lungs. On the other hand, GB showed a viral distribution mainly in lymphoid organs (i.e., lymph nodes and tonsils). The fact that these animals presented mild and most likely transient brain lesions suggests that the virus was cleared in most of the infected organs with the exception of

lymphoid tissues. As previously reported by Cabezón *et al.* (2011), the seropositive infected chamois (GA-2/GB-2) tested negative in all tissue samples.

In addition, the RNA load detected in group A was also higher than in group B, underlining differences in tropism, replication and infectious capacity of the high-virulence BDV Cadi-6 strain. Viral neurotropism observed in infected adult chamois from GB-1 was demonstrated, although the mild forms of presentation and the lack of viral RNA genome detection at the end of the experiment may indicate a clearance of virus in this organ. Many studies in pestivirus distribution in tissues pointed to the kidney as a reliable tissue for detecting the highest viral RNA loads (Hurtado *et al.*, 2009; Postel *et al.*, 2016). However, this does not agree with our study because only one chamois in GA-1 presented RNA genome in the kidney, although viral RNA was detected in urine in four out of five chamois in this group.

Effects on reproduction

In the present study both BDV-4 strains showed negative reproduction effects in pregnant females without antibodies at the beginning of the experiment (GA-1/GB.1). The BDV strain Cadí-6 infecting GA-1 was detected in high RNA loads in the foetal sera, placenta, brain, thymus and in one out of two spleens. These results agree with a previous experimental infection (Martin *et al.,* 2013) where the authors found viral genome in all studied organs. Moreover, Hurtado *et al.* (2009) presented similar results when infecting pregnant ewes with BDV and confirmed that placenta could be an interesting sample for BDV detection in pregnant ewes. Another study infecting ewes with BVDV-2 found high viral antigen presence in placentomes. The authors suggested that replication in placentomes preceded and was possibly required for congenital spread of the virus (Scherer *et al.,* 2001). Despite showing lower viral RNA loads, the placenta, brain and thymus of at least one of the foetuses from GB-1 presented viral RNA. In addition, all foetuses from GA-1 and GB-1 chamois had microscopical lesions in placentomes and the brain indicating that both strains caused foetal death. The moment when BDV foetal infection occurs determines reproductive success and foetal development (Nettleton *et al.*, 1998; Schweizer and Peterhans, 2014). In the present study, it is likely that at the time of inoculation the foetus have reached the immunological competence and that abortion was feasible as described in other BDV infections (Loken *et al.,* 1995; Nettleton *et al.,* 1998). The BDV strain

Cadí-6 caused foetal death with a haemorrhagic diathesis also affecting the reproductive organs and the foetus itself. The Freser-5 strain caused mild lesions in pregnant females but caused necrosis of the placentomes and foetal death resulting in mummification or abortion of the foetuses. Interestingly, one seropositive pregnant female (GA-2) had a viropositive foetus without antibodies against pestivirus, which could indicate that this animal was a PI. The existence of this epidemiological feature in chamois has not been demonstrated in the wild, but has been suggested in some studies and demonstrated in an experimental infection with a single chamois (Vautrain and Gibert*,* 2008, Marco *et al.*, 2012, Marco *et al.,* 2015, Beauneé *et al.,* 2015). The Freser-Setcases NHR, where the chamois were captured, is characterized by high BDV seroprevalence, low-virulence virus circulation and increasing population numbers without BDV cases or outbreaks. This supports the hypothesis that viral persistence in this area is determined by PI animals and that the pathogenesis is similar to that of BDV infections in domestic ruminants.

Viral shedding and epidemiology implications

Constant shedding of BDV-4 was detected by RT-qPCR in nasals swabs in GA-1 chamois from 8 dpi onwards. The same viral excretion was observed in previous experimental infections of chamois (Cabezón *et al.,* 2011; Martin *et al.,* 2013). Lower shedding duration was detected in rectal swabs when compared with the results of the two previous experimental infections in chamois. The most interesting findings in viral shedding were seen when comparing the two studied strains. Only in five samples from three GB-1 chamois was viral genome detected, between 12 dpi and 15 dpi and in much lower RNA loads than in GA-1. Moreover, no viral RNA was detected in rectal swabs during the experiment in the group infected with the low-virulence strain Freser-5. These differences were observed also in urine RT-qPCR analysis, where four out of five chamois of GA-1 presented viral shedding and none of the GB-1 chamois showed viral presence. The fact that a GA-2 animal presented viral RNA in nasal swabs at 15 dpi may be attributed to a timely virus replication in the nasal cavity due to viral transmission from a GA-1 viraemic chamois. Chamois Rp 3 in GA-1 only shed once at 12 dpi through the nasal and rectal routes, with a lower viral load than the rest of the group. These differences were also observed in viral RNA distribution, only found in lymphoid tissues and sera, only on two sampling days. Also, the same chamois presented mild microscopic brain

lesions, with few multifocal glial nodules, while the other chamois infected with the same strain presented more severe brain lesions. The evolution of infection seen in this chamois was similar to that previously described in an experimentally-infected chamois that was able to clear the virus (Cabezón *et al.*, 2011). These cases showed the importance of individual differences and suggest that a low percentage of chamois may overcome infection with a high-virulence BDV strain. Genetic diversity of wild populations may shed some light on that issue, as suggested previously (Cavallero *et al.,* 2012).

These results are essential for the management of chamois populations. The knowledge of the circulating strains in a particular area and the seroprevalence at the population level may provide some clues to predict the epidemiology of the infection and the risks of disease outbreaks. In some cases, the efforts to eliminate BDV in wild populations could be counterproductive if a high-virulence strain invades these areas with a naive population. Although it provide useful information , the 5'UTR sequence is the only fragment for BDV phylogenetic analyses and is not sufficient to observe clades grouped by virulence, as has been seen in other pestiviruses (Leifer *et al.* 2013). Continuing with this approach, the genetic relationships of different strains such as the recent BDV from the genogroup 8 that was reported to cause mortality in chamois, may be of concern. Phylogenetic investigations on other regions of the genome (i.e., E2) could shed light on viral strain virulence factors and help in the virological understanding of BDV implications in livestock and wildlife.

Conclusions

The present study highlights the pathological and epidemiological implications of two close phylogenetically-related strains. On the one hand, the Cadí-6 virus was confirmed to be a high-virulence BDV strain, inducing long-lasting viraemia, with wide tissue distribution, brain lesions and foetal death. The long-lasting viraemia and the high RNA loads detected mainly in nasal swabs may indicate the possibility of horizontal transmission in severe outbreaks. On the other hand, the Freser-5 strain has been confirmed as a low-virulence strain for chamois, despite inducing foetal death. This strain of BDV may persist in the chamois population through PI animals, similar to BD in sheep. Moreover, the effective cross-protection of chamois infected with the low-virulence strain (Freser-5) in the face of the high-virulence strains (Cadí-6) is of

importance in pestivirus epidemiology. This fact, together with the diverse epidemiological scenarios seen in the field, strongly suggests that infections with a low-virulence strain prevent the chamois population from disease outbreaks caused by a virulent BDV strain. The present study highlights that BDV strain diversity plays a key role in the epidemiological heterogeneity of pestivirus infections in chamois populations.

4. Study II

The European hare (*Lepus europaeus*) and Alpine marmot (*Marmota marmota*) as potential wild reservoirs for ruminant pestiviruses

Alpine marmot study has been added modifying the scientific publication:

Colom-Cadena, A.; Cabezón, O.; Rosell, R.; Fernández-Aguilar, X.;, Blanch-Lázaro, B.; Tetas, E.; Lavín, S.; Marco, I. (2016) The European hare (*Lepus europaeus*) as a potential wild reservoir for ruminant pestiviruses. Preventive Veterinary Medicine 131 60–63

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Introduction

The epidemiology of pestiviruses (Fam. *Flaviviridae*) in free-ranging multi-species systems is far to be understood. Their capability to cross the species barrier and overcome the host immune response lead to diverse epidemiological situations that may be modulated by several factors such as climatology, hosts densities or interactions with other pathogens (Fernández-Sirera *et al.*, 2012b; Schweizer and Peterhans, 2014).

Ruminant pestivirus species, Bovine Viral Diarrhoea Virus (BVDV) type 1 and type 2, and Border Disease Virus (BDV) have been described affecting several wild and domestic artiodactyl species (Passler and Walz, 2010; Ridpath, 2010; Vilček and Nettleton, 2006). This wide range of potential hosts, the high mutation rate of the virus and its capability to originate persistently infected (PI) animals allow these viruses to persist in ruminant populations (Dubois et al., 2008; Nettleton *et al.*, 1998). Reports on potential wild reservoirs and the confirmation of independent domestic and sylvatic cycles of pestivirus infections has led to develop more studies on pestivirus presence in wildlife (Passler and Walz, 2010; Marco *et al.*, 2009a). Hence, this host diversity has become of importance mainly in countries where BVDV plans are implemented (Casaubon *et al.,* 2012).

Moreover, the interest of potential non-ungulate hosts for pestivirus has increased. BVDV and BDV studies have indentified in last years the European rabbit (*Oryctolagus cuniculus*) (Frölich and Streich, 1998; Bachofen *et al.*, 2014; Grant *et al.*, 2015) and Bennett's Wallaby (Munday, 1972) as free-ranging pestivirus susceptible hosts. In addition, Seong *et al.* (2015) reported susceptibility in mice (*Mus musculus*) experimentally infected with BVDV.

Since 2001, the populations of the Pyrenean chamois (*Rupicapra p. pyrenaica*) (NE- Spain, Andorra and SE-France) have suffered outbreaks of disease causing losses up to the 80% in some populations (Marco *et al.*, 2009b). The etiological agent of these infections was classified into the BDV-4 genogroup (Arnal *et al.*, 2004). Fifteen years after the first outbreak, pestivirus are still circulating among Pyrenean chamois, suggesting an endemic and asymptomatic virus circulation in some areas (Marco *et al.*, 2015). Although Pyrenean chamois' sympatric ruminant species have been suspected to be involved in the maintenance of the virus in the Pyrenees, low antibody

seroprevalences, absence of PI individuals and no clinical infections have been reported (Fernández-Sirera *et al.*, 2012b).

In order to amplify the knowledge of the potential role of sympatric species in the maintenance of pestivirus in the ecosystem, epidemiological research of pestivirus focused in non-ruminant species, such as the European hare (*Lepus europaeus*) and the Alpine marmot (*Marmota marmota*), commonly and widely distributed in alpine pastures (Smith and Johnston, 2008; Ballesteros, 2012; Cassola, 2016), is of interest. The main goal of the present work was to assess the pestivirus exposure in the European hare from alpine and non alpine areas, and in Alpine marmot in an alpine area.

Materials and methods

Blood samples were obtained by intracardiac puncture from 94 hunted European hares. All hares were sampled in the North of Catalonia, North-Eastern Spain and collected by wildlife-rangers or veterinarians from carcasses of hunted hares, legally hunted during the period from October to February 2014–2015. Within this region, two areas were differentiated according to ecosystem characteristics: Pyrenean (alpine and subalpine ecosystems) versus Non Pyrenean (non alpine and subalpine ecosystems) areas (Fig. 4.1). Alpine marmots were captured by means of two-door live traps baited with dandelions (*Taraxacum densleonis*). Traps were placed near the entrance of the main burrow of each group. Each individual captured was tranquilized using Zoletil 100 (0.1ml/Kg), and blood samples were obtained by femoral venipuncture. Marmots were captured in the Pyrenees (Cadí-Alt Urgell NHR), in an area were the Pyrenean chamois population suffered the highest mortality by pestivirus infection ever described (a drop of 80% in 2005) (Marco *et al.* 2009b) (Fig. 4.1). A total of 49 Alpine marmot samples were included in the present study. Blood from hares and marmots were placed in sterile serum separator tubes and centrifuged at 1200g for 15 min. Sera were stored at −20ºC until analysed.

Sera were tested for the presence of neutralizing antibodies against pestivirus by means of the Virus Neutralization Test (VNT). The test was performed following the procedure described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2008) using Madin-Darby bovine kidney (MDBK) cells. Neutralizing antibody titres were expressed as the reciprocal of the highest dilution that neutralized 100 tissue culture infective doses (100 $TCID_{50}$)

in all cultures, calculated according to Reed and Muench's method (Reed and Muench, 1938). Titers of 1:10 and higher were considered positive. Viral replication was monitored by the Immuno-Peroxidase Monolayer Assay (IPMA). VNT was performed against a reference pestivirus BVDV1 strain NADL (Acc. No. M31182) and a BDV-4 isolated from chamois (BDV Cadi-6; Acc. No. AM905924). As we expected to find BDV infection in Pyrenean areas, significance between both viruses was established when changes in titres were up to two or more folds (OIE, 2008).

The presence of viral RNA in sera samples was assessed by Reverse transcription-polymerase chain reaction (RT-PCR). The RNA was extracted using a commercial kit (Nucleospin Viral RNA Isolation, Macherey-Nagel, Düren, Germany). The RT-PCR was performed using previously described panpestivirus primers 324 and 326 (Vilček *et al.*, 1994; Vilček and Belák, 1996) and a commercial kit (One-Step PCR kit, Qiagen, Hilden, Germany).

Statistical analyses to determine significant differences between pestivirus prevalence between areas were carried out with a contingency table Chi-square test. The statistic was assessed by means of the statistical software R version 3.2.2 (R Development Core Team, 2014).

Results

A total of 34 out of 94 (36.2%; $CI_{95\%}$: 26%–46%) sera from European hares presented neutralizing antibodies against pestivirus (Table 4.1). Significant differences were found between BVDV-1 and BDV-4 titres in 3 and 4 samples respectively. Twenty seven sera presented antibodies classified as compatible with both ruminant pestiviruses as no significant differences were found between titres. Titres of antibodies in front of ruminant pestiviruses ranged between 1/20 and 1/160 $TCID_{50}$ (Table 4.2). Seroprevalences in the Pyrenean area (43.1%; $CI_{95\%}$: 30%–56%) had no statistically significant differences when comparing to Non-Pyrenean area (25%; CI_{95} 10%–40%) (Table 1) by means of Chi-square test (p-value=0.076). No neutralizing antibodies were found in Alpine marmot sera samples (Fig. 4.1). RT-PCR analysis of the 94 sera samples from European hares and of the 49 sera samples of Alpine marmot resulted negative.

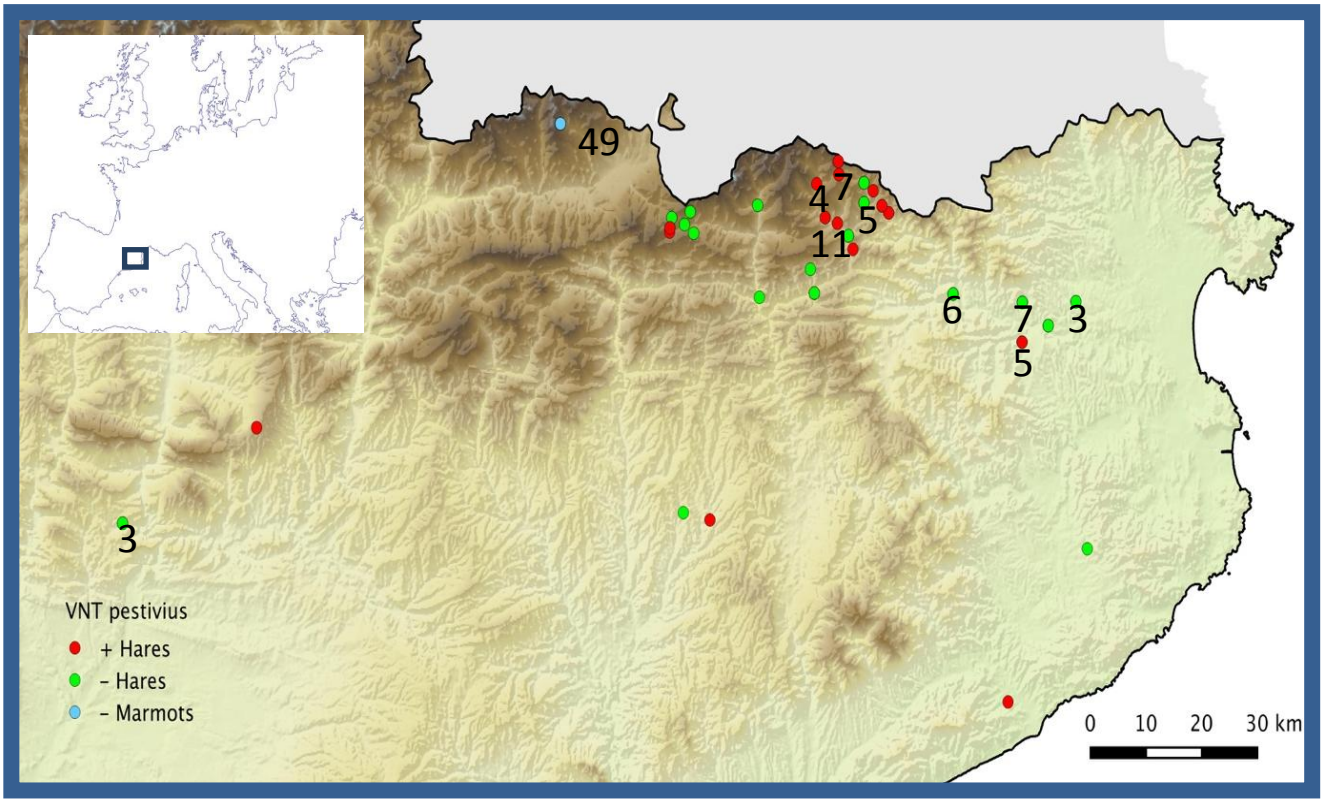

Figure 4.1 Spatial distribution of sera samples from European hares and Alpine marmots. The numbers represent samples with the same geographical coordinates or too close to be differentiated on the scale map, and with the same result in Virus Neutralization Test.

Zone	N	SNT positive (%)
Pyrenees (Regions: *Ripollès, Alta cerdanya, Baixa cerdanya, Berguedà*)	58	25 (43.1)
Non Pyrenees (Regions: *Garrotxa, Pla de l'estany, Maresme, Bages, Osona, Alt Empordà, Gironès, Noguera*)	36	9 (25)
Total	94	34 (36.2)

Table 4.1 Results of the presence of antibodies against pestivirus (BVDV-1 and BDV-4) in European hare (*Lepus europaeus*) by means of Virus Neutralization Test.

ID	BDV4 [a]	BVDV1 [b]	ID	BDV4	BVDV1
LE14003 (P)	20	0	LE15008 (P)	0	20
LE14004 (P)	40[c]	0	LE15013 (NP)	80[d]	0
LE14006 (P)	40[c]	0	LE15020 (P)	0	20
LE14009 (P)	20	20	LE15024 (NP)	0	20
LE14012 (P)	20	40	LE15025 (NP)	0	20
LE14015 (NP)	0	20	LE15039 (NP)	0	10
LE14025 (P)	40	20	LE15040 (P)	40	NL
LE14026 (P)	0	20	LE15041 (P)	0	40[c]
LE14027 (P)	0	20	LE15042 (P)	0	20
LE14028 (P)	40	40	LE15043 (P)	0	10
LE14029 (P)	0	10	LE15045 (P)	80[d]	0
LE14030 (P)	0	20	LE15052 (P)	20	0
LE14032 (NP)	20	0	LE15057 (P)	0	10
LE15001 (NP)	0	10	LE15058 (P)	160	160
LE15004 (P)	0	40[C]	LE15060 (P)	40	40
LE15005 (P)	0	20	LE15063 (NP)	20	0
LE15007 (P)	0	80[d]	LE15065 (NP)	20	20

NL: not lecture; P: Pyrenean region; NP: non-Pyrenean region
[a] BVDV-1 NADL, Acc. No. M31182
[b] BDV-4 isolated from chamois (BDV-Cadi6; Acc. No. AM905924)
[c]Two folds difference.
[d]Three folds difference.

Table 4.2 Results of antibody titres obtained by Virus Neutralization Test from positive European hares.

Discussion

Pestiviruses cause important economic losses in livestock (Ridpath, 2010) and the importance of sympatric wildlife has been widely studied (Vilček and Nettleton, 2006). It is essential to consider wild hosts in epidemiological studies of pestivirus and especially for the implementation of eradication plans (Stahl and Alenius, 2012). In the Pyrenees, the epidemiological role of wild hosts has the additional value of understanding the BDV cycle in alpine areas, associated with high mortality outbreaks in Pyrenean chamois. Although several alpine artiodactyls have been suspected to play a role in the epidemiology of chamois viral disease (Fernández-Sirera *et al.*,

2012b) for the moment none has clearly been identified to participate in a hypothetic sylvatic cycle of pestivirus in the Pyrenees.

The data reported in this study underline the importance to increase the knowledge about the role of non-artiodactyl species in pestivirus infections. Since only two wild species of not even-toed animals have been reported to date having antibodies against pestivirus (Munday, 1972; Frölich and Streich, 1998; Grant *et al.*, 2015;) the results obtained in the present study supposes the inclusion of the European hare as the third free-ranging non-artiodactyl species being susceptible to pestivirus infection.

The positive serological results in European hares are similar with those reported in free-ranging European rabbits by Frölich and Streich (1998). However, in contrast with this last study, Grant *et al.* (2015) presented a serological analysis of rabbits from Scotland and Northern England with a very low seroprevalence (1.2%). The high seroprevalence observed in our study could be related to a higher pestivirus circulation in the study areas. The Pyrenean chamois populations of this alpine area show high prevalence of antibodies against BDV, strongly suggesting a sylvatic cycle and a BDV circulation (Marco *et al.*, 2009a). In addition, despite the lack of specific surveillance program of pestivirus in livestock from the study area, some authors confirmed a high prevalence of antibodies against pestiviruses in cattle and sheep, and along with the high reported densities of domestic ruminants in the alpine pastures represents a chance for virus maintenance (Alba *et al.*, 2008; Marco *et al.*, 2009a; Fernández-Sirera *et al.*, 2012a,b).

The titres of neutralizing antibodies observed in our study were low (1/20–1/160 $TCID_{50}$). However, Bachofen *et al.* (2014) reported similar titres (1/11–1/32) of neutralizing antibodies in experimentally BVDV infected rabbits by intravenous and oronasally routes, and by contaminated hay.

Molecular analyses of the sera from the European hares did not detect pestivirus RNA, similarly to previous studies from free-ranging lagomorphs (Frölich and Streich, 1998). In this sense, due to the high infection rate observed in this study, further molecular research on lymphoid tissues or spleen homogenates would provide more information regarding the virus strain and its pathogenesis. However, experimental pestivirus infection in species other than ungulates such as mice (Seong *et al.*, 2015) reported the infection in several tissues showing significant histopathological changes; but they did not evaluate antibody production during infection.

Moreover, Grant *et al.* (2015) demonstrated trans-placental infection of the offspring after pregnant rabbit infection, which potentially enables important epidemiological figures for pestivirus persistence within populations. In the present study we tried to detect antibodies in another rodent, the Alpine marmot, supported by the fact that pestivirus infection was reliably established in other rodent species, although no positive results were found in antibody or viral genome detection analyses. The abovementioned studies and our results indicate that some lagomorphs and rodents are susceptible hosts for pestivirus infection and could play a role in the epidemiology of ruminant pestiviruses. We couldn't demonstrate virus excretion and the experiments in rabbits and mice did not clearly show this process, so more studies of pestivirus infection and excretion in lagomorphs and rodents could give light in viral maintenance in the field. Moreover, regarding Alpine marmot sampling at the present study, the analysis of samples from other areas in Pyrenees could report different scenarios. The high seroprevalences presented here may indicate virus transmission between European hares and hypothetical maintenance into wild hares populations.

The results shown in the present study indicate that the European hare is susceptible to pestivirus infection and that could be involved in the epidemiology of ruminant pestiviruses. More field research and experimental studies are needed to evaluate the role of European hare and Alpine marmot in pestivirus epidemiology.

5. Study III

New insights on pestivirus maintenance in transhumant sheep and sympatric Pyrenean chamois (*Rupicapra p. pyrenaica*)

Introduction

Pastoral practices are of importance in the contact between domestic species and between domestic and wild species. Some studies have focused on the transhumance in order to control parasitic and infectious diseases (Eckert and Hertzberg, 1994; Macpherson, 1995). In this context, viral infections caused by pestivirus are of concern in alpine meadows (Braun *et al.*, 1998; Presi and Heim, 2010).

Border Disease Virus (BDV) belongs to the genus *Pestivirus* (Fam. *Flaviviridae*). Postnatal BDV infections in sheep are usually mild with pyrexia and transient lymphopenia. Infections in pregnant ewes before immunocompetence of the foetus can cause abortions and mummifications, but also the birth of a persistently infected (PI) offspring. The latter are characterized by specific BDV immunotolerance, absence of antibodies, and shedding of virus throughout their life. PI animals play a key role in pestivirus epidemiology (Nettleton *et al.*, 1998).

BDV infection has also been associated with high mortality rates in sheep from the Aveyron region, France (Chappuis *et al.*, 1984) and from northeastern Spain (Vega *et al.*, 2015). BDV and BVDV infections have been described to affect over 50 species of free-ranging wild and domestic artiodactyl species (Vilček and Nettleton, 2006). Thus, the pestivirus known as "ruminant pestivirus" (BVDV, BDV) is not species-specific and the role of different species in its maintenance is of concern, especially for BVDV eradication plans (Casaubon *et al.*, 2012).

Aside from the impact on livestock, ruminant pestivirus has been of importance in wildlife population health surveillance since 2001, when high mortality outbreaks caused by BDV-4 were described in the Pyrenean chamois (*Rupicapra p. pyrenaica*) at the border between France, Spain and Andorra (Marco *et al.*, 2009b).

Pestivirus transmission cycles in domestic and wildlife species are of importance in the epidemiology of infections. Pestivirus transmission between domestic species (Krametter-Froetscher *et al.*, 2007b; Braun *et al.*, 2013b), intraspecific maintenance of BVDV in white-tailed deer (*Odocoileus virginianus*) (Passler and Walz, 2010) and of BDV in Pyrenean chamois (Marco *et al.*, 2009a; Fernández-Sirera *et al.*, 2012b) have been confirmed. Less typical is the interspecific

transmission between domestic and wildlife ruminants although this has been demonstrated in a few cases (Casaubon *et al.*, 2012; Martin *et al.*, 2015; Passler *et al.*, 2016).

In the Pyrenees, the susceptibility of Pyrenean chamois to BDV infections highlights the importance of understanding the role of sympatric livestock in the epidemiology of pestivirus. Different epidemiological scenarios have been described in Pyrenean chamois populations (Fernández-Sirera *et al.*, 2012b; Marco *et al.*, 2015) identifying areas where BDV is ruled by frequent epizooties and others where BDV is endemic but no epizootic outbreaks occur. In these ecosystems, the role of transhumant sheep is still unclear.

In order to shed light on that issue, we developed an epidemiological study of transhumant sheep herds that share grazing areas with Pyrenean chamois in two different epidemiological scenarios. The main goal was to determine if both sheep and chamois are capable of sustaining independent cycles of pestivirus infection.

Materials and Methods

Five transhumant sheep farms in the Central and Eastern Pyrenees were included in the study (Fig. 5.1). Three of them (Farm-1-3) grazed seasonally in the Val d'Aran and Pallars Sobirà counties (zone A) where BDV infection in chamois is frequent and high mortalities have been reported since 2001. This population has not yet reached the densities of chamois before the first outbreak. Two other transhumant farms (Farm-4 and -5) are in the Freser-Setcases National Hunting Reserve (zone B), a high chamois-density area where BDV is highly prevalent in chamois but where no mortality outbreaks linked to the virus have been reported to date.

A total of 751 sheep (347 lambs, 404 adults) were sampled before grazing in alpine meadows (April-May) and sampling was repeated when all were stabled again at the farm of origin (October - November). In each farm, similar numbers of adults that had been in alpine pastures previously and lambs that had never been off the farm were selected (Table 5.1). The sampling protocol was carried out in 2014 in three farms (Farm-2, -4, -5) (first transhumant season), and in 2014 and 2015 in Farm-1 and -3 (first and second transhumant season). Blood samples were extracted from the jugular vein by venipuncture. Samples were centrifuged at 1200g for 15 minutes and sera were stored at -20ºC until analysis. Age and location of farms and grazing areas

were recorded and each animal was identified for re-sampling after transhumant periods. In farms where some animals could not be re-sampled (because of death or loss), other sheep with the same age condition were included to maintain the sampling size. Animals sampled twice, before and after transhumance, represented 86.4% of the total in the first season and 66.2% in the second season.

Samples of Pyrenean chamois from zones A and B where obtained during the hunting season (September to February) during three consecutive years (2013-2015). Samples consisted of sera and/or spleen tissue. Blood samples from hunted animals were obtained by intracardiac puncture and placed and processed as described before in sheep samples. A total of 421 chamois were included in the present study.

All sera samples were tested for the presence of antibodies against pestivirus using a commercial competition ELISA assay (BVD/MD/BD P80 Ab, IDEXX, Montpellier, France). Positive sera were tested with a comparative virus neutralization test (VNT) for the detection of neutralizing antibodies against the pestivirus strains BVDV-1 NADL, two BDV strains of domestic animal origin from Spain, BDV-5 "Esp-97" and BDV-4 "pig-SP-2007", and a chamois BDV-4 strain different for each farm depending on the alpine meadows used in the transhumance (zone A: BDV-4 "ARAN-15" and BDV-4 "PALLARS-8"; zone B: BDV-4 "FRESER-5"). These BDV-4 chamois strains were isolated from BDV viraemic Pyrenean chamois between 2014 and 2016. A Virus Neutralization Test was performed using procedures described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2008) with Madin–Darby bovine kidney (MDBK) cells. Neutralizing antibody titres (positive ≥ 1:10) were expressed as the reciprocal of the highest dilution that neutralized 100 tissue culture infective doses (100 $TCID_{50}$) in all cultures (Reed and Muench, 1938). Viral replication was monitored by the Immuno-Peroxidase Monolayer Assay (IPMA) using a home-made polyclonal pestivirus-specific serum.

Total viral RNA was extracted directly from 200 µl of sera samples using a MagAttract 96 *cador* Pathogen Kit (Qiagen, Venlo, Netherlands) as per manufacturer's instructions. A one-step reverse transcription-PCR kit was used with SYBR® Green-based real-time PCR (Thermo-fisher Scientific, Waltham, Massachsetts, USA). Positive results were considered for threshold cycle values (Ct) less than 40. Samples in which fluorescence was undetectable were considered

negative. The primers 324 and 326 were used for the amplification reaction (Vilček *et al.*, 1994; 1996).

The positive PCR sample was inoculated in Madin-Darby bovine kidney (MDBK) cells in order to isolate the virus. Single passage was performed and the isolate was stored at −80°C. The isolated virus was sequenced and the 5' untranslated region (5' UTR) was characterized. The phylogenetic tree was calculated by the neighbour-joining method using an automatic root location. To test the reliability of the branches in the tree, a bootstrap analysis of 1000 replicates was performed by creating a series of randomly selected bootstrap samples.

True prevalences and confidence intervals (CI) for single proportions were calculated in all the farms before and after transhumance, and in lambs and adults in the highly seropositive farm. Statistical analyses to determine significant prevalence differences before and after transhumance, and between ages (Farm-1) were carried out with a contingency table chi-square test. Each sera titre obtained by VNT was checked individually to detect three-fold differences against different strains. Titre differences at the flock level were pairwise compared with the Wilcoxon signed-rank test. The limit of significance was defined as $P \leq 0.05$. The statistical analyses were assessed by means of the statistical software R version 3.4.0 (R Development Core Team, 2016).

Results

The seroprevalence in sheep farms obtained by detecting specific antibodies against pestivirus is presented in Table 5.1. Overall seroprevalence of pestivirus antibodies ranged between 0 and 91.1% at the flock level from the zone A grazing area and between 4 and 16.7% in zone B. Specific antibodies against pestivirus were found in Pyrenean chamois in the two studied areas and the three consecutive years (Fig. 5.1). Prevalence by zone and year were: in zone A in 2013 (77.7%; 95% CI: 59.2-89.4), 2014 (69.2%; 95% CI: 50.0-83.5) and 2015 (66.7%; 95% CI: 39.6-86.2); in zone B in 2013 (53.9%; 95% CI: 44.3-63.3), 2014 (52.9%; 95% CI: 44.6-60.9) and 2015 (57.9%; 95% CI: 48.7-66.6).

In the first season, statistically significant differences in prevalence before and after transhumance at the flock level were found only in Farm-1 ($P<0.0001$), while for the other four

farms no seroconversion upon return from alpine meadows was detected (Fig. 5.2a). In light of these results, a second season included only Farm-1 and a second farm as a control (Farm-3). Statistically significant differences were detected when comparing seroprevalences before and after the second transhumance season at the flock level from Farm-1 (P=0.013), as they increased from 69.6% (95% CI: 58.0-79.2) to 91.1% (95% CI: 75.3-96.5) when returning from transhumance.

Differences in seroprevalences associated with age were found in Farm-1. The lambs presented statistically significantly higher antibodies after transhumance in the first season (P<0.0001) and in the second one (P=0.002) (Fig. 5.2b).

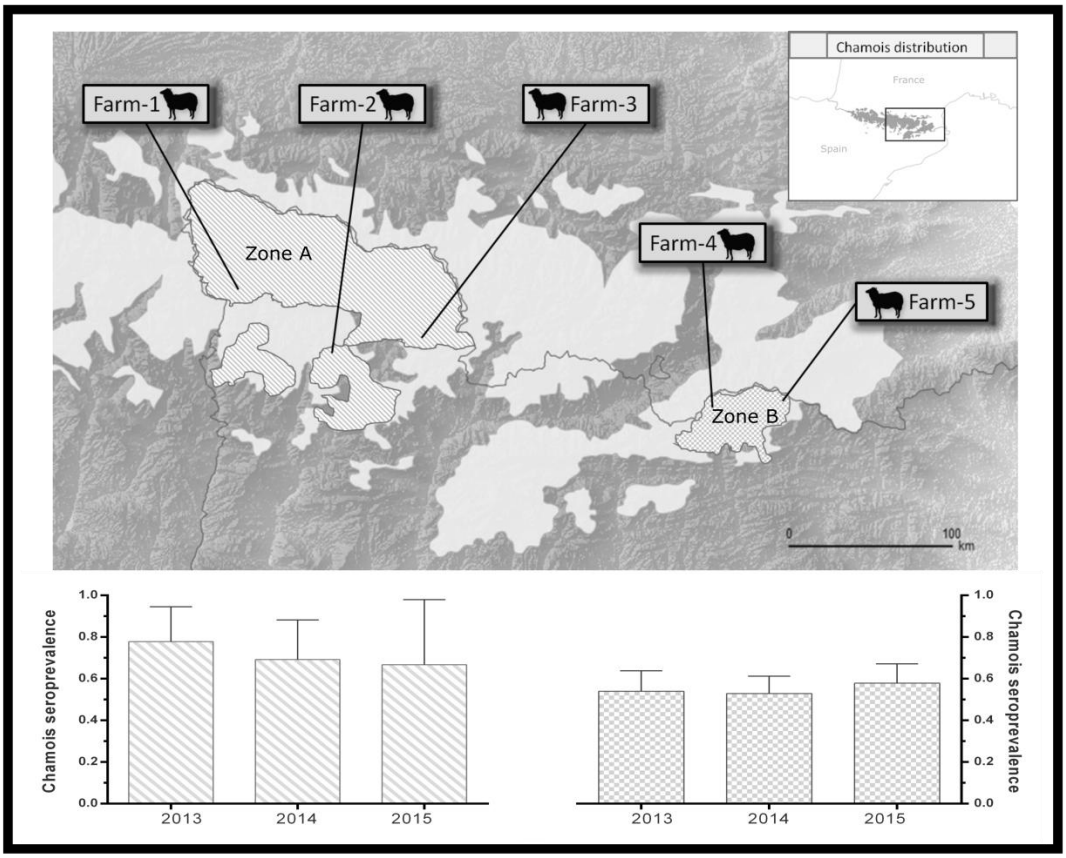

Figure 5.1 Map showing the distribution of Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*), sheep farms and boxplots of chamois pestivirus seroprevalence. The area where chamois sera and spleen were sampled and the seroprevalence of this population represented in boxplots are shown in stripes for the Alt-Pallars area (zone A) and squares for Freser-Setcases NHR area (zone B).

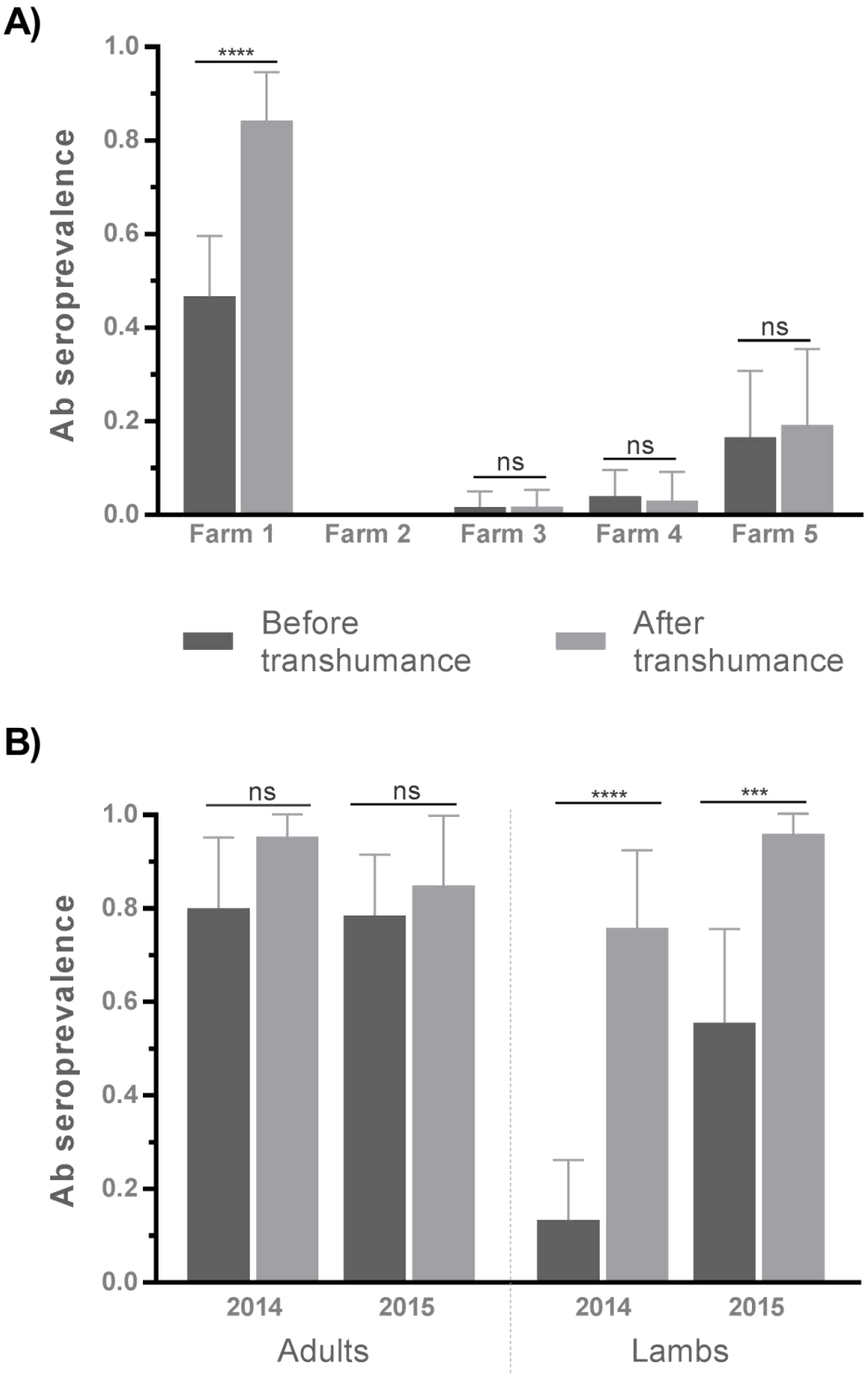

Figure 5.2 Antibody seroprevalences with 95% CI with differences indicated as statistically significant *** (P<0.005) or not (NS) in **A)** Studied farms before and after transhumance during the first season, and in **B)** Farm-1 during two transhumant seasons with seroprevalences presented separately by age (lambs and adults).

Mean, median and range of antibody titres against each strain obtained by VNT are summarized in Table 5.2. In Farm-1, VNT was performed for 159 sera. Three-fold differences in titres were found mainly in domestic BDV-4 strains (27 sera), BDV-4 strains vs BVDV (11 sera) and BVDV-1 NADL (2 sera). Statistically significant differences ($P<0.05$) between pairwise comparisons were made at the flock level by time (before and after transhumance) and in the two seasons (a total of 4 sampling moments). Significance was established between BDV-4 chamois and BDV-4 livestock strains in all time points and between BVDV-1 and BDV-4 livestock strains in all time points with the exception of post-transhumance samples in the second season. In the other three farms, VNT was performed in 14 sera. Significantly higher titres of BDV-4 domestic strains were found in Farm-3 (one out of four sera) and of BDV-4 chamois strain (two out of three sera) in Farm-5.

From all 751 sheep sera, only one sample was positive for virus detection by means of PCR and the 5'UTR region was sequenced. This sample belonged to a lamb from Farm-1, sampled before the second transhumance season, but it was not possible to isolate it in cell culture and use it for VNT. This positive sample establishes the prevalence of virus presence the Farm-1 at 0.4%. From all 421 sera and spleen samples from chamois, four were PCR positive. With this data, the virus prevalence in the chamois population from 2013 to 2015 was 4.6% (IC 95%: 1.6-12.7) in zone A and 0.3% (IC 95%: 0.01-1.6) in zone B. The 5'UTR region of the four chamois strains were sequenced. The 5'UTR sequence of these viruses, when compared to previously selected strains from GenBank, classified them into the BDV-4a subgroup. Moreover, geographical distribution patterns can be observed in chamois sequences (Fig. 5.3).

		Before transhumance				After transhumance			
		N	ELISA-ab Prev % (95% CI)			N	ELISA-ab Prev % (95% CI)		
	Farm size	(Lamb/Adult)	Farm	Lambs	Adults	(Lamb/Adult)	Farm	Lambs	Adults
First season									
Zone A Farm-1	4200	60 (30/30)	46.7* (34.6-59.1)	13.3 (5.3-29.7)	80.0 (62.7-90.5)	51 (29/22)	84.3* (72.1-92.0)	75.9** (57.9-87.8)	95.5 (78.2-99.8)
Zone A Farm-2	800	58 (29/29)	0	0	0	59 (30/29)	0	0	0
Zone A Farm-3	600	59 (31/28)	1.6 (0.9-8.9)	0	3.6 (0.2-17.8)	56 (28/28)	1.8 (0.9-9)	0	3.6 (0.2-17.8)
Zone B Farm-4	500	50 20/30	4.0 (1.1-13.5)	0	6.7 (1.8-21.3)	33 (12/21)	3.0 (0.2-15.3)	0	4.8 (0.2-22.7)
Zone B Farm-5	50	30 (15/15)	16.7 (7.3-33.6)	13.3 (3.7-37.9)	20.0 (7.0-45.2)	26 (14/12)	19.2 (8.5-37.9)	14.3 (4.0-39.9)	25.0 (8.9-53.2)
Second season									
Zone A Farm-1	4200	69 (27/42)	69.6* (58.0-79.2)	55.6 (37.3-72.4)	78.6 (64.1-88.3)	45 (25/20)	91.1* (79.3-96.5)	96.0** (80.5-99.8)	85.0 (63.9-94.8)
Zone B Farm-3	600	85 (33/52)	1.2 (0.06-6.4)	0	1.9 (0.1-10.1)	70 (24/46)	1.4 (0.07-7.7)	0	2.2 (0.1-11.3)

Table 5.1 Seroprevalences of antibodies against pestivirus obtained by ELISA in all sampled sheep farms and periods. * Statistically significant differences before and after transhumance in flock level prevalence were found in Farm-1 in the first season (P<0.0001) and the second one (P=0.013). Also in Farm-1, lambs presented statistically significantly higher antibodies (**) after transhumance in the first season (P< 0.0001) and the second one (P=0.002).

Farm ID	Transhumance	BDV-4 (Sympatric chamois)[1]			BVDV-1 (NADL)			BDV-4 (esp97)			BDV-4 (pig-SP-2007)		
		Mean	Median	Range	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range
Farm-1	BT-1	47.9	40	0-320	30	20	0-160	212.9	160*	20-640	137.9	80*	20-640
	AT-1	58.6	40	0-160	146.4	40	0-2560	312.9	160*	20-2560	150.9	160*	0-320
	BT-2	77.1	60	0-640	107.9	80	0-640	359.4	160*	10-2540	312.9	160*	20-2560
	AT-2	55.4	40	10-160	141.2	40	0-640	175.6	160	10-640	151.7	80	10-640
Farm-3	BT-1	20	20	---	0	0	---	80	80	---	40	40	---
	AT-1	20	20	---	0	0	---	0	0	---	0	0	---
	BT-2	20	20	---	0	0	---	0	0	---	0	0	---
	AT-2	20	20	---	0	0	---	160	160	---	160	160	---
Farm-4	BT-1	800	800	320-1280	10	10	0-20	30	30	20-40	25	25	10-40
	AT-1	80	80	---	0	0	---	40	40	---	160	160	---
Farm-5	BT-1	536	20	10-2560	100	40	20-320	146	10	0-640	68	0	0-320
	AT-1	260	0	0-1280	96	40	40-320	80	40	0-320	42	0	0-160

Table 5.2 Titres obtained by virus neutralization test of ELISA positive samples. All sera were tested in two domestic BDV-4 strains (BDV-4 esp97 and BDV-4-pig-SP-2007), a reference BVDV strain (BVDV-1 NADL) and a different sympatric chamois BDV-4 strain depending on transhumant the alpine grazing area for each farm. [1](Farm-1= ARAN-15; Farm-3= PALLARS-8; Farm-4 and 5= FRESER-5). BT (before transhumance); AT (after transhumance). Numbers 1 and 2 in the transhumance column indicate sampling season (1-first season; 2-second season). Data with no range included only one sample. * Statistical significance at the flock level was established between BDV-4 chamois and BDV-4 domestic strains in all sampling times ($P<0.01$), and between BVDV-1 and BDV-4 domestic strains in all sampling times ($P<0.01$) with the exception of the post-transhumance sampling in the second season.

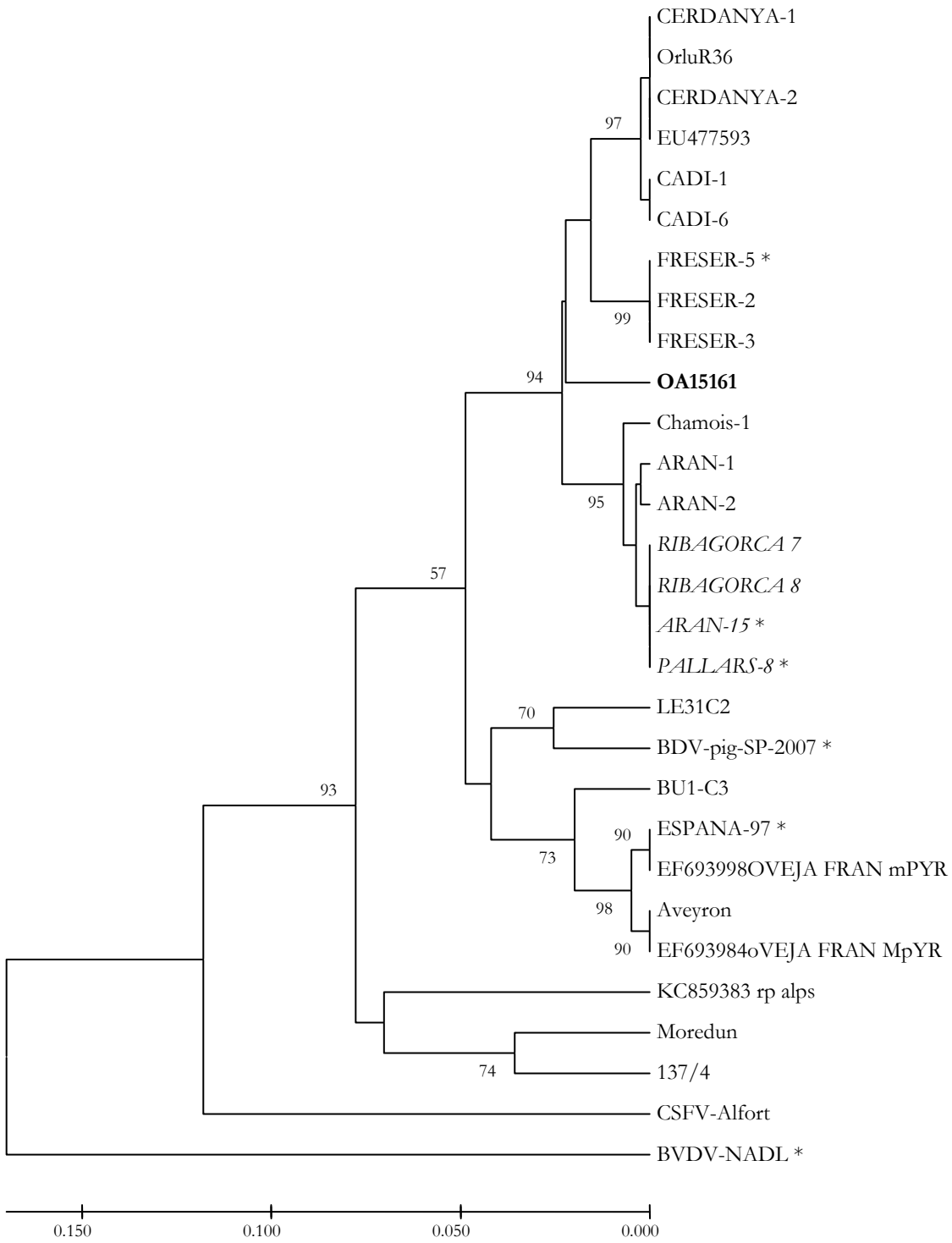

Figure 5.3 Unrooted neighbor-joining phylogenetic tree based on the 5' untranslated region (UTR) sequence among Pestiviruses. The evolutionary history was inferred using the UPGMA method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances

used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 29 nucleotide sequences. Evolutionary analyses were conducted in MEGA 6. Chamois strains found in the present study (in italics) were enclosed with ovine strain (in bold) in a differentiated group into Border Disease Virus genotype 4 (BDV-4) with other chamois BDV strains. The numbers on the branches indicate the bootstrap values (in percent; 1,000 replicates) only when values were ≥70. Sequences were taken from GenBank. * Strains used in VNT analysis.

Discussion

Pestivirus infection cycles in sheep and Pyrenean chamois were investigated in two geographic alpine areas where previous studies described frequent BDV infection in chamois populations but with different epidemiological scenarios (Fernández-Sirera *et al.*, 2012b; Marco *et al.*, 2015). Viral infection in chamois has been maintained as shown by the high seroprevalence observed. In zone A, where BDV has caused large mortality outbreaks in 2001 and 2002, antibodies against pestivirus were present with high prevalence between 2013 and 2015. This seroprevalence was similar to that found in zone B in the same years, where no mortality outbreaks had been detected, and are in accordance with those found in some French Pyrenees areas (Pioz *et al.*, 2007).

More heterogeneity was found when studying pestivirus seroprevalence in transhumant sheep flocks grazing in the same alpine areas during the same period. The presence of antibodies in sheep flocks seasonally settled in zone A (Farms 1-3) ranged from 0 to 91.1%, whereas the seroprevalence in sheep grazing in zone B (Farms 4 and 5) was between 4 and 16.7%. Farm-1 had the highest rate of infection during the study period, with a seroprevalence as high as 91% indicating endemic infection with active circulation of virus, most probably maintained by PI animals (Schweizer and Peterhans, 2014). While we could not confirm it, the virus-positive lamb was most likely a PI animal. In Farms 3 to 5, the seroprevalence was low, between 1.2% and 19.2%, with no changes over time. This could be attributed to a lack of current viral circulation, or determined by unusual infections.

In Spain, BDV infection has been reported to be widespread with heterogenic antibody seroprevalences ranging from 17.9% to 93% (Berriatua *et al.*, 2006; Valdazo-González *et al.*, 2008). Seroprevalences of antibodies against pestivirus in alpine grazing sheep also showed highly heterogeneous epidemiological scenarios in Europe. The data ranged from moderate to high pestivirus seroprevalence in the Spanish Pyrenees (23-69%) (Marco *et al.*, 2009a), and Austrian, French and Italian Alps (26-90%) (Gaffuri *et al.*, 2006; Krametter-Frotscher *et al.*, 2007a; Fernández-Sirera *et al.*, 2012c; Martin *et al.*, 2015) to low seroprevalence reported in the Cantabrian Mountains (Spain) (5.9%) (Fernández-Aguilar *et al.*, 2016) and Swiss Alps (6.9%) (Braun *et al.*, 2013a).

The use of VNT to understand the origin of sheep infections showed an interesting diversity between zones A and B after confronting neutralizing antibodies against different BDV strains. In zone A, 27 sheep sera from Farm-1 showed significant statistical differences, with higher titres to BDV strains of livestock origin, when compared to the BDV-4 chamois strain from the same zone and a reference strain of BVDV-1. This may indicate that it is unlikely that sheep are infected with Pyrenean chamois strains, as suggested before by Marco *et al.* (2009a). However, other VNT studies in Pyrenean chamois did not find antigenic differences between BDV strains of domestic and wildlife origin (Pioz *et al.*, 2007; Fernández-Sirera *et al.*, 2012a).

In zone B, Farm-4 presented two of the three positive sera with significantly higher titers against the local BDV strain circulating in chamois. This may indicate that interspecific transmission of BDV strains is possible between sheep and Pyrenean chamois. This is of special interest as zone B is the only area in the Catalan Pyrenees where BDV has been detected since 1996, but no mortality outbreaks have ever been reported (Marco *et al.*, 2015). One factor that could explain the differences observed between the two studied zones is chamois density. In zone A, the chamois population dramatically declined (approximately a 40% decrease after the first outbreak (Marco *et al.*, 2007), and as of yet, has not recovered, while in zone B chamois densities are the highest in the Catalan Pyrenees. This may increase the

probability of contacts between sheep and chamois during transhumance in the alpine areas. However, although transhumance facilitates pathogen sharing(Eckert and Hertzberg, 1994; Macpherson, 1995), pestivirus transmission may not be efficient because direct contact is needed (Schweizer and Peterhans, 2014) and this seldom happens between sheep and chamois (Ryser-Degiorgis *et al.*, 2002; Rüttimann *et al.*, 2008; Casaubon *et al.*, 2012). However, Martin *et al.* (2015) found two isolates from chamois and sheep in same area in the French Alps presenting a 92% identity, according to the 5' UTR region. These results may be indicative of interspecific transmission.

The movement of sheep to alpine meadows facilitates pestivirus infection at the flock level as has been demonstrated in Farm-1, when seroprevalence increased after transhumance from 46.7% to 84.3% during the first season, and from 69.6% to 91.1% during the second season. Similar results were obtained by Krametter-Froetscher *et al.* (2007b) reporting an increase in BDV seroprevalence in sheep after communal alpine pasturing (from 67.6% to 83%). Braun *et al.* (1998) also presented similar results when studying BVDV seroprevalence in cattle farms with PI animals (an increase from 63.3% to 80.1% after going to alpine pastures). The fact that in our study only one out of five farms presented seroconversion after transhumance may be attributed mostly to farm size and by joining feedlots to alpine meadows.

Most of the research on pestivirus in transhumant livestock is focused on BVDV and cattle because of eradication plans in some European countries. However, knowledge of BDV presence in sheep and wildlife plays a major role in the monitoring of eradication programs as these animals could infect cattle (Braun *et al.*, 2013b) and BDV from sheep may impair serological BVDV surveillance (Kaiser *et al.*, 2017).

Viroprevalence in Farm-1 is in accordance with previous studies in which BDV viral prevalences ranged from 0.3-0.7% (Valdazo-González *et al.*, 2006; 2008; Braun *et al.*, 2013a). The removal of these animals (especially PI animals) is essential for the control and eradication of border disease (BD) and also for bovine viral diarrhea (BVD) programs (Braun *et al.,* 2013b, Casaubon *et al.,* 2012). Bodmer *et al.* (2008)

and Presi and Heim (2010) presented studies where PI animals are removed to reduce BVDV infections. Although the first could not completely prevent pestivirus infections and the former was only a first attempt to implement an eradication program, both studies confirmed that removing PI animals are efficient measures to reduce pestivirus infections in transhumant livestock.

Regarding the phylogenetic analyses of 5' UTR sequences, the six strains from chamois and the one from sheep clustered into a BDV-4a subgroup. In addition, they all grouped within the same geographical area of the previously sequenced BDV in the Pyrenees (Luzzago *et al.*, 2016). The phylogenetic proximity of domestic and wild BDV strains suggests that chamois and sheep can share BDV as proposed previously by Martin *et al.* (2015). However, the lack of more isolates from sheep in this region calls this into question. Moreover, the fact that sheep antibodies from Farm-1 had more antigenic affinity to domestic BDV strains may indicate that other strains different from the one sequenced are infecting the majority of sheep in this farm. More BDV strains of Pyrenean ovine origin and genetic analysis on other regions of the genome (i.e., E2 region) may shed light on the putative BDV-4 chamois cluster or the BDV-4 Pyrenean cluster.

Conclusion

In the study area, an epidemiological scenario where chamois and sheep maintain pestivirus infections independently is likely. However, differences in pestivirus epidemiology and ecology in chamois populations may favour circumstantial contacts with sheep flocks and eventual interspecific viral transmission. Transhumance facilitates virus circulation at the flock level in farms where BDV is present. Joining feedlots to alpine meadows is a main cause in viral transmission. BDV strains from Pyrenean chamois and sheep are closely phylogenetically related and fit into the putative BDV-4 chamois cluster, although antibodies from sheep flocks seem to have a stronger antigenic relationship with domestic BDV strains.

Part III: General discussion and conclusions

6. General discussion

Natural ecosystems are highly complex, and infectious diseases can play an important role in their dynamics. In the last years, research into the ecology of wildlife diseases has improved our understanding of the factors that modulate their transmission and spread. Host and pathogen genetic variability, immunology, environmental characteristics and interspecies pathogen transmission have risen as important factors on wildlife diseases epidemiology.

RNA viruses are characterized by high mutation rates and this fact needs to be taken into account when extrapolating the epidemiology of a disease caused by them. Evolutionary patterns observed in different genus of RNA viruses present a complex and challenging picture (Grenfell *et al.,* 2004; Lobo *et al.,* 2009; Pibus *et al.,* 2008). The factors that may intervene on viral selection pressure are only partially known and difficult to predict. Most studies of RNA viruses have focused on influenza virus, HIV, dengue virus and Hepatitis C Virus (HCV) because of their relevance for human health. However, in spite of the huge investment and the great advances made, even today many issues remain unknown and new genetic variants continue to cause unexpected epidemiological scenarios (Grenfell *et al.,* 2004; Pibus *et al*., 2008).

In 1904, the CSFV was one of the first viruses discovered, and many years after it was included into the *Pestivirus* genus (Schwenitiz and Dorset, 1904). Since then, new disease presentations, viral species, and susceptible hosts have been associated to the *Pestivirus* genus (Schweizer and Peterhans, 2014; Tautz *et al.,* 2015; Hause *et al.,* 2015). In 2001, a new disease and host was associated to a Border Disease Virus (BDV) in Pyrenean chamois in Spain, France and Andorra (Arnal *et al.,* 2004; Hurtado *et al.,* 2004). In light of the complex interactions between the pathogen, the host and the areas where it inhabits, the present thesis was designed to contribute to a better understanding of the factors that govern the epidemiology of pestivirus infection in chamois.

In Study I, we aimed to unravel the pathogen diversity that could explain the different epidemiological scenarios. An experimental infection study with two chamois BDV strains: a high-virulence strain, namely "BDV Cadí", and a strain

considered of low virulence, namely "BDV Freser-5" was designed. The knowledge of genetic variability of BDV circulating strains from a certain geographic range is relevant to predict the outbreak's impact on the population and thus for wildlife management decisions. As shown in previous BDV experimental infections in chamois, high-virulence strains, like "BDV Cadí", developed a long-lasting viraemia (Cabezón *et al.,* 2011; Martin *et al.,* 2013). In these infections, the immune compromise, brain lesions, and high viral shedding were of concern (Fig. 3.3, 3.4, 3.6, 3.7. Study I). The present experimental infection confirmed the high virulence of BDV Cadí strain and demonstrated its negative effects on adult individuals and their reproduction. The foetal mortality in all pregnant females occurred during the first two weeks of infection. The epidemiological consequences of these high-virulence strains have been exemplified in field studies by the reports of high mortality outbreaks in free-ranging populations. The highest mortality was recorded in 2005, when a BDV Cadí-like strain caused a drop of about the 86% of the chamois population in the Cerdanya-Alt Urgell NHR (Marco *et al.,* 2009b). Thus, the identification of these strains in a wild population should go hand in hand with a strict monitoring of the population in order to assess the disease impact. Study I demonstrates that, in horizontally infected chamois, high RNA loads are excreted by nasal route at least during 18 days (Fig. 3.7. Study I). This, together with the findings of previous reports demonstrating vaginal, rectal and oral routes as a source of virus (Cabezón *et al.*, 2010a; Cabezón *et al.*, 2011; Martin *et al.*, 2013), highlights the importance of horizontal transmission in naturally infected chamois.

The chronicity of high-virulence BDV strain infection may explain differences between field and experimental clinical disease presentations. The main clinical alterations seen in naturally infected chamois are neurological signs (Marco *et al.,* 2007). However, in Study I and in previous experimental BDV infections (Cabezón *et al.,* 2011; Martin *et al.,* 2013), no neurological signs were observed. Nonetheless, the use of long-acting tranquillizers may be masking the neurological clinical manifestations. A clinical presentation of BDV infection seen exclusively in experimental infections in chamois is the haemorrhagic diathesis observed, as previously reported (Cabezón *et al.* 2011; Martin *et al.,* 2013). The origin of this

presentation is associated to thrombocytopenia (Cabezón *et al.,* 2011). The fact that these lesions have not been found in naturally infected chamois may be due to the acute course and death of affected chamois. In the wild, those animals may die in isolated places or be predated after death, making it very difficult to locate them. In contrast, chamois with a more chronic and progressive disease could develop encephalitis and the neurological signs that allow capturing them.

In order to understand virulence, we also have to analyse its genetic diversity. As has been described for other pestiviruses (Risatti *et al.,* 2005; Leifer *et al.,* 2013; Wang *et al.,* 2015), the identification of virulence-related viral genome regions could be essential for the prevention and management of infections. Luzzago *et al.* (2016) demonstrated that the isolated BDV chamois strains are distributed in a geographical pattern. This pattern seems to be also partially related to virulence in both strains assessed in the Study I. The importance of genetic diversity in regions such as E2 may clarify the phylogenetic relations between strains within a pathogenic perspective. The fact that a BDV belonging to genogroup 8 was identified as the causative agent of chamois death in Italian Alps, increase the interest on genetic patterns (Peletto *et al.,* 2016; Caruso *et al.,* 2017). Although the phylogenetic analysis suggests that the isolates from Alps and Pyrenees are distantly related, the identification of highly conserved regions in their genomes would increase the knowledge on the virulence determinant regions of pestiviruses.

In contrast to the high-virulence BDV strains, we demonstrated the existence of low-virulent strains (i.e. the Freser-5 strain) in the Pyrenees, which is in accordance with the epidemiological scenario in some areas. In Freser-Setcases NHR no mortality outbreaks have been observed although BDV has been present at least since 1996 (Marco *et al.,* 2011). The only diseased chamois reported in this area was found to be infected with a BDV named Freser-1. Interestingly, the phylogenetic analysis of its 5'UTR sequence clustered with the high-virulence isolates group from previous epizootics recorded in the Pyrenees. Importantly, this animal was found in the border between Freser-Setcases NHR and the Cadí-Alt Urgell NHR.

Far from being a high-virulence strain, these apparently low-virulence BDV strains confirmed to cause a transient viraemia cleared after the specific humoral immune response. Study I also demonstrated that the low-virulence BDV virus was maintained in a low RNA load in lymphoid organs. However, the existence of few glial nodes in the brain of all infected chamois demonstrates the neurotropism of this BDV strain (Fig. 3.3. Study I). Although these lesions are not sufficient to cause the neurological signs described in naturally diseased chamois, this situation could worsen in an immunocompromised animal. The aforementioned epidemiological situation of Freser-Setcases NHR, together with the experimental infection, clinical and virological findings, points out that the most probable route of viral maintenance is the vertical transmission, hypothetically maintained by PI animals. This fact is in contrast with the areas were high mortality outbreaks occurs, where the long-lasting viraemic chamois infected horizontally play a key role in pestivirus epidemiology. Although two pregnant females inoculated in Study I with the low pathogenic strain aborted or had a mummified foetus, it is a classical outcome of all (high- and low-virulence) pestivirus infections. It is well known that pestiviruses, depending on the stage of gestation, can cause abortions, stillbirth, mummifications and malformations. This reproductive failure is more severe during early stages of gestation. In BDV infections in pregnant ewes, the most severely affected lambs were those infected from 16 to 90 days of gestation (Sawyer *et al.,* 1991; Scherer *et al.,* 2001; García-Pérez *et al.*, 2009). The fact that the infection in Study I was probably after the onset of immunological competence of the foetus was not enough to prevent the resulted failure in the gestation. BDV infections up to mid-pregnancy and before the last third of gestation commonly can result in abortion (Loken, 1995; Nettleton *et al.,* 1998). Also, the infective dose could vary the severity of disease in foetuses as was demonstrated in ewes (Richardson *et al.*, 1990). Staying on this subject, the key role in low-virulence pestiviral infections (in BVDV and BDV) epidemiology are the persistently infected (PI) animals. These animals born without antibodies against the virus but shedding virus throughout his life is a consequence of a pestivirus infection before the onset of foetal immune competence (Loken, 1995; Nettleton *et al.,* 1998). In chamois, Vautrain and Gibert (2008) reported an experimental infection that seemed to demonstrate the existence

of this epidemiological figure in chamois. The results obtained in Study I reinforce this possibility due to the finding of an infected foetus without antibodies from a naturally infected animal. The presence of PI animals in the areas where no pestivirus mortality outbreaks occur could explain the high seroprevalence observed, as shown in Study III.

The antibody cross-reaction between pestivirus species has been widely studied and demonstrated (Paton, 1995). In Study I and in the former conducted by Cabezón *et al.* (2011), virus inoculation of a highly pathogenic BDV strain in antibody positive chamois demonstrated effective cross-protection between BDV-4 strains. These inoculated chamois did not present viraemia, viral shedding or viral RNA in tissues all over the challenge period. Moreover, the previously acquired humoral immunity protected the foetus from a heterologous BDV. Interestingly, those animals were captured in the Pyrenean area were the low-virulence strain is circulating, Freser-Setcases NHR. The high antibody seroprevalence in this population (demonstrated in the Study III and in Marco *et al.* (2009b)) could be hindering the entrance in that area of more pathogenic strains. Since the first cases of diseased chamois appeared, the disease progressed from west Pyrenean areas to the east. As said before, one diseased chamois was found in the western border of Freser-Setcases NHR. The fact that most of the population was hypothetically protected with antibodies against a low-virulence strain may block the spreading of the high-virulence strains to this area. The existence of this efficient cross-protection could be relevant for vaccine development. However, vaccination in wildlife is tricky and need a careful approach (Cross *et al.,* 2007). For example, the cost of developing a vaccine to treat the few chamois that can be captured may not be justifiable in terms of its impact on the wild population.

Another factor influencing the epidemiology of pestivirus infections may be the existence of "resistant" populations. This hypothesis was proposed when field studies confirmed the circulation of pestivirus at some chamois populations without abnormal mortalities. An interesting study of the polymorphisms at MHC class II DRB1 exon 2 locus showed that the chamois population from the mentioned

Freser-Setcases NHR had higher genetic diversity than the other studied chamois populations (Cavallero *et al.*, 2012). Although viruses trigger the MHC class I polymorphism, the authors suggested that MHC class II polymorphism in chamois could be indirectly related to BDV disease presentations. Previously, Álvarez-Bustos *et al.* (2007) detected lower genetic diversity on the western populations of Pyrenean chamois. So, studies on MHC class I in Pyrenean chamois may contribute on genetic diversity bases with implications on chamois immunology. However, studies on both MHC genetic regions are highly complex. The selected genes of study, the spatial sampling distribution, the timescale of sampling and the correlations between genetic diversity and diseases correlations may lead to erroneous interpretations (Piertney and Oliver 2005; Sommer, 2005; Acevedo-Whitehouse and Cunningham, 2006; Radwan *et al.* 2010).

The present thesis has not focused on studying the host genetics diversity as a factor in pestivirus infections epidemiological scenarios. Nonetheless, some findings of Study I may address this issue. In our experimental infection and in the one developed by Cabezón *et al.* (2011), all the animals infected were from Freser-Setcases NHR and all the infected individuals with the high-virulence strain developed lesions similar than the ones seen in other chamois populations. These findings may highlight that virus strain could be the main factor determining the epidemiology. Nevertheless, one chamois from Study I of the present thesis infected with the high-virulence Cadí-6 strain seemed to clear the virus in sera, presented low RNA load in studied tissues and shedding routes, and had mild lesions on brain. The relation between genetic diversity and resistance to pestivirus infections was not assessed. However, these results highlight the possibility of the existence of a low percentage of animals that are able to overcome a high virulent pestivirus infection.

Dealing with a disease affecting a wild host in a complex ecosystem, interspecific relations may emerge as an important factor of epidemiology variations. It has been reported that, in the early years of pestivirus research, several animal models (horse, cat, dog, guinea pig, mouse, chicken and rabbit) were infected with pestiviruses in

order to assess their susceptibility (Baker *et al.*, 1954; Bachofen *et al.*, 2014). Individuals of these species were inoculated with BVDV and, interestingly, rabbits were the only species successfully infected. Since then, more than 50 species of wild ungulates have been found to be susceptible to pestivirus infection (Vilček & Nettleton, 2006; Passler and Walz, 2010; Ridpath, 2010).

In the Pyrenees, all sympatric ungulates of Pyrenean chamois have been studied in order to understand their role in pestivirus mortality outbreaks (Marco *et al.*, 2009a; Marco *et al.*, 2011; Fernández-Sirera *et al.*, 2012b;). Although it has been proven that some mouflons and red deer had been in contact with the pathogen, the role of sympatric ungulates in chamois pestivirus epidemiology seems to be anecdotic. Research in non-artiodactyl hosts confirmed the susceptibility of the common rabbit (*Oryctolagus cunniculus*) (Frölich and Streich, 1998; Bachofen *et al.,* 2014; Grant *et al.,* 2015), and expanded it to Bennett's wallaby (*Macropus rufogriseus*) (Munday *et al.,* 1972) and mice (*Mus* sp.) (Seong *et al.,* 2015), thus highlighting the interest of sympatric host role. Although the former one was only demonstrated under experimental conditions, the inclusion of a new mammalian order could be of importance in wild hosts from the same taxa. In light of these findings, Study II was designed to assess the susceptibility of two Pyrenean species belonging to the *Lagomorpha* (European hare, *Lepus europaeus*) and *Rodentia* (Alpine marmot, *Marmota marmota*) orders abundantly present in the chamois habitat. The results in Alpine marmot were negative. Given that the area of study was limited, additional studies including other Alpine marmot populations are needed. Strikingly, the European hare was confirmed as the third non-artiodactyl wild host shown to be susceptible to pestivirus infection. Seroprevalence of 36% of studied animals were very similar to those found in free-ranging rabbits in Germany (40%) (Frölich and Streich, 1998). The fact that antibodies against pestiviruses were detected in a species of hare widely distributed in Catalonia region and not limited to the Pyrenean area, raises new and exciting questions (Fig. 4.1. Study II). On one hand, these results suggest that pestivirus may be widely distributed in domestic and/or wild mammals in that area. To author's knowledge, there is only one study on pestivirus seroprevalence in domestic species across the Catalonia region. Alba *et al.* (2008)

detected pestivirus seroprevalences of 49.3% in sheep flocks. Moreover, the high seroprevalence in hares may imply a high rate of direct or indirect contact between animals, previously unreported in the scientific literature.

Since the first description of disease related to BDV infection in Pyrenean chamois, sheep was considered as a possible source of transmission. It is the species in which the disease was described for the first time in 1959 and since then it has distributed worldwide (Vilček and Nettleton, 2006; Valdazo *et al.,* 2006; Tautz *et al.,* 2015). However, few studies regarding its economic impact exist due to the high variability in factors such as breeds, management conditions or vaccine strategies across regions and farm systems. For example, high impact by pestivirus infection was estimated regarding the relationship between growth retardation and economic losses in experimental animals (Sweasey *et al.,* 1979). Also, Sharp and Rawson (1986) calculated a reduction of 20% of income in commercial flocks by pestivirus infections. More recently, García-Pérez *et al.,* (2009) estimated that pestivirus-related losses in a standard flock of 250 ewes in the Basque Country could be of about 3000 Euros.

Sheep has been always at the centre of the debate concerning the epidemiology of pestivirus infections in chamois. As has happened with many other diseases, farmers, wildlife researchers, conservationists and hunters blame each other for the infectious outbreaks. Previous studies have demonstrated that pestivirus could be maintained independently into sheep and chamois populations (Krametter-Froetscher *et al.,* 2007a; Marco *et al.*, 2009a; Fernández-Sirera *et al.*, 2012b). Moreover, other studies have shown that occasional infection between domestic and wild hosts occurs (Casaubon *et al.*, 2012; Martin *et al.*, 2015; Passler *et al.*, 2016). Less clear is whether these interspecific infections play a role in the maintenance of BDV in the Pyrenees (Bertin-Cavarait, 2006). An example of the relevance of that issue is the management measures proposed in France. Recently, some researchers have proposed the vaccination with a BVDV vaccine of all the sheep flocks to prevent the transmission of the disease to chamois (personal communication). Their statement is based on the belief that if the virus is not present in sheep, there will

be a decrease in pestivirus infections in chamois populations living in the same area. This proposal has both supporters and detractors in a debate that implies a huge effort and economic inversion. Interestingly, it is probable that the strains that are circulating nowadays in chamois populations have an ovine origin. Following this hypothesis, Luzzago *et al.* (2016) reconstructed the onset and spread of the chamois outbreaks. They showed how the high mutation rate of the RNA virus can evolve it in many strains. The authors even geo-referenced the hypothetical starting point of the outbreaks.

Study III aimed to better understand the role of sheep in the epidemiology of BDV infection in chamois. The approach was to assess if transhumant Pyrenean sheep flocks are infected with BDV, the effect of transhumance in pestivirus maintenance, and if there is transmission between sheep and sympatric chamois by identifying circulating BDV strains in both species. Sheep flocks were selected focusing in two grazing areas relevant for chamois epidemiology. Based on previous studies (Fernández-Sirera *et al.,* 2012b; Marco *et al.*, 2015), two different epidemiological areas were selected: the first one with historical/recurrent high mortality outbreaks and a decrease in population trend, and the second with absence of disease and mortality and high density with increasing population trend (Fig. 5.1. Study III). In both areas, BDV is actively circulating according to mortality reports in one area and high antibody seroprevalence in both of them. Among the five sheep farms studied, only one showed high seroprevalence. In this particular farm, differences in lambs and adults antibody seroprevalence before and after transhumance highlight that it is in alpine meadows where most of BDV transmission, between individuals of the same flocks or between different flocks, occurs. Although the only BDV strain sequenced in this farm was closely related to the Pyrenean chamois strains, the VNT analysis of all seropositive sheep indicated that the BDV strain circulating is more likely to be from ovine origin. Hence, our results demonstrate that in farms with constant virus circulation joining feedlots is the main factor for viral transmission, and that transhumance is a pastoral practice that facilitates pestivirus maintenance. An epidemiological scenario where chamois and sheep BDV infection is maintained independently is more likely than a scenario with sharing BDV infection cycles.

However, the fact that the VNT results in one farm showed more antigenicity to chamois BDV strains and the genetic similarity between chamois strains and the aforementioned ovine strain could indicate sporadic transmission between domestic and wild animals.

The confirmation that pastoral practices are a main factor in virus maintenance is of importance in livestock management. Hence, in countries where pestivirus eradication plans are being implemented, the health status of flocks before transhumance is a clue in control strategies, as has been proposed before (Bodmer *et al.*, 2008; Presi and Heim 2010). Nevertheless, management measures may not be focused only in a simple pathogen-host perspective. Environmental and social actors should be included in decision processes. In the case of transhumance, as remarked by Liechti and Biber (2016), the decline of this old pastoral practice may leads to ecological, economic and socio-cultural losses.

Returning to the subject of debate in France, the present thesis may contribute with new insights for management decisions. The diversity on pastoral practices, orography and chamois densities in each Pyrenean area may difficult the extrapolation of conclusions to other areas. However, the present results indicate that BDV vaccination of sheep flocks to avoid BDV infection in chamois may have limited effects. Moreover, regarding the difference of virulence between strains confirmed in the Study I, the knowledge of BDV genogroups and strains circulating in domestic and wild animals, and the monitoring of the seroprevalence, are the clue to predict the impact of BDV in Pyrenean chamois populations. Also, studies on contact rates between chamois and domestic hosts in different pastoral systems could be important in these predictions. Other management measure currently under debate is chamois translocation. Though being justified as a way to increase densities and genetic diversity, these kind of measures need to be carefully planned. The effects of these exogenous introductions are usually uncontrollable and could be even detrimental (Woodford and Rossiter, 1993; Crestanello *et al.,* 2009; Gortázar *et al.*, 2015).

The fact that Freser-like BDV may have protected the chamois populations from the invasion of a more pathogenic strain is of interest. Thus, the circulation of a low-pathogenic BDV in wild animal populations could be beneficial in some special cases. Nevertheless, it should be taken into account that the epidemiological scenario could rapidly change due to the high mutagenic rate of RNA viruses. The low-pathogenic strain circulating in Freser-Setcases NHR may be a consequence of virulence attenuation. Some authors have proposed that genetic changes in the open reading frame (ORF) of BVDV occur faster in a single PI than in many acute infections (Neil *et al.,* 2011). This may lead to an increase in the number of virus' genetic variants in areas maintained mainly by persistent infections than in those where transient infections are common. The study of BVDV genetic diversity have underlined that the low-virulence strains are better adapted to the host and are thus more prone to persist in natural conditions. Hence, most of BDV and BVDV isolates are of low virulence strains. However, periodic emergence of virulent pestiviruses occurs. A selection of viral mutants, that replicate more than the parent virus, would facilitate the emergence of virulent strains causing extensive tissue damage and a burst of viral shedding (Bolin and Grooms, 2004). This evolutionary pattern may be the one present in the Central and Western Pyrenees where high mortality outbreaks affect chamois populations.

Luzzago *et al.* (2016) proposed the putative Chamois BDV cluster when classifying different BDV subgroups. The fact that Study 3 includes an ovine isolate into that cluster may change the nomenclature to a putative Pyrenean BDV cluster. However, Dubois *et al.* (2008) identified an ovine BDV in Pyrenees closely related to BDV genogroup 5. The rational classification of groups and subgroups according to the pestivirus phylogeny is an important point that should be improved in future studies. The use of new molecular technologies and bioinformatic tools should help the field of virus research to make a qualitative leap forward.

The present thesis has focused on some factors that influence the epidemiology of pestivirus infections in chamois. However, studies on other factors such as the environment, other sympatric wild species, population genetics, immunology and

coinfections are necessary to complete the picture of pestivirus epidemiology (Alzier *et al.,* 2003, Delahay *et al.,* 2009; Daszak *et al.*, 2013). Also, many anthropogenic factors have to be taken into account. Demographic and anthropogenic changes are important drivers of disease emergence. Ecological, political and socioeconomic drivers need an interdisciplinary approach to understand and control diseases (Daszak *et al.*, 2013). Implementing control and monitoring plans lacking a scientific base could worsen the already fragile epidemiological scenarios. Along these lines, joining efforts across the Pyrenean area would be a big step forward. Standardization of sampling protocols and analyses, a fluent sharing and diffusion of results would have a greater beneficial impact than any experimental infection or thesis in pestivirus infections of chamois. Unluckily this collaboration does not exist yet. According to this, a Transpyrenean research group formed by scientists, administrations and civil society could be crucial for an optimal control and management of pestivirus infections. Moreover, the scientific community and specially their financers and the politics that determine them may be also considered as an anthropogenic factor influencing pestivirus epidemiology. Encouraging an interest in wildlife health, and raising awareness of its economic impact on human beings, may improve the current knowledge in many fields and prevent future threats. Nowadays the Pyrenean chamois is classified by the IUCN as "least concern" status of conservation (Herrero *et al.*, 2015). However, the ecological, social and economic value of this species is usually underestimated. Moreover, Serrano *et al.* (2015) suggested that pestivirus infections in Pyrenean chamois could drive certain chamois populations to extinction. So, actually efforts on conservation of Pyrenean chamois should be addressed today in order to avoid a probable worsening of their conservation status in the near future.

7. Conclusions

1. Cadí-6 is a high-virulence BDV strain in experimentally infected antibody negative Pyrenean chamois that widely distributes in tissues of adult animals and foetuses, causing a long-lasting viraemia and death.

2. Antibody negative Pyrenean chamois infected with Cadí-6 BDV strain develop a humoral immune response that is not able to produce viral clearance.

3. Freser-5 is a low-virulence BDV strain for non-pregnant Pyrenean chamois that distributes mainly in lymphoid tissues, causes mild brain lesions and a humoral immune response that leads to serum viral clearance.

4. Freser-5 BDV strain causes foetal mortality in the middle of gestation in Pyrenean chamois and causes mild brain lesions in females that seroconvert and clear the virus from serum and remains only in low RNA load in tonsil after 26 days post inoculation. Hence, the pathogenesis of Freser-5 BDV strain in Pyrenean chamois is similar to that of classical border disease in sheep.

5. The low-virulence Freser-5 BDV strain, which circulate in the Freser-Setcases area, causes an effective cross-protection against the infection with high-virulence (Cadí-6) strains in adult Pyrenean chamois.

6. The presence of high- and low-virulence BDV strains in Pyrenean chamois populations agrees with the epidemiology of the disease reported in the Pyrenees since the appearance of the first outbreak in 2001. These findings strongly suggest that strain diversity is the main factor that drives the diversity of epidemiological scenarios.

7. The European hare is the third wild non-artiodactyl species with documented susceptibility to pestivirus infection, while the Alpine marmot susceptibility has not been demonstrated.

8. Transhumance is a pastoral practice that favours BDV transmission in sheep flocks by joining feedlots in alpine meadows in the Pyrenees.

9. BDV transmission between sheep and Pyrenean chamois occurs occasionally in alpine meadows, although transhumant sheep and Pyrenean chamois have independent pestivirus cycles.

Part IV: References and annexes

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9. Annexes

Wild species included in the studies

Pyrenean chamois

Order: Artiodactyla
Family: Bovidae
Subfamily: Caprinae
Genus: Rupicapra
Species: R. pyrenaica
Subsp.: R. p. pyrenaica

Habitat and diet

Pyrenean chamois can be found between 400-2800 meters above sea level (García-González and Herrero, 2002; Herrero *et al.*, 2015). Chamois are highly adapted to alpine and subalpine habitats, moving around rocky areas, alpine meadows and forested valleys. Seasonal altitudinal migrations occurs in late fall and winter moving to lower lands in a range of about 300-800 meters (Lovari *et al.*, 2006; Crampe *et al.,* 2007). The composition of the diet depends on the availability of grasses and forbs, although in winter season chamois vary their diet to more woody plants (Rayé *et al.,* 2011). Diet competition with domestic or other wild ungulates in the same niche could move chamois to lower food availavility areas (Chirichella *et al.,* 2013).

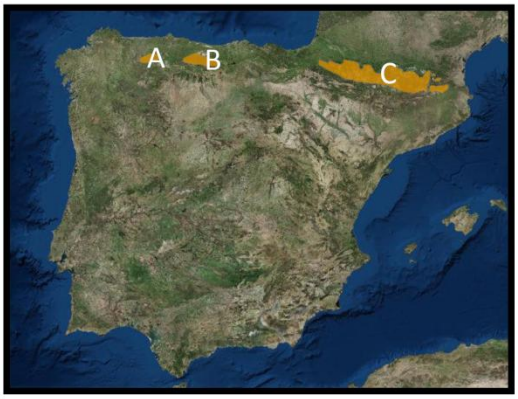


Figure 1. Distribution of Pyrenean chamois populations in the Iberian Peninsula. Subspecies *R. p. parva* inhabits A and B zones. Subspecies *R. p. pyreanica* inhabits in zone C. (Herrero *et al.* 2015)(www.IUCNredlist.com).

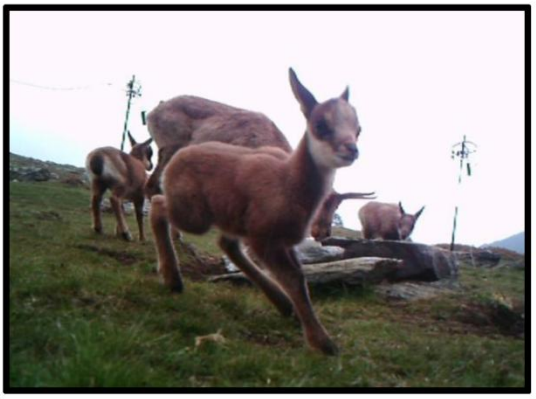


Figure 2. New born Pyrenean chamois and adult in a salt lick. Freser-Setcases NHR. Photo by Andreu Colom Cadena.

Life cycle and behaviour

Chamois are nearly monomorphic with the exception of the mating season. Males tend to be 4% heavier in spring while in autumn differences between males and females can be of 40% in body weight. In that moment, males exhibit a marked dorsal ridge of longer hair. Pyrenean chamois have two different seasonal coats, ruddy brown in summer and dark brown in winter.

Social behavior of Pyrenean chamois is mostly gregarious but can vary from solitary individuals to groups of diverse size. Males are more often solitary than females. Sexual segregation is common except during the rut, which take place between the end of October to the begining of December. This *Rupicaprae* reach the sexual maturity at 18-20 month of age although rarely contributes to reproduction before three years of age (Catusse, 1996). Female chamois are basically monotocous (170 days gestation period, 1 offspring per year rarely twins) with a moderate degree of polygyny (Loison *et al.,* 1999). The highest mortality (42%) is in kids (<1 year). For the rest of age classes survival rates are more fluctuate and there are no sexual differences (Gonzalez and Crampe, 2001; Bocci *et al.*, 2010; Corlatti *et al.,* 2012).

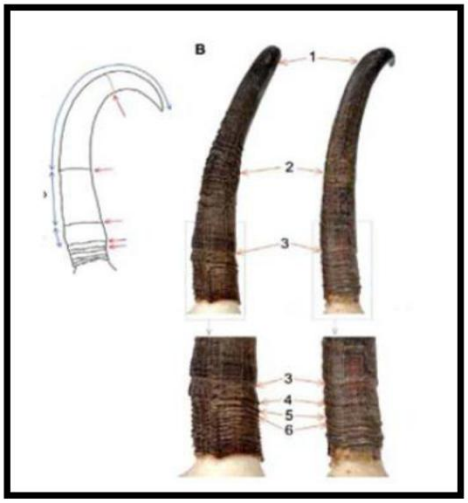
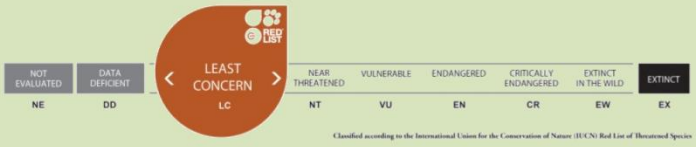


Figure 3. Chamois age determination by horn growth. Adapted from Pérez-Barbería *et al.* (2010).

Figure 4. Pyrenean chamois Border Disease Virus infection. Skin hyperpigmentation, alopecia and hyperkeratosis are observed. Photo: Ignasi Marco Sánchez.

Major threats

Nowadays the major threat of Pyrenean chamois is the mortality outbreaks caused by Border Disease Virus (BDV) (Arnal *et al.,* 2004; Hurtado *et al.*, 2004). Since the first apparition of the epizootic in 2001, many Pyrenean chamois poupulations in the Spanish, French and Andorran area of Pyrenees has decreased in numbers. Space and food competition with livestock can be also a threat to small and isolated chamois populations (Herrero *et al.*, 2015). Chamois is a major game species in Spain and France. Hunting has an important social and economical implication in local communities when hunting is carefully managed.



European hare

Order: Lagomorpha
Family: Leporidae
Genus: Lepus
Species: L. europaeus
Subsp.: L. e. europaeus

Habitat and diet

The European hare is a highly adaptable species that can be found from sea level up to 2300m (Smith and Johnston, 2008). Hares are abundant in many types of habitats and so it is distributed in almost all Europe, south-western Asia and Northern Russia. Moreover, it has been introduced in many other countries (i.e. Chile, USA and Australia). In the northern half of the Iberian Peninsula the subspecies known as European hare is the *L. e. europaeus*. This lagomorphs are vegetarians consuming mostly grass although seasonally can feed on flowers, fruits and fungus (Smith and Johnston, 2008; Ballesteros, 2012).

Figure 1. Distribution of European hare in the Iberian Peninsula and Southern France (Smith and Johnston, 2008). (www.IUCNredlist.com)

Figure 2. European hare eating maize. Photo: © Manfred Danegger / www.photoshot.com

Life cycle and behaviour

The sexual dimorphism in European hares is only represented by low weight differences in females that tend to be heavier than males. The total length of *L. europaeus* is 48.0-70.0 cm (Macdonald and Barrett, 1993) and the body weight is between 3-4.4Kg. Hares are covered by a brownish-yellow coat with the exception of a white coat that covers a low extension of the ventral area. Season changes are found in winter when the coat is greyish. The tail is white and black.

Population densities range from 0.1/ha to 3.4/ha. Hares social and feeding behaviour is mainly developed in the twilight and the night. Although feeding is the main activity, they have complex social interactions based on hierarchy (Lorenzo *et al.*, 2015). Sexual maturity in females is around 7-8 month and in male at 6 month of age. European hare have a broad reproductive season with collective rut at night. Averages from one to four litters per year have been described (Macdonald and Barret, 1993; Smith and Johnston, 2008). After 40 days of gestation, from one to three leverets are born and get independent from the mother one month after. The maximum age described in the wild is of 12.5 years although the average life expectancy is of 1.04 years (Macdonald and Barrett, 1993).

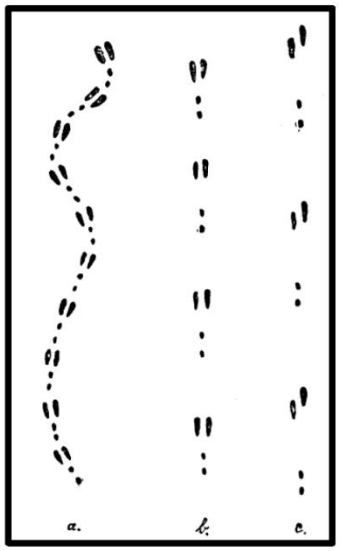


Figure 3. European hare feet track. a) Grass track; b) Normal walk; c) Flight. From www.escademic.com

Figure 4. Fighting European hares in reproductive season. Photo: Eliott Neep.

Major threats

The major threats of European hare are the intensified agro-practices that have reduced his food source (Reichlin *et al.*, 2006). Declines in European hare populations have been described in many European regions (Smith *et al.*, 2005; Lorenzo *et al.*, 2015). Moreover, the indiscriminate hunting in some areas has affected hare populations. The most important diseases challenging hare populations are the European Brown Hare Syndrome caused by a Caliciviurs and the Tularemia caused by the bacteria *Francisella tularensis* (Ballesteros, 2012).

Alpine marmot

Order: Rodentia
Family: Sciuridae
Genus: Marmota
Species: M. marmota
Subsp.: M. m. marmota

Habitat and diet

Populations of Alpine marmot disappeared from most parts of Europe in the early Holocene (Besson, 1971). Their presence in the Pyrenees date around 1948-1988 when 400 Alpine marmots from French Alps were introduced (López *et al.,* 2010). Alpine marmot is highly adaptable in alpine and sub-alpine ecosystems and can be found in a range from 1000 to 3000 meters above sea level with preference on slope areas. Marmot diet is based on a high diversity of plant species (Rudatis and Battisti, 2006). Regarding the phenological state of the plants, marmots preference is towards flowers of dycotelonae and the inflorescence of grasses (García-González *et al.,* 2014).

Figure 1. Distribution of Alpine marmot in the Iberian Peninsula and Southern France (Cassola *et al.,* 2008). (www.IUCNredlist.com)

Figure 2. Alpine marmot pup. Photo: Marie-Léa Travert. From Tafani *et al.* (2013)

Life cycle and behaviour

Alpine marmot is a monomorphic species. Changes of weigh throughout the year are related to hibernation process. At the end of hibernation (mid-April) the adult weight can be of 2.2Kg while at the entry of hibernation (mid-October) they can weight 6.5Kg (Körtner and Heldmaier, 1995). The total length without the tail is 45-68 cm. The tail length is 14-16 cm and is brown coated with a black extreme. The body coat is brown with orange markings on the back and white beige to orange on the belly. A white band covers a space between eyes, nose and ears.

Alpine marmot is a social species that live in family groups from 2 to 20 individuals (Allainé, 2000). The family group territory range between 0.9 to 2.8ha. Marmots reach the sexual maturity at two-years old and the rut begins just after hibernation. The gestation lasts 33 days and the litter is between 2-3 young although a 7 offspring has been recorded (García-González *et al.* 2014). Hierarchy rules the social structure in family groups and the main dispersal period is just after hibernation. In the early summer dominant and dispersal marmots from the same sex use to have aggressive encounters. Hibernation lasts approximately 200 days (Körtner and Heldmaier, 1995). The Alpine marmot lifespan in the wild is around 7 years.

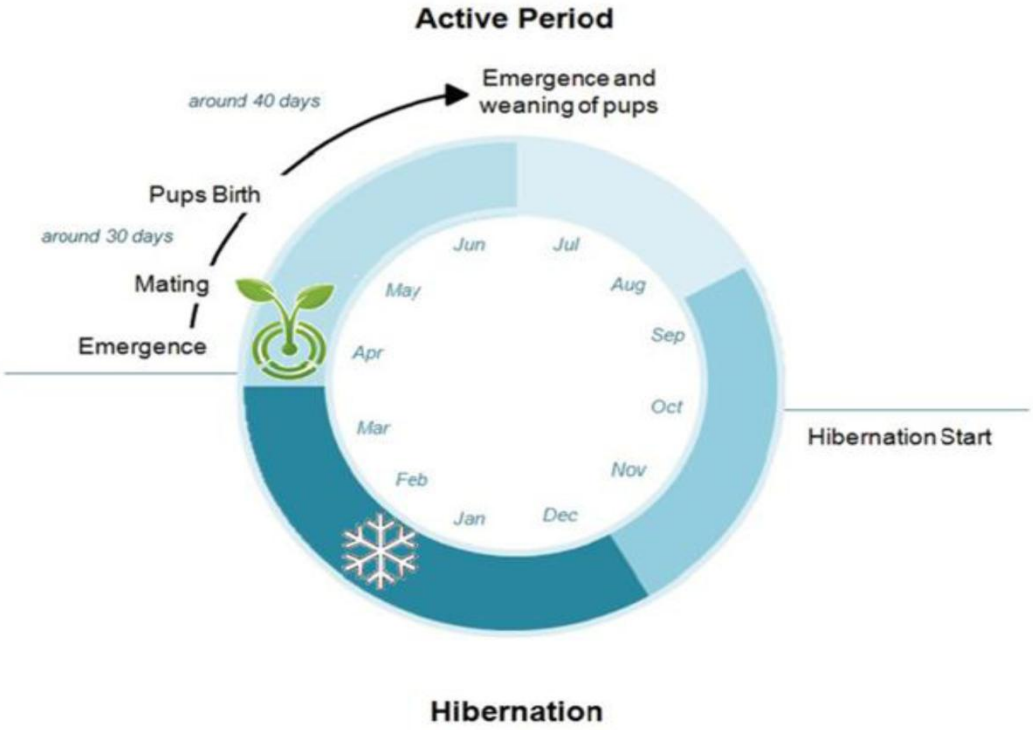


Figure 3. Alpine marmot life cycle: annual (cycle) and for the entire life (blue strip). Figure from Tafani *et al.* (2013).

Major threats

The Alpine marmot has not major threats described yet (García-González *et al.,* 2014). Their population are in expansion and no important diseases are described affecting them. However, climate change can be a factor of variation on these issues. The increase risk of infectious diseases due to climate change is a future threat with hypothetical more severe effect on hibernating species (Geiser *et al.,* 2013; Hernandez *et al.*, 2013; Cassola, 2016).

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