EPIDEMIOLOGY OF PESTIVIRUS INFECTION IN PYRENEAN CHAMOIS (*Rupicapra pyrenaica pyrenaica*) AND OTHER WILD AND DOMESTIC RUMINANTS

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Tesi doctoral
Departament de Medicina i Cirurgia Animals
Facultat de Veterinària
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Els Doctors **IGNASI MARCO SÁNCHEZ** i **SANTIAGO LAVÍN GONZÁLEZ,** Professor Titular i Catedràtic d'Universitat respectivament de l'Àrea de Coneixement de Medicina i Cirurgia Animal de la Facultat de Veterinària de la Universitat Autònoma de Barcelona,

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Que la memòria titulada "Epidemiology of pestivirus infection in Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) and other wild and domestic ruminants", presentada per LAURA FERNÁNDEZ SIRERA per a l'obtenció del grau de Doctora en Veterinària per la Universitat Autònoma de Barcelona, s'ha realitzat sota la nostra direcció i, un cop considerada satisfactòriament finalitzada, autoritzem la seva presentació per tal que sigui avaluada per la comissió corresponent.

I perquè així consti als efectes que siguin oportuns, firmem el present informe a Bellaterra, el 29 de maig de 2012.

Firmat: Ignasi Marco Sánchez

Firmat: Santiago Lavín González

D'où vient qu'en Tyrol et qu'en Suisse
Où je suis allé par hasard
Il n'est pas un chamois qui puisse
Me sembler beau comme un izard?

Edmond Rostand – Les Musardises

Luchon 1887

Agraïments

Muchas son las personas que me han acompañado en este *via crucis* así que me temo que me voy a extender un poco.

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Y per continuar amb el PESTITIM dedico unes línees a la Rosa, que ha estat com una mare al laboratori. És una tranquil·litat treballar amb una persona com tu a prop! Sobretot quan surten fotos amb bandes sospitoses o els virus se'ns descontrolen… tu sempre trobes una solució pràctica per a tot! Gràcies.

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A Nora por todas la risas compartidas (y cotilleos, para qué nos vamos a engañar). Por los viajes a Lleida o desde Lleida, saltándonos todos los desvíos.

A Ester! La Master Beer. Perquè em caus rebé i totes les estones amb tu son bones.

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TABLE OF CONTENTS

1. ABSTRACT

1. ABSTRACT

Since 2001 several outbreaks of a new disease associated to Border Disease Virus of genotype 4 infection (BDV-4) have caused important declines in the Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) populations. The main goal of the present research work was to study the epidemiology of BDV infection in Pyrenean chamois and other wild and domestic ruminants, since pestiviruses are not strictly host-specific.

The main study areas consisted of the Catalan and Andorran Pyrenees, but also different areas from the Ports de Tortosa i Beseit and Sierra Nevada Spanish mountains and from the Italian and Swiss Alps. The studied species were the Pyrenean chamois, European mouflon (*Ovis aries*), red (C*ervus elaphus*), roe (*Capreolus capreolus*), and fallow deer (*Dama dama*), Alpine chamois (*Rupicapra rupicapra*), Alpine (*Capra ibex*) and Iberian ibex (*Capra pyrenaica*), and sheep, goats and cattle that share the habitat with chamois.

Sera were tested for the presence of antibodies against pestivirus with a commercial blocking ELISA assay. Comparative virus neutralization tests were performed in positive sera by using a bovine viral diarrhoea virus (BVDV) strain and reference and local BDV strains. Viral detection in wild ruminants was performed by reverse transcription-polymerase chain reaction (RT-PCR) using the panpestivirus primers 324 and 326 and/or with a commercial sandwich ELISA assay. As last, the sequence analyses of the 5'UTR region from positive samples and virus isolation were performed.

The study of pestivirus epidemiology in Pyrenean chamois populations previously affected by BDV-infection outbreaks revealed that in some populations the disease has become endemic and BDV circulates frequently in the chamois population, possibly having a negative impact on host population dynamics. Contrarily, in some populations BDV does not seem to circulate after the disease outbreak. The study of a Pyrenean chamois population from the Eastern Pyrenees unaffected by disease outbreaks revealed that BDV-4 is self-maintained in this population, apparently without causing negative population dynamic effects. The circulation of weakly virulent strains could have been the cause of the high seroprevalence detected, which could hinder the spread of more virulent strains within this chamois population and the appearance of disease outbreaks. The seropositive Pyrenean chamois had BDV antibodies in all the studied populations, and most of them had higher titres against the BDV-4 strains.

All the wild ruminants sharing habitat with Pyrenean chamois showed a low seroprevalence. The seroprevalence in sheep and goats, varied depending on the geographical origin, while in cattle it was constantly high. Sheep had higher antibody titres against the BDV-4 strains, cattle against BVDV, while goats showed higher titres against both BVDV and BDV strains.

The study of pestivirus epidemiology in the south-western Italian Alps revealed a high seroprevalence in Alpine chamois, suggesting that members of the genus Rupicapra are likely to be infected with pestiviruses. Otherwise, Alpine and Iberian Ibex from all zones had a low seroprevalence, and most of the positive ibex had BDV antibodies. In Switzerland, the analyzed red deer and one Alpine chamois, had antibodies to BVDV, and the rest of chamois had antibodies to BDV. This result indicates that wild ruminants from this country can be infected by both pestivirus species.

2. RESUM

2. RESUM

Des de l'any 2001, diferents brots d'una malaltia associada a la infecció per un virus de la malaltia de la frontera de genotip 4 (BDV-4, de l'anglès *border disease virus*) han causat reduccions importants en el nombre d'efectius d'isard (*Rupicapra pyrenaica pyrenaica*). L'objectiu principal d'aquesta tesi doctoral va consistir en estudiar l'epidemiologia de la infecció per BDV a l'isard i a altres remugants salvatges i domèstics, ja que els pestivirus no són hoste-específics estrictes.

La principal zona d'estudi va consistir en els Pirineus Catalans i Andorrans, però també es van estudiar diferents zones dels Ports de Tortosa i Beseit i Sierra Nevada, a Espanya, i dels Alps italians i suïssos. Les espècies estudiades van ser l'isard, el mufló europeu (*Ovis aries*), el cérvol (C*ervus elaphus*), el cabirol (*Capreolus capreolus*), la daina (*Dama dama*), l'isard alpí (*Rupicapra rupicapra*), l'íbex (*Capra ibex*) i la cabra salvatge (*Capra pyrenaica*), així com l'oví, caprí i vaquí que pasturen en els prats d'alta muntanya.

Es va determinar la presència d'anticossos enfront a pestivirus en sèrum mitjançant una tècnica d'ELISA. En els sèrums positius es va dur a terme un test de seroneutralització vírica comparada amb una soca del virus de la diarrea vírica bovina (BVDV, de l'anglès *bovine viral diaorrhea virus*) i diferents soques de BDV locals i de referència. La detecció vírica es va realitzar mitjançant una tècnica de reacció en cadena de la polimerasa inversa i/o un test ELISA. Per últim, es va seqüenciar la regió no codificant 5' dels virus detectats i es van aïllar.

L'estudi de poblacions d'isard prèviament afectades per brots de malaltia va indicar que la infecció per BDV ha esdevingut endèmica en determinades zones i que possiblement aquest fet està relacionat amb la manca de recuperació de certes poblacions. Tanmateix, en altres zones, el virus aparentment ha deixat de circular i la recuperació de la població d'isards després dels brots és bona. L'estudi a la reserva nacional de caça de Freser-Setcases, va establir que el BDV-4 és mantingut a la població d'isards sense causar cap efecte negatiu a la dinàmica poblacional. És possible que en aquesta població hi circulin soques menys virulentes de BDV-4, causant una elevada seroprevalença i evitant la dispersió d'altres soques més virulentes, i l'aparició d'un brot de malaltia. En totes les poblacions els isards seropositius van presentar títols més alts d'anticossos contra les soques BDV, i la majoria contra les BDV-4, concretament.

En tots els remugants salvatges que comparteixen hàbitat amb l'isard es va detectar una seroprevalença baixa. La seroprevalenca en oví i caprí va variar depenent de l'origen geogràfic, i al vaquí sempre va ser elevada. L'oví va presentar títols d'anticossos més alts enfront a les soques BDV-4, el vaquí a BVDV i les cabres enfront a BDV-4 i BVDV.

Als estudis realitzats al sud-oest dels Alps italians es va detectar una elevada seroprevalença a l'isard alpí. Aquest fet suggereix que la infecció per pestivirus és freqüent a les ambdues espècies del gènere Rupicapra. Per altra banda, als íbex i a les cabres salvatges de totes les zones es va detectar una seroprevalença molt baixa, i la majoria dels individus positius van mostrar anticossos enfront a BDV. A Suïssa, tots els cérvols analitzats i un isard alpí van mostrar anticossos enfront a BVDV, mentre que la resta d'isards van mostrar títols més alts enfront a BDV. Aquest resultat indica, que els remugants salvatges de Suïssa s'infecten amb BVDV i també amb BDV.

3. RESUMEN

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Desde el año 2001 diferentes brotes de una nueva enfermedad asociada a la infección por un virus de la enfermedad de la frontera de genotipo 4 (BDV-4, del inglés *border disease virus*) han diezmado varias poblaciones de rebeco pirenaico (*Rupicapra pyrenaica pyrenaica*). El objetivo de la presente tesis es estudiar la epidemiología de la infección por BDV en el rebeco pirenaico y en otros rumiantes salvajes y domésticos, dado que los pestivirus no son hospedador-específicos estrictos.

La principal zona de estudio consistió en los Pirineos catalanes y andorranos, pero también se estudiaron otras zonas de los Puertos de Tortosa y Beceite y Sierra Nevada, en España, así como diversas zonas de los Alpes italianos y suizos. Las especies estudiadas fueron el rebeco pirenaico, el muflón europeo (*Ovis aries*), el ciervo (C*ervus elaphus*), el corzo (*Capreolus capreolus*), el gamo (*Dama dama*), el rebeco alpino (*Rupicapra rupicapra*), el íbice (*Capra ibex*), la cabra montesa (*Capra pyrenaica*), y también ganado ovino, caprino y vacuno que pasta en las praderas de alta montaña.

Se determinó la presencia de anticuerpos en suero mediante una técnica de ELISA. Los sueros positivos se enfrentaron a una cepa de diarrea vírica bovina (BVDV, del inglés *bovine viral diaorrhea virus*) y a varias cepas de BDV locales y de referencia en un test de seroneutralización vírica comparada. La detección vírica se llevó a cabo mediante una técnica de reacción en cadena de la polimerasa inversa y/o mediante un test ELISA. Por último, se secuenció la región 5' no codificante de los virus detectados y se aislaron éstos.

El estudio de poblaciones de rebeco pirenaico previamente afectadas por brotes de enfermedad, demostró que la infección por BDV se ha convertido en endémica en algunas poblaciones y este hecho posiblemente esté relacionado con la falta de recuperación de las mismas. Sin embargo, en otras poblaciones que se están recuperando, se han encontrado evidencias de que el BDV ha dejado de circular. El estudio de la población de rebecos de la reserva nacional de caza de Freser-Setcases, demostró que el BDV-4 circula entre los rebecos sin causar ningún efecto negativo en la dinámica poblacional. Es posible que en esta población circulen cepas menos virulentas de BDV-4 que mantienen la seroprevalencia elevada, evitando así la dispersión de otras cepas más virulentas y la aparición de un brote de enfermedad. En todas las poblaciones estudiadas los rebecos presentaron títulos de anticuerpos mayores frente a BDV y la mayoría, más concretamente, frente a BDV-4.

Se detectó una seroprevalencia baja en todos los rumiantes salvajes que comparten hábitat con el rebeco pirenaico. La seroprevalencia en ovino y caprino varió dependiendo del origen geográfico, mientras que en vacuno se detectó una seroprevalencia elevada en todas las zonas. El ovino presentó títulos de anticuerpos más altos frente a las cepas BDV-4, el vacuno frente a BVDV y el caprino frente a BDV-4 y BVDV.

Los estudios realizados en el suroeste de los Alpes italianos revelaron una alta seroprevalencia en el rebeco alpino, sugiriendo que la infección por pestivirus es frecuente en ambas especies del género Rupicapra. En el íbice y la cabra montesa se detectó una seroprevalencia baja en todas las zonas y la mayoría de animales positivos mostraron anticuerpos específicos de BDV. En Suiza, los ciervos y un rebeco alpino mostaron anticuerpos frente a BVDV, mientras que el resto de rebecos mostraron anticuerpos frente a BDV. Este resultado indica que los rumiantes salvajes de este país se pueden infectar con ambas especies de pestivirus.

4. INTRODUCTION

4. INTRODUCTION

Pestiviruses are viruses that are distributed worldwide and cause important economic losses in cattle, swine and small ruminant production systems. Recently, one of these viruses has been associated for the first time with the decline of a wild species, the Pyrenean chamois *(Rupicapra pyrenaica pyrenaica)*. In 2001 and 2002 an outbreak of a previously unreported disease associated with border disease virus (BDV) infection was described in Pyrenean chamois from the Central Pyrenees. After the outbreak had subsided, the chamois population was found to have decreased by about 42%. Subsequent to this first outbreak, several epizooties took place in other chamois populations from the Pyrenees, causing some of them to collapse. The appearance of this new disease reveals a need to study from a conservationist and management standpoint the epidemiology of BDV infection in the Pyrenean chamois in order to improve the knowledge of the consequences of its appearance in this area and to describe the epidemiological scenario. However, pestiviruses are not strictly host-specific and consequently other Artiodactyls such as roe (*Capreolus capreolus*), red (*Cervus elaphus*) and fallow (*Dama dama*) deer, European mouflon (*Ovis aries*) and domestic ruminants that share habitat with Pyrenean chamois should also be included in any such study. These species could potentially become infected with the virus and play a role in the epidemiology of the infection.

The first part of this thesis focuses on the epidemiology of BDV in the Pyrenees. The presence of BDV was investigated after the disease outbreaks in two of the Pyrenean chamois populations that were most seriously affected by the disease. In addition, BDV epidemiology in the Pyrenean chamois population found in the Freser-Setcases National Hunting Reserve was analyzed. This is the only population on the southern face of the Pyrenees that has not yet been affected by an outbreak of this disease. Both of these studies include work on other sympatric wild and domestic ruminants. Finally, the pestivirus surveillance programme undertaken to monitor the Pyrenean chamois and other wild ungulates in the Principality of Andorra was described, as well as the outbreak of BDV that occurred in that country in 2009.

This is the first time that a pestivirus has been responsible for such high mortality in a wild ruminant species, a fact that has raised concern for other wild ungulate populations. The presence of pestiviruses was also investigated in the Alps, an area in which the chamois is common, by analyzing the presence of pestiviruses in the Alpine chamois (*Rupicapra rupicapra*), as well as in other wild and domestic

ruminants in the south-west of the Italian Alps that share pastures with Alpine chamois. The fear exists that other vulnerable wild ungulate species such as the Iberian (*Capra pyrenaica*) and Alpine (*Capra ibex*) ibexes, could be affected by this infection. It was thus essential to examine the role of pestiviruses in these two species and so the presence of pestiviruses in Alpine ibex from the Italian Alps and in Iberian Ibex from two Spanish protected areas, the Ports de Tortosa i Beseit National Hunting Reserve and the Sierra Nevada National Park, was investigated.

In recent years a number of European countries have established bovine viral diarrhoea virus (BVDV) eradication programmes, which has posed the question as to whether wild ruminants serve as a reservoir for this pestivirus and thus contribute to the maintenance of the infection in cattle. The last part of this thesis focuses on this topic with a study of the specificity of pestivirus antibodies in wild ruminants from Switzerland.

5. LITERATURE REVIEW

5. LITERATURE REVIEW

5.1. Mountain ruminants of Europe

Wild ungulates are the largest terrestrial mammals occurring in most European countries, playing an important role in the ecological dynamics. Within this group we find the wild ruminants, which share common ancestors with domestic ruminants, and they are all related phylogenetically. For this reason there exist several examples of pathogens which may be exchanged between different wild ruminant species and between domestic and wild ruminants (for a review, see Pastoret *et al.*, 1988). Next we summarize the main morphological and biological characteristics of different wild ruminant species that inhabit the mountains of Europe.

5.1.1. Chamois

The chamois, genus *Rupicapra*, belongs to the order Artiodactyla, family Bovidae, subfamily Caprinae, tribe Rupicaprini. These group-living ungulates inhabit diverse mountain regions of Europe and the Middle East (Figure 1). Fossil remains suggest an origin of chamois in Eastern Europe or south-west Asia (Lovari, 1987). Two species are considered in nearly all modern taxonomic revisions: the Pyrenean chamois, *Rupicapra pyrenaica*, with three subspecies, and the northern chamois, *Rupicapra rupicapra*, with seven subspecies. However, recently this separation has been questioned by some authors (Corlatti *et al.,* 2011).

Figure 1: Natural distribution range of *Rupicapra* spp. *Rupicapra pyrenaica*: (1) *parva*, (2) *pyrenaica*, (3) *ornata*. *Rupicapra rupicapra*: (4) *cartusiana*, (5) *rupicapra*, (6) *tatrica*, (7) *carpatica*, (8) *balcanica*, (9) *caucasica*, (10) *asiatica.* Adapted from Corlatti *et al.,* 2011.

Pyrenean chamois

The Pyrenean chamois (Figure 2; French: izard or isard, Italian: camoscio pirenaico, Spanish: rebeco pirenaico or gamuza, Catalan: isard, Aragonese: sarrio or chizardo, Occitan: sarri, craba), *Rupicapra pyrenaica*, is a wild ungulate endemic to south-west Europe, where it occurs as three subspecies: *R. p. ornata* in the Apennine Mountains, *R. p. pyrenaica* in the Pyrenees and *R. p. parva* in the Cantabrian Mountains (Shackleton, 1997; Pedrotti and Lovari, 1999a) (Figure 1).

R. p. pyrenaica and *R. p. parva* are listed as Least Concern by the IUCN (International Union for Conservation of Nature) Red List. However, *R. p. ornata* is assessed here as Vulnerable. The 2003 estimate for the total number of *R. p. pyrenaica* was around 53,000 (Herrero *et al.,* 2008). This is now likely to be an overestimate of the population, as many chamois populations have locally declined since then due to outbreaks of disease associated to pestivirus infection (Marco *et al.*, 2009b).

The coat of Pyrenean chamois is ruddy brown in summer and dark brown in winter. A dark mid-dorsal stripe is present. The top of the head and throat are paler in color, as are the shoulders, back of the lower neck, and rump. Both sexes wear horns, which are black and straight finishing in a hook directed caudal and ventral. A dark stripe extends from the base of the horns to the eyes and muzzle. Biometrical measures from Pyrenean chamois range: 100-110 cm of total length, 69-75 cm shoulder height and 25-40 kg weight (males), and 20-32 kg (females) (Pflieger, 1982; ANCGG, 1992; Weber, 2001).

Figure 2: Adult male of Pyrenean chamois (*R. p. pyrenaica*). Photo: I.Marco.

Sexual dimorphism is not very evident in Pyrenean chamois. Male is more heavily-built than female, with a deeper thorax. Width of the neck in the male is longer than length of the mandible, whereas in females the opposite relationship can be observed (Catusse *et al*., 1996) (Figure 3). Moreover, male horns show a strong oval section with a more acute angle than female, whose horns are thinner and round sectioned (Sáenz de Buruaga, *et al*., 1991; Ponti, 1992) (Figure 4). Winter coat is more contrasted in males than in females. Adult males have preputial hair and a mane over the dorsal midline, which is particularly evident in winter (Sáenz de Buruaga *et al*., 1991; Catusse *et al*., 1996) (Figure 5). The age of Pyrenean chamois can be determined easily and accurately by observing the horns, since each winter produces a ring (Pérez-Barbería, 2009) (Figure 6). On the basis of this, generally three age classes are established: kid (less than 1 year old, Figure 7), yearling (up to two years old, Figure 8) and adult (2 years or older).

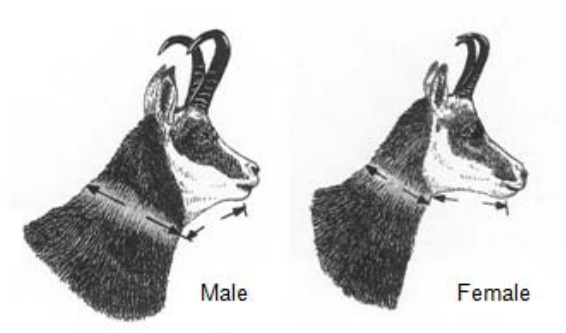

Figure 3: Neck and head in chamois male and female (Catusse *et al*., 1996).

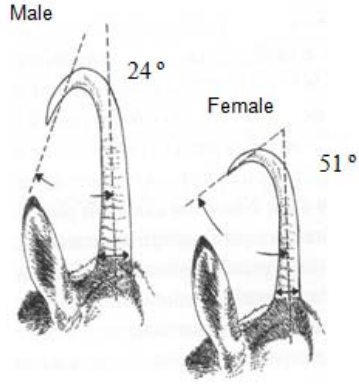

Figure 4: Differences in the angle between male and female horns (Catusse *et al*., 1996).

Figure 5: Pyrenean chamois adult male. Photo: J.P. Crampe.

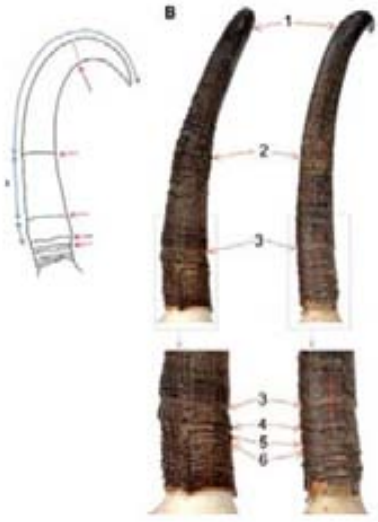

Figure 6: Chamois age determination by counting the rings oh the horns. Adapted from Pérez-Barbería, 2009.

Figure 7: Pyrenean chamois adult female and kid (Photo: J.P. Crampe).

Figure 8: Pyrenean chamois yearling (Photo: J.P. Crampe).

Pyrenean chamois are found at elevations of 400-2,800 m (Palomo and Gisbert, 2002) in alpine meadows, rocky areas, and forested valleys and lower slopes in mountainous regions. This species generally stays above 1,800 meters in alpine meadows during the warmer months of the year (Herrero *et al.*, 2008a). In late fall chamois descend, while usually staying on steep slopes, and in forested areas. Home range of Pyrenean chamois oscillates from 20 to 100 hectares in males to 50 to 500 hectares in females (ANCGG, 1992). Pyrenean chamois feeds predominantly on herbaceous plants (grass and pulses); although up to 300 different plant and several lichen species have been described in its diet (Catusse *et al*., 1996).

Pyrenean chamois is a gregarious species, and females, kids and young up to two years form herds (Figure 9). Young males also can form groups, although normally males older than two years are solitaries (Catusse *et al*., 1996). During rut, males join female groups, and try to keep females grouped in a small area which they defend from other males (ANCGG, 1992). Pyrenean chamois reaches sexual maturity at 18-20 months of age (Catusse *et al*., 1996). Rut takes place from the end of October to the beginning of December. Gestation takes 160 to 185 days and births are singles (Pflieger, 1982).

Figure 9: Pyrenean chamois herd integrated mostly by adult females, kids and yearlings.

Today, the most important threat for Pyrenean chamois is disease. Since 2001, the disease outbreaks associated to pestivirus infection have caused important declines in different populations from the Pyrenean subspecies. On the other hand, in the Cantabrian subspecies, sarcoptic mange outbreaks periodically cause local declines (Fernández-Morán *et al.,* 1997).

Most Pyrenean and Cantabrian populations are hunted (with the exception of those within the National Parks). Chamois is a major game species in Spain, France and Andorra and it is considered as an important economical source for rural communities (Herrero *et al*., 2008a).

Northern chamois

The northern chamois (Figure 10; French: chamois, Italian: camoscio alpino, Spanish: Rebeco norteño, Occitan: camóç), *Rupicapra rupicapra*, is native to several mountains in Europe, including the Carpathian Mountains of Romania, the European

Alps, the Tatra Mountains, and the Balkans. It is also native to parts of Turkey, and the Caucasus (Figure1). The chamois has also been introduced to the South Island of New Zealand. It occurs as seven subspecies: *balcanica*, *carpatica*, *cartusiana*, *rupicapra*, *tatrica*, *asiatica* and *caucasica* (Shackleton, 1997; Pedrotti and Lovari, 1999a). The northern chamois is widespread with a population of over 440000 individuals and it is assessed as Least Concern in the IUCN Red List (Aulagnier *et al*. 2008a). However, several chamois subspecies are considered as globally threatened (*R. r. tatrica* is Listed as Critically Endangered and *R. r. cartusiana* as Vulnerable). The alpine chamois *R. r. rupicapra,* comprises the bulk of the global northern chamois population, and is widespread and abundant in the mountains of Austria, Germany, Italy, Switzerland, eastern France, Slovenia and Croatia (Aulagnier *et al*., 2008a).

The summer coat of northern chamois has a reddish-brown color which turns to dark brown in winter. The face is white with pronounced black stripes below the eyes, a white rump and a black stripe along the back. Jaw, thin and throat are also white. Both sexes are similar in appearance, but there are some morphological differences (see Pyrenean chamois). Biometric measures from northern chamois are: 110-130 cm of total length, 70-85 cm shoulder height and 25-35 kg weight (females) and 30-45 kg (males) (Wilso and Mittermeier, 2011a).

Figure 10: Northern chamois (*Rupicapra rupicapra*) male. Photo: I. Marco.

Northern chamois occurs from 500 m to 3,100 m (Aulagnier *et al.*, 2008a). This ungulate inhabits steep, rocky areas in the mountains, utilizing a variety of habitats including alpine meadows, open rocky areas, mixed broadleaf woodland, and coniferous woodland (Pedrotti and Lovari, 1999a). Seasonal movements involve

migrations between low-elevation forests in winter and subalpine and alpine grasslands in spring. Northern chamois feeds on grasses, herbs, leaves of trees, buds, shoots, and fungi (Sägesser and Krapp, 1986).

Northern chamois is a gregarious species. During the non-mating season males tend to be solitary and females and young form separate herds. The rut takes place in late November/early December. Females gestate for 165-175 days, and have one offspring per pregnancy (Wilso and Mittermeier, 2011a).

The subspecies *R. r. balcanica*, *R. r. cartusiana*, and *R. r. tatrica* are threatened by the deliberate introduction of subspecies from other geographic areas (especially *R. r. rupicapra*) (Shackleton, 1997). Competition with domestic livestock and introduced species is a threat to the more vulnerable subspecies. *R. r. rupicapra* populations suffer periodic outbreaks of sarcoptic mange, occasionally causing up to 80% mortality (Rossi *et al.,* 1995). *R. r. rupicapra* is a major game species in several countries of Europe and most of the populations are hunted (with the exception of those within the National Parks).

5.1.2 Alpine and Iberian ibex

Ibexes belong to the order Artiodactyla, family Bovidae, subfamily Caprinae, tribe Caprini, genus Capra.

Alpine ibex

The Alpine ibex (Figure 11; French: bouquetin de Alpes, Italian: Stambecco, Spanish: íbice), *Capra ibex*, is endemic to Europe, where its native range is the Alps of France, Switzerland, Austria, Germany, and northern Italy (Shackleton, 1997). It has been introduced to Slovenia and Bulgaria (Pedrotti and Lovari, 1999b). The ibex was driven very close to extinction due to massive hunting in the early 19th century, and with the exception of the population in the Gran Paradiso National Park, all current populations originate from re-introductions or introductions. In the 1990s it was estimated that c.30, 000 ibex lived in the Alps (Pedrotti and Lovari, 1999b). This species is listed as Least Concern by the IUCN (Aulagnier *et al*., 2008b).

The winter coat of adult males is chestnut brown with white belly. Sides of the body, lower chest, and legs are dark and tail is black. Females are uniformly brown but with a black tail and dark stripe along lower portion of body. The summer coat of males and females is yellowish-brown, but males show paler neck, forehead, and flanks. The horns are present in both sexes and its length is 75-102 cm (males) and up

to 35 cm (females). The horns grow throughout life, forming a distinct growth segment (annulus) each year. The count of annulus allows determining the age. Biometric measures from Alpine ibex are: total length 115-135 cm (males) and 55-100 cm (females), shoulder height 65-95 cm; weight 70-120 kg (males) and 40-65 kg (females) (Wilso and Mittermeier, 2011b).

Figure 11: Alpine ibex (*Capra ibex*) adult male. Photo: I. Marco.

Alpine ibex occurs from 500 to 3,000 m (Pedrotti and Lovari, 1999b). This species typically inhabit open, rocky habitats at high altitude, above the tree line. Ibexes prefer steep, south-facing slopes with rugged topography and grassy vegetation. Below the tree line, at subalpine levels, ibexes are usually found in open, sunny woodland interspersed with rocky outcrops (Nievergelt and Zingg, 1986; Pedrotti and Lovari, 1999b). They migrate seasonally to different altitudes, spending the harsher winter months at medium elevations. This species feed on alpine grasses, herbaceous plants and shrubs (Pedrotti and Lovari, 1999b.).

Alpine ibexes occur in maternal herds of 10-20 members, while males roam solitarily or associate with other males of similar age and social status. Mating occurs in December-January. Dominant males do most of the courting and mating and attempt to prevent other bucks from mating with the guarded females. Females gestate for about 165-175 days, and usually carry one kid per pregnancy (Aulagnier *et al.,* 2008b; Wilso and Mittermeier, 2011b).

The main threats for the Alpine ibex are the reduced genetic diversity, the founder effect and minimum viable populations (Shackleton, 1997; Maudet *et al.,*

2002). Alpine ibex are legally hunted in some areas (e.g. Bulgaria, Switzerland, Austria, Slovenia), although hunting is completely prohibited in several countries (Shackleton, 1997).

Iberian ibex

The Iberian ibex (Figure 13; French: bouquetin, Spanish: cabra montesa), *Capra pyrenaica*, comprises four subspecies: *C.p. pyrenaica*, *C.p. lusitanica*, *C.p. victoriae*, and *C.p. hispanica*. This species is endemic to the Iberian Peninsula. The subspecies *C.p. lusitanica* and *C.p. pyrenaica* are extinct. *C.p. victoriae* occurs in the central Spanish mountains (Sierra de Gredos), and has been re-introduced to a number of additional sites in Spain and northern Portugal (Palomo and Gisbert, 2002; Herrero *et al.*, 2008b.). *C.p. hispanica* occupies the mountains that run along the Mediterranean coast, from the Ebro river to the rock of Gibraltar (where it no longer occurs), as well as the Sierra Morena. It has been reintroduced in Montserrat and Sierra de Guara (García-González and Herrero, 1999). The most recent updated distribution of the Iberian ibex is shown in Figure 12 (Acevedo and Real, 2011).

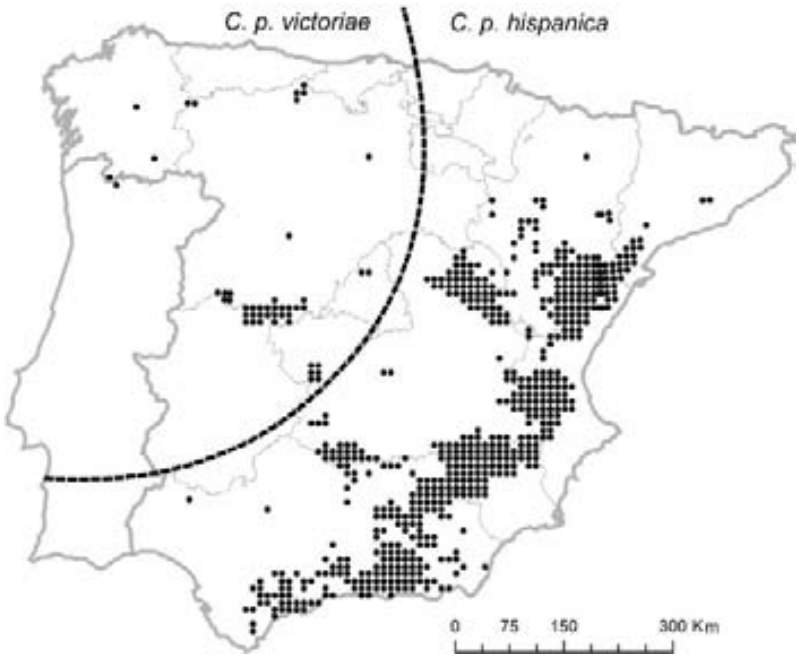

Figure 12: Current distribution of the Iberian ibex. The discontinuous line represents the distribution of the two subespecies, *C. p. victoriae* in the north-west, and *C. p. hispanica* in the south and east. Adapted from Acevedo and Real, 2011.

The Iberian ibex shows remarkable sexual dimorphism, males being greater in size and weight with larger horns than females (Fandos, 1991; Granados *et al*., 1997). The winter coat of adult males is greyish to pale brown to whitish on the side of head, throat, und upper front and sides of the neck, as well as on the upper sides of the body extending to hind quarters. The forehead, beard, front and back of the neck and front of the shoulder and legs are black and there is a dark mid-dorsal stripe. The summer coat is cinnamon-colored. Females show a uniformly brown coat, except for white underparts and a mid-dorsal stripe (Acevedo and Cassinello, 2009). The horns grow throughout life, forming a distinct growth segment (annulus) each year. The count of annulus allows determining the age. Biometric measures from Iberian ibex are: total length 108-155 cm (males) and 97-130 cm (females), shoulder height 65-85 cm (males) and 65-76 cm (females); weight 50-90 kg (males) and 31-41 kg (females) (Wilso and Mittermeier, 2011c).

Figure 13: Iberian ibex (*Capra pyrenaica*) adult male (left) and female (right). Photo: I. Marco.

The species is found from sea level to 3,400m (Palomo and Gisbert, 2002). It shows big habitat plasticity, but it invariably occurs in rocky habitats. Rocky areas on the coast may be used, although cliffs and screes interspersed with scrub or pine trees are the most typical habitats. The Iberian ibex is a mixed feeder (browser and grazer). This species shows high feeding plasticity, and the percentage of each type of resource consumed may vary altitudinally (Martínez, 1994), geographically (Granados *et al*., 2001) and seasonally (García-González and Cuartas, 1992).

The sexes are segregated for most of the year and here are male-only groups and mixed groups of females, juveniles and subadults. During the rutting season adult males and females come together (Granados *et al*., 2001). However, this pattern may

vary (Acevedo and Cassinello, 2009). Ibex altitudinal dispersion occurs according to resources availability, e.g. heading to rich, high altitude areas in summer, which are usually covered by snow in winter (Gonçales, 1982; Escós, 1988; Travesí, 1990).

Mating occurs in November-December. Dominant males do most of the courting. Gestation lasts 175-185 days, and usually one kid is carried per pregnancy.

The major disease threat for Iberian ibex is sarcoptic mange, caused by *Sarcoptes scabiei* (Shackleton, 1997; Leon-Vizcaíno *et al.*, 1999; Herrero and Pérez, 2008). Alteration and fragmentation of the habitat may also impact negatively upon certain Iberian ibex populations. The founder effect also represents a threat for the Iberian ibex populations (Pérez *et al*., 2002). The impact of hunting (predominantly for trophies) has not been scientifically assessed (J. M. Pérez, personal communication), but poaching of large dominant males might alter gene flow.

5.1.3 Other species

European mouflon

The European mouflon, *Ovis aries* (Figure 14; *Ovis orientalis musimon* in IUCN, 2004), belongs to order Artiodactyla, Family Bovidae, subfamily Caprinae, tribe Caprini, genus Ovis. It is a wild sheep endemic to Corsica and Sardinia. However nowadays it is no longer considered as a wild species and the IUCN has taken this species off the list. Recent studies conclude that this species descended from the domestic sheep, and it has been given the scientific name of *Ovis aries*. The European mouflon has been introduced in central Europe and the sole natural populations persist on the islands of Sardinia and Corsica (Santiago-Moreno *et al.*, 2004).

Mouflons have a red-brown short-haired coat with a dark back-stripe, light colored saddle patch. Horns are only present in males, but some females can present small rudimentary horns. The male's horns are curved in almost one full revolution (up to 85 cm). Mouflon biometric measures are: total length 120-140 cm, shoulder height 65-75 cm (females) and 70-80 cm (males), and weight 25-35 kg (females) and 35-55 kg (males) (Santiago-Moreno *et al.*, 2004).

The European mouflon lives in lightly wooded areas with plenty of under-growth on the middle slopes of mountains. Mouflons graze on short grasses, heather, and shrubs. These ungulates live in small or medium-large sized herds. In the summer, rams are solitary or are found in small groups apart from the ewes. For most of the year, the European mouflon is sedentary and travels no further than what is necessary

for grazing. The herd instinct is very strongly developed, and the herd tends to remain in one area.

Figure 14: European mouflon (*Ovis aries*) adult male.

Mating occurs generally in October-January and gestation lasts 152-157 days (Santiago-Moreno *et al.*, 2004). The main threat for European mouflon is the intermixing with domestic sheep. The trophy of the rams is strongly appreciated by hunters and mouflon hunting is a common activity in many countries of Europe.

Red deer

The red deer, (Figure 15; French: cerf, Italian: cervo, Spanish: ciervo, Catalan: cèrvol), *Cervus elaphus,* belongs to family Cervidae, tribe Cervini, genus Cervus. It contains six subspecies (*C.e. elaphus*, *C.e. bactrianus*, *C.e. barbarus*, *C.e. corsicanus*, *C.e. maral*, and *C.e. yarkandensis*). This species has a large global distribution extending from Europe and North Africa through central Asia, Siberia, the Far East and North America (Koubek and Zima, 1999). Only *C.e. elaphus* is naturally distributed in Continental Europe. This species is listed as Least Concern in the IUCN red list (Lovari *et al.,* 2008a).

The summer coat is reddish or reddish-brown, with grayish legs and whitish belly. The winter coat is grayish-brown. Adult stags have a neck mane. Newborn calves have a brown coat with scattered white spots. Antlers of adult stags are long, cylindrical, and typically well branched. Biometric measures are: total length 180-205

cm (stags) and 165-180 cm (hinds), shoulder height 105-130 cm (stags) and 95-115 cm (hinds), and weight 110-220 kg (stags) and 75-120 kg (hinds) (Wilso and Mittermeier, 2011d) .

Figure 15: Red deer (*Cervus elaphus*) hinds with their offspring.

It inhabits open deciduous woodland, upland moors and open mountainous areas (sometimes above the treeline), natural grasslands, pastures and meadows (Koubek and Zima, 1999). In woodland, its diet consists mainly of shrub and tree shoots, but in other habitats it also consumes grasses, sedges and shrubs.

The basic social unit is the matrilineal family group, with a dominant old hind associated with her daughters and their dependent offspring. Stags live singly or form male herds. Rutting season is in September-October. Competition among stags is high and they are involved in roaring contests, dominance displays and overt fights with a high risk of serious injury. Calving occurs in June-July following a gestation of 235 days. Females drop single calves in late spring (Wilso and Mittermeier, 2011d).

The introduction of animals from North America to Europe has resulted in the spread of parasites and diseases to previously unaffected subpopulations. Red deer hunting is regulated in most of the existing populations in Europe (Lovari *et al*., 2008a).

Roe deer

Roe deer (Figure 16; French: chevreuil, Italian: capriolo, Spanish: corzo, Catalan: cabirol, Occitan: cabròl), *Capreolus capreolus,* belongs to order Artiodactyla, family Cervidae, tribe Capreolini, genus Capreolus. The following subspecies are recognized

C.c. italicus, *C.c. garganta*, *C.c. capreolus,* and *C.c. coxi*. It is found through most of Europe (with the exception of Ireland, Iceland and Mediterranean Islands), Caucasus and Near East. Roe deer is listed as Least Concern in the IUCN red list. However, subspecies *C.c. italicus* is rare and faces serious threats (Lovari *et al*., 2008b).

This species is a small deer. The winter coat is greyish. Bucks have a large white kidney-shaped rump patch. In does this rump patch is heart-shaped and there is a tuft of hairs close to the vulva. The area around the muzzle is black, the lips and chin are white. Antlers are short (16-23 cm in adult bucks). Biometrical measures for roe deer are total length 107-127 cm, shoulder height 65-84 cm, weight 20-30 kg (adult bucks) and 17-29 kg (adult does). The roe deer found in the Mediterranean are smaller than those found in the East (Wilso and Mittermeier, 2011e).

Figure 16: Roe deer (*Capreolus capreolus*) doe. Photo: O. Giordano

Roe deer occurs from sea level up to 2,400 m in the Alps (von Lehmann and Sägesser, 1986). It occupies a wide variety of habitats, including deciduous, mixed or coniferous forests, moorland, pastures, and arable land. It prefers landscapes with a mosaic of woodland and farmland (Stubbe, 1997). Roe deer are typical selective feeders or concentrate selectors, preferring soft food rich in soluble carbohydrates and proteins.

Roe deer are not very social, living alone or in small groups. Bucks are solitary during the territorial period (spring and summer), does in the last part of their

pregnancy and parturition time. Roe deer are typically sedentary. The rutting season takes place from mid-July to mid-August. Implantation of the embryo occurs in January and the gestation period is of 150 days. Does give birth to 1-3 fawns (Wilso and Mittermeier, 2011e).

Fallow deer

The Fallow deer (Figure 17; French: daim, Italian: daino, Spanish: gamo, Catalan: daina), *Dama dama*, belongs to order Artiodactyla, family Cervidae, tribe Cervini, genus Dama. This species was formerly distributed in Anatolia and Turkey. It has been introduced into Europe from ancient times and later into many other countries in north and South America, South Africa, Australia, New Zealand and Fiji Islands (Masseti and Mertzanidou, 2008).

The summer coat is reddish-brown, with white spots on the back and the upper half of the flanks. Winter coat is gray-brown. Biometric measures are: total length 145-155 cm (bucks) and 130-145 cm (does), shoulder height 85-95 cm (bucks) and 70-80 cm (does), weight 50-80 kg (bucks) and 35-45 kg (does). Antlers which are found only in males are characteristically palmated (Wilson and Mittermeier, 2011f).

Figure 17: Fallow deer (*Dama dama*) female. Photo: SEFaS.

Fallow deer is a highly adaptable species that can survive in a wide range of habitats, including forest, scrubland, grassland, pastureland and plantations. It is a preferential grazer, feeding on grass and ground vegetation among trees, and herbs and forbs in neighbouring fields.

Males and females live apart the most part of the year. The basic social unit is a group of one or two females with their fawns and yearlings does. Female groups can coalesce in larger herds. Males are solitary or form less stable male groups. Rutting season is October; gestation length is 229-234 days and does give birth to one fawn (Wilson and Mittermeier, 2011f).

5.2. Pestivirus

5.2.1. Taxonomy

The genus Pestivirus, Flavivirus, and Hepacivirus constitute the family Flaviviridae (Pringle, 1999). Pestiviruses are enveloped spherical viruses, 40–60 nm in diameter approximately. The genome consists of a positive single-stranded and nonpolyadenylated RNA molecule, 12.3 kb in length that contains one large open reading frame flanked by 5' and 3' noncoding regions (NCR) (Collett *et al.*, 1988; Meyers *et al.*, 1989; Ridpath and Bolin, 1995; Becher *et al.*, 1998). In the virus-encoded polyprotein, the viral proteins are arranged in the following order (from the N to the C terminus): N-terminal autoprotease (N^{pro}); capsid protein C and envelope proteins (NS2, NS3, NS4A, NS4B, NS5A AND NS5B) (Meyers and Thiel, 1996) (Figure 18).

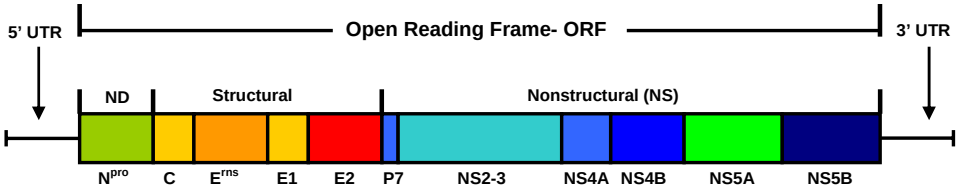

Figure 18: Representation of final protein products of the single open reading frame (ORF) and 5' and 3' untranslated (UTR) regions of a non-cytopathic border disease virus.

Four species of pestiviruses are officially accepted by the International Committee on Taxonomy of Viruses (ICTV): bovine viral diarrhea virus 1 (BVDV-1); bovine viral diarrhea virus 2 (BVDV-2); border disease virus (BDV) and classical swine fever virus (CSFV). The viruses were named after the disease that they cause, so traditionally pestiviruses isolated from sheep and goats were termed BDV, those from cattle BVDV, and those from swine CSFV (Nettleton, 1990). Ruminant pestiviruses, i.e.

BVDV and BDV, are not strictly host-specific and they have the ability to cross species barriers and to infect a wide range of Artiodactyla species (Vilcek and Nettleton, 2006). On the other hand, CSFV has not yet been identified outside the natural host, and it is restricted to domestic pigs and wild boars (Liu *et al.*, 2009). A fifth tentative species is represented by the strain H138, which was isolated from a giraffe in Kenya (Becher *et al.*, 2003). When an ICTV Subcommittee is uncertain about the taxonomic status of a new species or about assignment of a new species to an established genus, the new species is listed as a tentative species (ICTV, 2002).

In addition to the established species, there are three groups of recently identified but unclassified pestiviruses. These are Pronghorn virus, isolated from a blind pronghorn antelope in the United States (Vilcek *et al.*, 2005a); Bungowannah virus, isolated from an Australian outbreak of myocarditis syndrome in swine (Kirkland *et al*., 2007), and the HoBi-like viruses, isolated from cattle (Bauermann *et al.*, 2012).

All pestiviruses are antigenically related but criteria for virus species demarcation include: nucleotide sequence relatedness, antigenic relatedness as measured in cross-neutralization assays, and host of origin (Nettleton *et al.,* 1998). A virus species is defined by the ICTV as a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche. A "polythetic class" is one whose members have several properties in common, although they do not necessarily all share a single common defining property (ICTV, 2002). Different regions of pestiviral genomes have been employed to study their genetic diversity and classification into genotypes and species mostly relies on phylogenetic analysis, usually performed after alignment of 5'UTR, Npro or E2 sequences (Becher *et al.*, 1999).

Currently, BVDV-1 is divided into 11 genetic subgroups (Vilcek *et al.,* 2001), BVDV-2 into two subgroups (Vilcek *et al.*, 2005b), BDV into seven subgroups (Figure 18) (Giammarioli *et al.*, 2011) and CSFV into three (Greiser-Wilke *et al.*, 2006). However, taxonomy of pestiviruses, especially ruminant pestiviruses, is in a dynamic state and uncertainty exists in the classification of pestiviruses (Liu *et al.*, 2009).

5.2.2. Bovine viral diarrhoea virus

Bovine viral diarrhoea virus is a worldwide distributed virus which comprises two species or genotypes: BVDV type-1 (BVDV-1) and BVDV type-2 (BVDV-2). The two genotypes have similarities but are different genetically (Ridpath, 2003). BVDV-2 is widely distributed in North-America, but infection with these viruses have been reported occasionally in Germany, Italy, France, Belgium and Austria (Wolfmeyer *et al.*,

1997; Pratelli *et al*., 2001; Couvreur *et al.,* 2002; Vilcek *et al.,* 2003). BVDV infection causes high economic losses and for this reason several European countries have started eradication programs (Houe *et al.*, 2006). The host range for BVDV includes most even-toed ungulates, including swine, but domestic cattle seem to be the primary host. Both BVDV genotypes contain cytopathic (cp) and non-cytopathic (ncp) biotypes. These biotypes are identified by presence or absence of cytopathic effect in susceptible cell cultures (Bolin, 1995).

Infection of cattle with BVDV induces three disease conditions; acute bovine diarrhoea (BVD), congenital persistent infection, and mucosal disease (MD). Acute BVD occurs after birth and is induced by primary postnatal infection with either cp or ncp BVD. Clinical signs are variable and consist of respiratory, enteric and/or reproductive disease and fever. The severity of clinical signs ranges from clinical unapparent to fatal, depending of the virulence of the viral strain and other factors (host's health and reproductive status, and presence of secondary pathogens). Acute infection with some ncp BVDV-2 causes a severe clinical disease consisting of a haemorrhagic syndrome. This form of BVDV is called severe acute BVD and it is characterized by prolonged pyrexia, thrombocytopenia and leukopenia (Ridpath, 2003). Acute infections with BVD result in seroconversion and antibodies probably persist for life (Fredriksen *et al.*, 1999).

The second disease condition, congenital persistent infection, is a consequence of a prenatal infection with nc BVD between the second and fourth month of gestation. At this period, the immune system of the foetus is not developed, and the newborn will recognize the virus as a self-antigen. It will be a persistently infected (PI) animal, characterised by a highly specific immunotolerance to the virus. PI animals shed large quantities of virus throughout their lives and play a key role in maintaining the virus in the population (Houe, 1999). However, in quantitative terms persistent infections are rare and the estimated incidence of PI animals in the cattle populations uses to be around 1% or lower (Peterhans and Schweizer, 2010). Persistent infections may be associated with poor performance and an increased frequency of other infections, but some PI cattle may be normal in health and development.

Persistently infected cattle eventually develop a third form of disease: MD. It is characterised by severe diarrhoea, mucosal erosions, massive destruction of lymphoid tissue in the gastrointestinal tract, and fever. This form of disease is invariably fatal (Liebler-Tenorio *et al.*, 2000). Pairs of cp and ncp viruses which differ only in one structural protein are isolated from these animals (Ridpath, 2003). The cp BVDV that

induces MD in a persistently infected animal may originate by spontaneous mutation from the persistent ncp BVDV or it may be created by superinfection of PI animals with a cp BVDV (Peterhans and Schweizer, 2010).

5.2.3. Border disease virus

Border disease (BD) was firstly reported in 1959 in sheep from the border region between Wales and England (Hughes *et al.,* 1959). The aetiological agent of this disease, BDV, has a worldwide distribution, and it causes disease mainly in sheep although disease occasionally has been reported in goats (Nettleton *et al.*, 1998a). The economic importance of BD lies in the reproductive failure and the abortions caused by the infection, and the low survival rate and low carcass scores of affected lambs (García-Pérez *et al*., 2008).

BDV genetic diversity is greater than other pestivirus species and currently BDV isolates segregate into seven phylogenetic subgroups (Valdazo-González *et al*., 2007). BDV-1 has been isolated in sheep from the USA (Sullivan *et al.*, 1997), UK (Vilcek, 1997), Australia (Becher, 1999) and New Zealand (Vilcek, 1998); BDV-2 in ruminants in Germany (Becher, 2003); BDV-3 in Switzerland (Stalder *et al.*, 2005) and Austria (Krammeter-Froetscher *et al.*, 2007); BDV-4 in Spain (Arnal *et al*., 2004; Valdazo-González *et al.,* 2007) and BDV-5 and -6 in France (Dubois *et al.*, 2008, Figure 19). Pestivirus isolates from Turkey seem to form the seventh subgroup (Oguzoglu *et al.*, 2009). Recently, a novel group, putatively BDV-7, has been isolated in small ruminant flocks in Italy (Giammarioli *et al.,* 2011).

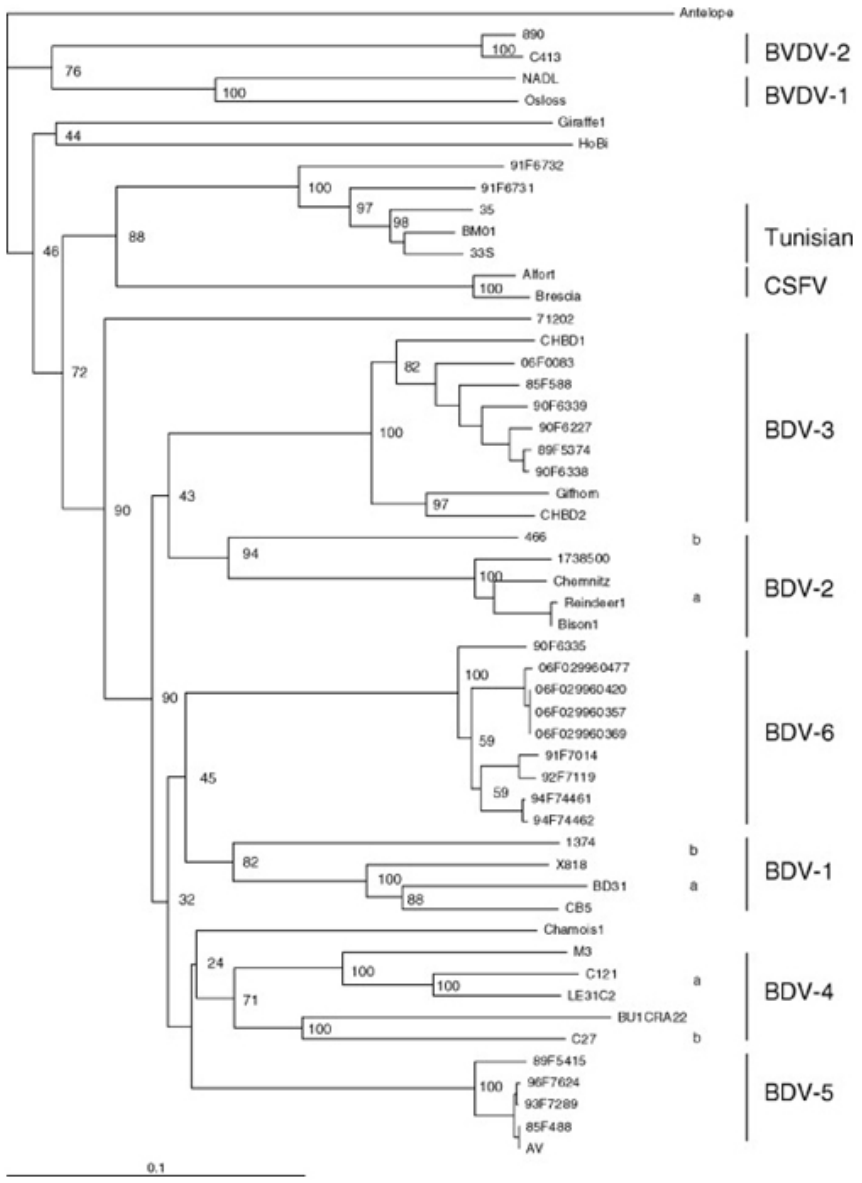

Figure 19: Neighbour-joining phylogenetic tree present in the study of Dubois *et al*. (2008). The tree has been constructed using 489 nt from the Npro region and proposes new BDV subgroups.

BDV postnatal infections are transient and they are also called acute infections. Horizontal transmission between animals follows the oro-nasal route and generally virus can be detected in serum between days 4 and 11 post infection. After a short-period of viraemia, neutralizing antibodies appear in serum and after this the animals remain immune. Postnatal infection tends to be mild and is characterized by a mild pyrexia and transient lymphopaenia (Nettleton, 1990). However severe outbreaks of

disease with high mortality have been reported occasionally in relation with BDV acute infections (Chappuis *et al*., 1984) and a mucosal disease syndrome has been described in PI sheep (Monies *et al.*, 2004).

When infection of pregnant ewes occurs, they suffer a transient infection as described above. But like all pestiviruses, BDV has the ability to cross the placenta and infect the foetus with variable consequences. The infection can cause embryonic and foetal death in any stage of gestation, resulting in reproductive failure. But if infection occurs before day 60 of gestation, before foetal immunocompetence, and the foetus survives, the newborn will be a PI (as it has been described before with BVDV). PI lambs can show several clinical features. They appear as small and weak animals and show a range of neurological signs, such as tremors. The affected lambs typically have alterations of fleece, which appears pigmented and straight. The abnormal fleece together with the tremors has given the familiar name of "hairy shaker" to these lambs (Nettleton *et al.,* 1998). Their appearance is weak, but they can also be apparently normal. Generally they show a poor growth and lower life expectancy. If the infection occurs in the late gestation, when the immune system of the lamb is already developed, the foetus will clear the virus and the newborn will have pre-calostral antibodies against the virus (Nettleton *et al.*, 1992).

Regarding to BDV epidemiology, the studies indicate that horizontal transmission is dependent on the degree of contact between infected and non-infected animals and may be highly efficient in housed sheep (Barlow *et al.,* 1980; Nettleton *et al.*, 1992) but less so in sheep raised on pasture (Bonniwell *et al.*, 1987). Since no commercial BDV vaccines are available, BD control is based on identifying and eliminating PI animals and preventing infection of susceptible pregnant ewes (Berriatua *et al.*, 2004).

Serologic surveys have demonstrated widespread natural infection with pestivirus in goats in many countries (Nettleton *et al.*, 1998). However, BD occasionally has been described in this species (Loken *et al.*, 1982; De Mia *et al.*, 2005). The affected kids showed body tremors and locomotor dysfunction. It seems that acutely infected goats usually do not transmit pestivirus to other animals and, in natural infections there are very few live born PI kids. For this reasons it has been suggested that PI sheep are the main reservoir for BDV in goats (Loken, 1995).

5.2.4. Classical swine fever virus

Classical swine fever (CSF), also known as hog cholera is a highly contagious viral disease of swine. CSFV can be divided phylogenetically into three genotypes: 1, 2, and 3, every genotype consisting of three to four subgenotypes (Paton *et al.,* 2000). CSFV has a nearly worldwide distribution, but it's not present in North America and Australia. In Europe CSFV is endemic in wild boar in Italy, Germany, and parts of France and Switzerland (Gregg, 2002). Clinical features of CSF vary widely and depend of virus virulence but also with the age, breed and condition of the host. Acute, chronic and prenatal forms of CSF can be distinguished. The incubation period is also variable, and ranges from three to 15 days. Nowadays most outbreaks are associated with moderately virulent strains. The acute form of disease caused by these strains, consists of high fever, mild lethargy, anorexia, diarrhoea, alopecia, skin lesions, mild haemorrhages in lymphoid organs, transient leukocytopenia (or none), and low mortality. On the other hand, the classic virulent disease is characterized by high fever, extreme lethargy, anorexia, conjunctivitis, constipation followed by diarrhoea, haemorrhages in several organs, neurological signs, leukocytopenia, immunosupression and high mortality (within one to three weeks) (Moennig *et al.*, 2003). However, nowadays this form of disease is uncommon since the late 1960' moderate-virulence and low-virulence strains have become more predominant in Europe and Central America (Gregg, 2002).

The chronic form of CSF develops when pigs are not able to establish an effective immune response against the infection. Initial signs are similar to the acute infection. Later, predominantly non-specific signs, like wasting and chronic enteritis, are observed. The course of disease is always fatal, and animals may survive for 2–3 months. Pathological changes are less typical, especially the lack of haemorrhages on organs and serosae. In animals displaying chronic diarrhoea, necrotic and ulcerative lesions on the ileum, the ileocaecal valve and the rectum are common (Paton *et al.*, 2000; Moennig *et al.*, 2003).

Congenital infection of foetuses uses to cause sequelae, including, abortion, stillbirth, mummification, or birth of weak and dying piglets. However some piglets exposed to CSFV in the first trimester may be born healthy, but become PI with CSFV (Van Oirschot, 1979). These pigs are likely to have secondary infections, and may live for weeks or for a year, shedding virus throughout their life, playing a key role in spreading CSFV (Gregg, 2002).

5.3. Ruminant pestivirus infections in wildlife

As described before, ruminant pestiviruses are not strictly host-specific. In addition to cattle, sheep, their natural hosts, natural infection with ruminant pestiviruses occur in other domestic ungulates like goats and pigs, but also in a wide range of species of captive and free-living ruminants (Loken, 1995; Vilcek and Nettleton, 2006)

5.3.1. Border disease in Pyrenean chamois

In 2001 and 2002 an outbreak of a previously unreported disease associated to BDV infection (Arnal *et al.*, 2004; Hurtado *et al.,* 2004) was described in Pyrenean chamois from the Central Pyrenees, concretely in the Alt Pallars-Aran National Hunting Reserve (NHR). After the outbreak, the population was found to have decreased by about 42%, most probably due to the disease. This was the first time a BDV had been associated with an outbreak of a high-mortality disease in a wild species (Marco *et al.*, 2007).

In all affected animals a pestivirus was detected by ELISA of antigen detection and RT-PCR tests. Moreover, immunohistochemistry studies detected positive staining in several tissue samples. A BDV was isolated, characterized and defined as the etiological agent of the disease and phylogenetic analysis typed the chamois pestivirus as BDV-4 genotype (Arnal *et al.,* 2004; Hurtado *et al.*, 2004).

After this first disease outbreak several epizooties took place in other chamois populations from the Pyrenees. During 2005 a disease outbreak led to collapse the chamois population from the Cerdanya-Alt Urgell NHR. The estimated cumulative rate of decrease was 85.6%. In June of 2005, the disease spread to the nearby Cadí NHR (Figure 20) and private hunting areas, causing an estimated cumulative rate of decrease of 63% (Marco *et al.*, 2009b). During the summer of 2011 a disease outbreak was described in the Benasque valley, in Aragon. The mortality of this episode has not been estimated yet.

Figure 20: During the disease outbreak in the National Hunting Reserve of Cadí, a high mortality was described and several chamois carcasses were found in valley floors and couloirs.

Clinical manifestations of the affected chamois are variable. Most of the diseased animals have depression, weakness and movement difficulties. Presence of abnormal behaviour is common, with lack of fear to humans and absence of flight reaction. A typical sign are the different degrees of alopecia with an associated skin hyperpigmentation. At necropsy cachexia is described in all animals, and secondary infections are commonly observed. The most frequents are pneumonia but also various degrees of different parasitism, and abscesses. Hystopathological studies reveal microscopic lesions in the brain, mainly edema, gliosis, espongiosis, and cariorrexis. Skin lesions consist of follicular atrophy, mild to moderate epidermal hyperplasia with orthokeratotic hyperkeratosis and follicular hyperkeratosis, and hypermelanosis (Marco *et al.,* 2007). The affected chamois also have alterations in different haematological and biochemical parameters. Most remarkable changes are anemia and lymphopenia. This late would lead to immunosuppression and explain the high rate of secondary infections observed in the chamois affected by this disease (Fernández-Sirera *et al.*, 2011b).

Figure 21: Pyrenean chamois showing the typical signs of BDV infection: lack of fear in front of humans, alopecia and skin hyperpigmentation.

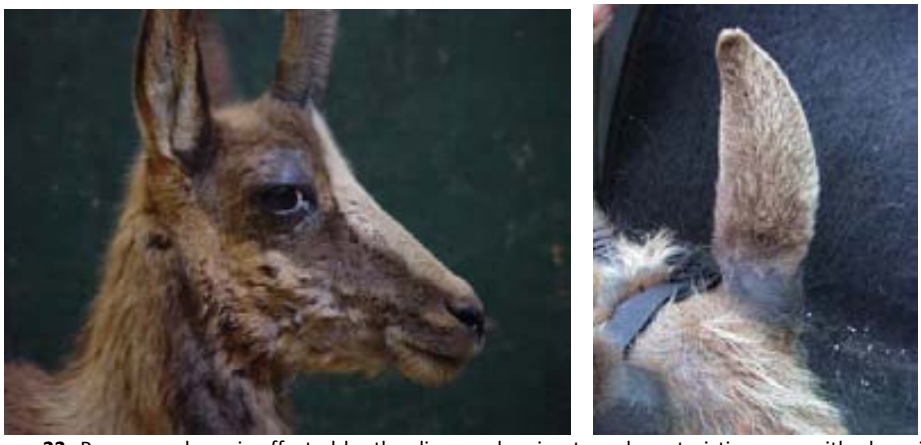

Figure 22: Pyrenean chamois affected by the disease showing two characteristic zones with alopecia: periorbicular (left) and periauricular (right).

Figure 23: Two different cases of Pyrenean chamois affected by the BDV associated disease. Both animals have cachexia. The degree of alopecia varies between cases.

As a consequence of the disease outbreaks, several studies raised with the aim to investigate the presence of pestiviruses in the Pyrenean chamois populations. (Marco *et al.*, 2011) performed a retrospective study in the Catalan Pyrenees with samples collected between 1990 and 2000 with the aim to detect evidence of pestivirus infection. This study revealed that BDV infection has been present in the chamois population since at least 1990, 11 years before the first outbreak of disease. Moreover, this study revealed a high seroprevalence (48.6%) of pestivirus antibodies in the analyzed Pyrenean chamois. An epidemiological survey conducted from 1995 to 2004 at Orlu, in the French Pyrenees, detected also high seroprevalence (70.3%) and viroprevalence (10.2%) (Pioz *et al.*, 2007). Results of this study corroborate the results of Marco *et al.*, (2011) demonstrating that a pestivirus had been present in the chamois population of Orlu since 1995, before the first outbreak of disease in the Pyrenees.

Despite all these studies, there is a general lack of information about the pathogenesis of BDV infection in the chamois. With the aim to increase the knowledge in this area, (Cabezón *et al.,* 2011) inoculated seronegative and seropositive chamois with a BDV isolated from naturally infected chamois. This represents a turning point in the study of the chamois pestivirus because this experiment confirmed that BDV is the primary agent of the disease in the Pyrenean chamois. This study also determined that previously acquired humoral immunity is protective against the infection. Another experimental study showed that the inoculation of pregnant chamois females lead to birth to a PI offspring that died prematurely (Gilbert, Schelcher and Vautrain, unpublished observation). This study leaves the door open to the existence of PI chamois in nature and their epidemiological role.

Recently, Vilcek *et al.* (2010) described the full-length genome sequence of the pestivirus strain H2121 which was isolated from Pyrenean chamois by Arnal *et al.* (2004).

5.3.2. Ruminant pestivirus infections in wildlife other than Pyrenean chamois

Regarding BVDV, different studies of the 70' and the 80' have demonstrated the contact of free-living ruminants with this virus. BVDV antibodies have been detected in several members of the Cervidae family, roe deer, red deer, fallow deer, mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*) and caribou (*Rangifer tarandus*). BVDV antibodies were also detected in buffalo (*Syncerus caffer*), giraffe (*Giraffa camelopardalis*) (Hamblin and Hedger, 1979), bighorn (*Ovis*

Canadensis), eland (*Taurotragus oryx*) and pronghorn antelope (*Antilocapra americana*) and in different members of the Camelidae family (Nettleton, 1990; Vilcek and Nettleton, 2006).

However, BVDV and BDV infections in free-ranging wild ruminants have been relatively poorly studied in Europe. The outbreaks of the previously unrecorded disease caused by BDV infection in the Pyrenean chamois populations across the Pyrenees (Arnal *et al.*, 2004) raised the need to deal with the epidemiology of pestivirus infection in mountain ruminants from Europe. However, the published studies focus mainly in the chamois and less in other wild ruminant species. One study performed in the Catalan Pyrenees with samples of ungulates other than chamois revealed 1.7% antibody positive red deer and 3.5% mouflons (Marco *et al.*, 2011).

Some pestivirus surveys have been performed recently in wild ruminants from the Alps. A survey performed in 1999 in the Italian Alps detected seroprevalences of pestivirus antibodies of 25.5% in Alpine chamois and 5.9% in red deer but no antibodies were detected in 73 roe deer samples tested (Olde Riekerink *et al.*, 2005). Another study carried between 2003 and 2007 in the French Alps detected 45.9% Alpine chamois positive to pestivirus antibodies (Martin *et al.,* 2011a).

Despite all these studies, the role of wild animals in the epidemiology of pestivirus infection is still unclear. Moreover, isolation of pestiviruses from these species is rarely reported. To date, free-ranging wild species with BVDV isolation are roe deer, eland, buffalo, alpaca (*Lama pacos*), red deer, pudu (*Pudu puda*) (Vilcek and Nettleton, 2006), serow (*Capricornis crispus*) (Harasawa *et al*., 2006), camel (Gen. Camelus) (Intisar *et al.,* 2010), mule deer (Van Campen *et al.*, 2001), and sika deer (*Cervus nippon*) (Gao *et al.*, 2011). BVDV have also been isolated from several captive wild ruminants (Doyle and Heuschele, 1983; Nettleton, 1990; Becher *et al.*, 1999; Uttenthal *et al.*, 2005; Nelson *et al.*, 2008). BDV has been isolated in three wild ruminant species. Two of them were captive animals in a German zoo: reindeer and European bison (*Bison bonasus*) (Becher *et al.*, 1999). Both strains were allocated into BDV-2 group. The only free-ranging wild species were from which BDV has been isolated is the Pyrenean chamois (Hurtado *et al.*, 2004). This late strain was allocated into BDV-4 group (Arnal *et al*., 2004).

5.3.3. Influence of communal alpine pasturing on the spread of pestiviruses

Summer communal pasturing is an important part of the Pyrenean and Alpine domestic ruminant farming. This century-old farming practice involves the pasturing of cattle, sheep and goats from different farms on meadows at high altitude in mountain regions, generally from May or June until September. In the fall, the animals return to their respective home farms. Mountain transhumance was initially performed at walking-distance. Nowadays, flocks are moved by cattle-trucks, allowing long-distance transportations of more animals; alpine meadows are overgrazed and the probability of contacts with wildlife increases (Martin *et al.*, 2011b)

Several studies have investigated the influence of communal alpine pasturing in the spread of different pathogens (Belloy *et al.*, 2003; Richomme *et al*., 2006; Siegwart *et al.*, 2006; Krametter-Froetscher *et al.*, 2007). Communal pastures are potential encounter points for several domestic and wild ruminants and that's why this farming practice could interfere in pestivirus epidemiology in different ways.

Firstly, different studies have demonstrated the influence of communal alpine pasturing in the spread of pestiviruses between domestic ungulates sharing pastures. Braun *et al.* (1998) and Siegwart *et al.* (2006) determined that this farming practice increases the risk of infection with BVDV in cattle, and facilitates the spread of the virus. It has been determined that communal Alpine pasturing does play a key role in the spread of BDV in Austria (Braun *et al.*, 1998; Krametter-Froetscher *et al.,* 2007). Due to the mixing of animals from different herds, cattle and sheep of uninfected herds can contact with pestivirus infected animals and introduce subsequently the infection in their naïve herd of origin.

Secondly, domestic ruminants grazing in alpine pastures could be infected by pestiviruses from wild ruminants. As stated before, ruminant pestiviruses are not strictly host-specific (Vilcek and Nettleton, 2006) and therefore virus transmission between different species is possible. This would be a remarkable problem in countries carrying on BVDV eradication programs or if infection of pregnant cattle in the critical period of gestation occurs.

Thirdly, domestic ruminants grazing in alpine meadows could be a source of pestiviruses for wild ungulates. This situation is more likely, since pestiviruses are widely distributed in cattle and sheep from Europe. Moreover spillover of disease from domestic to wild-living ungulates has been largely reported (Foreyt and Jessup, 1982; Callan *et al.*, 1991; Frolich *et al.*, 2002) and has been related to appearances of a range of emerging infectious diseases in wildlife (Daszak *et al.*, 2000). On the other hand, the

alpine pastures also could serve as an encounter point for different wild ruminants and therefore pestiviruses could be potentially transmitted between different wild species.

The risk of a pathogen to cross the species barrier depends on the species susceptibility but also on the rate of efficient contacts between the species. However the definition of "efficient contact" is controversial. Some authors consider as efficient contacts for Mycoplasma horizontal transmission the encounters between two individuals at a distance less than 20 m (Richomme *et al.*, 2006). However this distance can vary with the meteorological conditions and the pathogen involved. Some practices involved in alpine pasturing can increase the probability of efficient contacts to occur. One example are the salt deposits, which have been described as "epidemiological dangerous points" in the transmission of brucellosis between domestic and wild ungulates (Richomme *et al.,* 2006).

The studies that focused in the co-existence of wild and domestic herbivores in pastures show controversial results. Some of them demonstrate that wild ungulates tend to desert the area used by the domestic species (Austin *et al.,* 1983; Skovlin *et al.* 1983). A study performed with Pyrenean chamois and domestic sheep, described that chamois leave the areas grazed by the sheep flock, resulting in an almost total segregation (Rebollo *et al*., 1993). Dubost also described in 1986 segregation of Pyrenean chamois by domestic sheep. This kind of behavior would limit the transmission of pathogens. However, studies performed in Alpine chamois showed opposite results. Rüttimann *et al.* described in 2008 that the presence of sheep in alpine meadows had little effect on the behavior and feeding habits of Alpine chamois. This study described encounters in which the two species come closer than 50 m to each other, but with absence of body contact. Some authors suggest that the behaviour expressed by the chamois could be influenced by sheep density and faecal contamination (Fankhauser *et al.,* 2008). A study performed in the Swiss Alps determined that ibexes and sheep generally don't graze in the same area simultaneously. However this situation changed in presence of salt licks. Then the same study described encounters < 50m between sheep and ibexes, but also between chamois and sheep and chamois and ibexes. Interestingly this study describes direct body contact between a chamois kid and an ibex kid (Ryser-Degiorgis *et al.*, 2002). Regarding to cattle, Bassano *et al.* (1996) reported that the contemporary presence of alpine chamois or ibexes with cattle can rarely be observed despite a significant overlap of ranges. Pyrenean chamois generally tend to desert the areas grazed by

cattle, although this segregation do not occur in some individuals (personal observation).

In conclusion, alpine communal pastures play a role in the epidemiology of pestivirus infection. However, there is a general lack of information about this topic and more studies are necessary to fully understand if this farm practices increases interspecies pestivirus transmission.

6. HYPOTHESIS AND OBJECTIVES

6. HYPOTHESIS AND OBJECTIVES

Since 2001 different outbreaks of a disease associated to BDV-4 infection have caused important declines in several Pyrenean chamois populations. The appearance of this new disease reveals a need to study the epidemiology of BDV infection in the Pyrenean chamois in order to improve the knowledge of the consequences of its appearance and to describe the epidemiological scenario.

At the beginning of this research work several hypothesis were formulated. The first is that BDV infection has become endemic in the Pyrenean chamois populations previously affected by disease outbreaks and that BDV will spread to unaffected Pyrenean chamois populations. Moreover, ruminant pestiviruses are not strictly host-specific and consequently interspecific transmission of pestiviruses occurs between chamois, other wild ruminants and livestock. As last, sheep plays a key role in the epidemiology of infection maintaining and spreading BDV.

The main purpose of the present thesis was to study the epidemiology of pestivirus infection in Pyrenean chamois and other wild and domestic ruminants in the Pyrenees and in other European mountain areas. Methodological and specific objectives related to this main purpose were:

1. To study the epidemiology of pestivirus infection in Pyrenean chamois populations after the severe outbreaks associated to BDV infection.
2. To study the epidemiology of pestivirus infection in Pyrenean chamois populations not affected by these severe epizootics.
3. To investigate the epidemiology of pestivirus infection in other wild and domestic ruminants sharing habitat with Pyrenean chamois.
4. To study the epidemiology of pestivirus infection in Alpine chamois and other wild and domestic ungulates in the Italian and Swiss Alps and in two Spanish mountain systems.

7. STUDIES

7.1. STUDY I

New scenarios of border disease virus infection in Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*)

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ABSTRACT

Since 2001 several outbreaks of a new disease associated with border disease virus (BDV) infection have caused important declines in Pyrenean chamois (*Rupicapra pyrenaica*) populations in the Pyrenees. The goal of the present study was to analyze in the long term the post-outbreak BDV epidemiology in the first two areas (VAPS and CAUBS) affected by disease with the aim to establish if the infection has become endemic. In addition, we investigated if BDV infected wild and domestic ruminants sharing habitat with chamois. Since these disease outbreaks, the CAUBS chamois population has recuperated quickly, unlike the VAPS population, which has not recovered as expected. In chamois from VAPS, prevalence was high (73.47%) and constant throughout the whole study period and did not differ between chamois born before and after the BDV outbreak; in all, BDV was detected by RT-PCR in six chamois. In CAUBS, prevalence was lower (52.79%) and decreased during the study period; as well, prevalence was significantly lower in chamois born after the disease outbreak. No BDV were detected in this population. A comparative virus neutralisation test showed that all the chamois had BDV-specific antibodies. Pestivirus antibodies were detected in all the analyzed species, with low prevalence values in wild ruminants and moderate values in domestic ruminants. No viruses were detected in these species. These results confirm the idea that outbreaks of BDV infection mainly affect the Pyrenean chamois, although other wild ruminants can occasionally be infected. Two different scenarios have appeared since these disease outbreaks: in some chamois populations BDV circulates frequently, possibly having a negative impact on population dynamics, while in others BDV seems to have stopped circulating, a finding that could be related to the quick recovery of these chamois populations.

INTRODUCTION

The Pyrenean chamois *(Rupicapra pyrenaica pyrenaica)* is a mountain ungulate endemic to the Pyrenees (Northern Spain, Andorra and Southern France) that belongs to the order Artiodactyla. In 2001 and 2002 an outbreak of a previously unreported disease associated with BDV infection was described in Pyrenean chamois from the Central Pyrenees, specifically in the Alt Pallars-Aran National Hunting Reserve (NHR). Phylogenetic analysis of the 5'UTR region typed the chamois pestivirus as BDV-4

genotype (Arnal *et al*., 2004; Hurtado *et al*., 2004). After the outbreak, the population was found to have decreased by about 42%, most probably due to the disease. This was the first time that a BDV had been associated with an outbreak of a high-mortality disease in a wild species and the clinicopathological aspects were described (Marco *et al.*, 2007). Subsequently, several disease outbreaks associated with the same virus occurred in other Pyrenean chamois populations (Marco *et al*., 2009b).

Along with bovine viral diarrhoea virus 1 and 2 (BVDV-1 and -2) and classical swine fever virus (CSFV), BDV belongs to the genus Pestivirus (Fam. Flaviviridae). Ruminant pestiviruses (BDV and BVDV) are not strictly host-specific and transmission between different Artiodactyla species has been widely described (Nettleton, 1990; Vilcek and Nettleton, 2006). BDV is distributed worldwide and causes disease mainly in sheep, but also in goats. Postnatal infection in sheep tends to be mild and is characterized by mild pyrexia and transient lymphopaenia, followed by seroconversion (Nettleton *et al.*, 1998). However, severe outbreaks of disease with high mortality have been reported occasionally in cases of acute BDV infections in sheep (Chappuis *et al*., 1984); as well, a mucosal disease syndrome has been described in persistently infected (PI) sheep (Monies *et al.*, 2004).

Like all pestiviruses, BDV has the ability to pass through the placenta and infect the foetus with varying consequences. If infection occurs before day 60 of the gestation period (i.e. before foetal immunocompetence) and if the foetus survives, the newborn will be a PI animal characterized by specific immunotolerance against BDV, an absence of pestivirus antibodies and the continuous shedding of the virus throughout its life. PI animals can appear normal but usually grow poorly and have lower life expectancy (Nettleton, 1990). PI individuals play a crucial role in maintaining pestiviruses in a flock.

After a decade of disease outbreaks in Pyrenean chamois populations, several questions remained unanswered. Marco *et al*. showed in 2008 that this infection had become endemic in the Alt Pallars-Aran NHR, two years after the first disease outbreak. The goal of the present study was to analyze in the long term the post-outbreak BDV epidemiology in the first two areas affected by disease, with the aim to establish if the infection has become endemic. In addition, we investigated if BDV infected wild and domestic ruminants sharing habitat with chamois.

MATERIALS AND METHODS

Study area

The presence and epidemiology of BDV in ruminant populations was studied in two areas of the Pyrenees (Figure 1), both in the central Catalan Pyrenees (NE Spain, 1°15'N, 42°37'E) on the border with France. The first study area consists of the regions of Val d'Aran and Pallars Sobirà (VAPS), which includes most of the Alt Pallars-Aran NHR and adjacent private hunting areas (HPA). The disease was described for the first time in this area and between 2001 and 2002 caused an estimated decrease in the local chamois population of 42% (Marco *et al*., 2007). After this outbreak, the population continued to fall, dropping from 3,526 chamois in 2003 to 2,441 chamois in 2011. The second study area is situated in the East of the first study area, in the regions of Cerdanya, Alt Urgell, Berguedà and Solsonès (CAUBS), and includes the Cadí and Cerdanya-Alt Urgell NHR and adjacent HPA. During 2005, a disease outbreak led to the collapse of the chamois population in the Cerdanya-Alt Urgell NHR, causing an estimated cumulative rate of decline of 85.6%. In June 2005, the disease spread to the Cadí NHR and private hunting areas, with a subsequent estimated cumulative rate of fall of 63% (Marco *et al*., 2009b). Nevertheless, after these outbreaks, chamois populations have recovered successfully in this latter area, rising from 133 chamois in 2006 to 384 chamois in 2011 in the Cerdanya-Alt Urgell NHR and from 1,224 chamois in 2007 to 2,066 chamois in 2011 in the Cadi NHR (Direcció General de Medi Natural i Biodivesitat, Generalitat de Catalunya).

Pyrenean chamois share habitat with other wild ruminant species. Roe (*Capreolus capreolus*), red (*Cervus elaphus*) and fallow (*Dama dama*) deer, along with European mouflon (*Ovis aries*) inhabit VAPS, while roe and red deer live in CAUBS. As well, both study areas are characterized by communal alpine pastures that are shared by livestock (sheep, goats and cattle). Communal alpine pasturing is a centuries-old farming practice that involves the pasturing of domestic ruminants from different farms on grassland at high altitude in the Pyrenees, generally from May or June through to September.

Figure 1: Location of the study areas: Val d'Aran and Pallars Sobirà = VAPS; Cerdanya, Alt Urgell, Berguedà and Solsonès = CAUBS (Spanish Pyrenees). Dark lines represent regional boundaries and thin lines municipal boundaries.

Animals and samples

Samples from apparently (clinically) healthy wild ruminants were obtained during the hunting season (September to February) over a five-year period between 2007 and 2011. In VAPS we studied samples from 114 Pyrenean chamois, 52 roe, 43 red and 25 fallow deer, and six mouflons. In CAUBS we studied samples from 115 Pyrenean chamois and 29 roe and 27 red deer. Samples consisted of sera and/or spleen tissue. Blood samples, obtained by intracardiac venipuncture from animals that had been hunted, were placed into sterile serum separator tubes and centrifuged at 1200 g for 15 minutes. Spleen tissue was also collected. Sera and spleen samples were stored at -20ºC until analysis.

Sera from domestic ruminants were obtained during annual sanitary campaigns conducted when livestock descend from summer pastures. In VAPS we obtained samples over a period of four years (2007-2010) from 360 sheep, 250 goats and 361 cattle, while in CAUBS we obtained samples from just two years (2007 and 2008) from 184 sheep, 96 goats and 175 cattle.

Serological tests

Sera were tested for the presence of antibodies against pestivirus with a commercial blocking ELISA assay (BVD/MD/BD P80, Antibody Screening, Pourquier,

Montpellier, France). This test has a minimum specificity of 99.2 % and an observed sensitivity of 100 % for testing BDV positive sheep sera.

In order to confirm the results of the ELISA test and to determine the specificity of the antibodies, part (depending on the sample availability) of the selected positive ELISA sera were subsequently tested with a comparative virus neutralization test (VNT) using BVDV-1 strain NADL (Collett *et a*l., 1988; Gen Bank accession number M31182), BDV-1 strain 137/4 (Vilcek *et al*., 1997; Gen Bank accession number U65052) and BDV-4 strain Esp97 (Vega *et al.,* 2002; Gen bank accession number FR714860). Sera from VAPS were also tested with BDV-4 strain Aran-1 (Marco *et al*., 2008; Gen Bank accession number AM765800) isolated from a diseased chamois found in 2001 in the Alt Pallars-Aran NHR. Sera from CAUBS were also tested with BDV-4 strain Cadí-6 (Cabezón *et al*., 2011; Gen Bank accession number AM905923), isolated in the Cadí NHR in 2006.

The VNT were performed according to 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 using Reed and Muench's method (1938). Titres of 1:10 and higher were considered positive. Viral replication was monitored by the Immuno-Peroxidase Monolayer Assay (IPMA) with polyclonal home-made pestivirus-specific serum.

Virus detection

Reverse transcription-polymerase chain reaction (RT-PCR) in wild ruminant samples was performed in spleen homogenates or in sera when no spleen samples were available. RT-PCR was performed using described panpestivirus primers 324 and 326 (Vilcek *et al*., 1994) and a commercial kit (One-Step PCR kit, Qiagen, Hilden, Germany). Before the RT-PCR, viral RNA was extracted using a commercial kit (Nucleospin Viral RNA Isolation, Macherey Nagel, Düren, Germany). Domestic ruminant sera were tested for the presence of pestiviral antigen with a commercial sandwich ELISA assay (Synbiotics, Lyon, France) according to the manufacturer's procedure.

Sequence analysis

Sequence analyses of the 243 bp fragment of the 5'UTR region from RT-PCR positive samples were performed using primers 324 and 326 (Vilcek *et al*., 1994). Purified amplicons (Minelute Gel Extraction Kit, Qiagen, Hilden, Germany) were analyzed with Big Dye Terminator v.3.1 Kit and the ABI 3130xl Genetic Analyzer (Applied Biosystems, Warrington, United Kingdom). The phylogenetic tree was constructed by the neighbour-joining method (Saitou and Nei, 1987) using automatic root location. A bootstrap analysis of 1,000 replicates was performed by creating series of bootstrap samples to test tree branch reliability.

Statistical analysis

We calculated true prevalence and the confidence interval (exact binomial method) (Altman, 2000) in all the studied species in each zone. We stratified the prevalence in chamois in terms of the year. We classified the chamois from each study area into two groups according to whether they were born before/during the outbreak (2001–2002 for VAPS and 2005–2006 for CAUBS) or afterwards. We calculated the prevalence in each of these groups using the methods described above.

In order to test for differences in the observed proportions of positive Pyrenean chamois in VAPS and CAUBS we computed a chi-squared and reported the odds ratio (OR) values with its 95% confidence interval. The same test was computed to test for differences in chamois in terms of their date of birth with respect to the pestivirus outbreak (before/during or after) and to test for differences according to sex. We also used this test to check for differences between the two study areas in the observed proportions of positive animals in the rest of the studied species. A *P*-value for the chi-squared statistic ≤ 0.05 was interpreted as a lack of homogeneity between the proportions and therefore as a statistically significant difference between the two groups.

We performed a Wilcoxon signed-rank test to assess whether the titres of the analyzed chamois, sheep, goats and cattle in each study area differed in terms of the viral strains. To test for differences in the mean ranks we compared pairwisely the antibody titres against each virus strain. The limit of statistical significance was defined as $P \leq 0.05$.

Due to the small number of samples tested by VNT, in wild ruminants other than Pyrenean chamois we checked each animal's titre individually in order to detect greater than two-fold differences against different strains. The OIE has defined a 'rule'

whereby a three-fold difference or more between end-points of two titrations should be regarded as decisive for infection by the virus species yielding the highest titre (OIE, 2008). However, to the authors' knowledge, no 'rule' exists when comparing different subgroups of the same pestivirus species or even the same genotype (e.g. the BDV-Esp97 and BDV-strain chamois).

For data analysis we used the functions for analysing epidemiological data included in the library epiR (http://cran.r-project.org/web/packages/epiR/epiR.pdf/) and the free statistical software R (http://www.r-project.org/). In this R package point estimates and confidence intervals are based on formulae provided by Rothman (2002).

RESULTS

Serological results

Specific pestivirus antibodies were detected in Pyrenean chamois and red and roe deer from both study areas, as well as in fallow deer and European mouflon from VAPS. Antibodies were also detected in sheep, goat and cattle from both zones. Prevalence values from all these species, along with prevalence in Pyrenean chamois per year, are given in Table 1. In VAPS prevalence remained constant during the study period, while in CAUBS it tended to fall. Prevalence was also calculated in chamois born before/during the outbreak and in chamois born afterwards. From these two groups, however, only 35 chamois from VAPS and 84 chamois from CAUBS could be classified, since age data were not always available. The prevalence in animals born before or during the outbreak was 77.04% (95% CI: 56.12-89.77) in VAPS and 64.43% (95% CI: 52.70-74.62) in CAUBS, while the prevalence in chamois born after the epizooty was 61.14% (95% CI: 34.87-82.11) in VAPS and 14.52% (95% CI: 3.35-41.65) in CAUBS.

VNT was performed on 81 chamois, 10 red, one roe and two fallow deer, one mouflon, 133 sheep, 67 goats and 84 cattle sera. Mean titres for Pyrenean chamois, sheep, goats and cattle, along with their median and range values, appear in Table 2. VNT in goats from VAPS showed the greatest individual variability in comparison with cattle and sheep from both study areas and goats from CAUBS (Table 3). VNT results from wild ruminants other than Pyrenean chamois are given individually in Table 4.

Table 1: Prevalence and 95% confidence interval (exact 95% binomial confidence intervals) of the pestivirus NS3 antibodies in all the analyzed species. In the Pyrenean chamois prevalence is classified according to year.

		Prevalence (95% CI[a])		Prevalence (95% CI)	
	Year	**VAPS**	**n**	**CAUBS**	**n**
	2007	62.12% (29.87-86.17)	8	19.19% (0.02-62.06)	5
	2008	53.37% (28.42-76.55)	13	69.25% (48.62-84.23)	23
Pyrenean	2009	52.46% (30.26-73.57)	17	68.91% (49.50-83.33)	26
chamois	2010	74.74% (52.65-88.70)	20	44.17% (29.44-59.89)	38
	2011	90.14% (77.22-96.10)	41	32.65% (14.31-57.86)	15
	Total	73.47% (63.93-81.21)	99	52.79% (43.30-62.06)	107
Red deer	Total	10.73% (4.11-23.71)	43	15.15% (5.45-33.99)	25
Roe deer	Total	1.13% (0.00-10.21)	47	3.58% (0.00-21.00)	22
Fallow deer	Total	8.60% (1.66-28.19)	21	ND	0
Mouflon	Total	19.19% (0.02-62.06)	5	ND	0
Sheep	Total	23.12% (18.96-27.83)	360	49.49% (42.27-56.71)	184
Goats	Total	13.47% (9.68-18.32)	258	32.65% (23.94-42.67)	96
Cattle	Total	70.34% (65.39-74.84)	361	65.94% (58.58-72.59)	175

[a] CI: Confidence interval

Table 2: Mean, median and range of titres obtained for each virus strain and statistical significance of each pairwise comparison.

Species	Strain 1	Mean	Median	Range	vs.	Strain 2	Mean	Median	Range	Significance[a]
Chamois VAPS n= 32	NADL	73.12	40	0-640	-	Esp97	254.06	160	10-1280	*
	NADL	73.12	40	0-640	-	ARAN-1	581.87	160	20-10240	*
	NADL	73.12	40	0-640	-	137/4	784.37	320	20-5120	*
	Esp97	254.06	160	10-1280	-	ARAN-1	581.87	160	20-10240	ns
	Esp97	254.06	160	10-1280	-	137/4	784.37	320	20-5120	ns
	137/4	784.37	320	20-5120	-	ARAN-1	581.87	160	20-10240	Ns
Sheep VAPS n=79	NADL	60.88	20	0-640	-	Esp97	242.53	80	0-2560	*
	NADL	60.88	20	0-640	-	ARAN-1	191.77	80	0-1280	*
	NADL	60.88	20	0-640	-	137/4	234.12	20	0-5120	ns
	Esp97	242.53	80	0-2560	-	ARAN-1	191.77	80	0-1280	ns
	Esp97	242.53	80	0-2560	-	137/4	234.12	20	0-5120	*
	137/4	234.12	20	0-5120	-	ARAN-1	191.77	80	0-1280	ns
Goats VAPS n=35	NADL	113.71	20	0-640	-	Esp97	138	20	0-2560	ns
	NADL	113.71	20	0-640	-	ARAN-1	104.85	10	0-1280	*
	NADL	113.71	20	0-640	-	137/4	21.42	0	0-160	*
	Esp97	138	20	0-2560	-	ARAN-1	104.85	10	0-1280	ns
	Esp97	138	20	0-2560	-	137/4	21.42	0	0-160	*
	137/4	21.42	0	0-160	-	ARAN-1	104.85	10	0-1280	ns
Cattle VAPS n=51	NADL	664.11	320	10-5120	-	Esp97	161.37	40	0-2560	*
	NADL	664.11	320	10-5120	-	ARAN-1	71.56	20	0-1280	*
	NADL	664.11	320	10-5120	-	137/4	107.64	10	0-2560	*
	Esp97	161.37	40	0-2560	-	ARAN-1	71.56	20	0-1280	ns
	Esp97	161.37	40	0-2560	-	137/4	107.64	10	0-2560	ns
	137/4	107.64	10	0-2560	-	ARAN-1	71.56	20	0-1280	ns
Chamois CAUBS n=49	NADL	165.51	20	0-5120	-	Esp97	297.95	160	0-2560	*
	NADL	165.51	20	0-5120	-	CADÍ-6	447.34	320	40-1280	*
	NADL	165.51	20	0-5120	-	137/4	410.61	80	20-10240	*
	Esp97	297.95	160	0-2560	-	CADÍ-6	447.34	320	40-1280	*
	Esp97	297.95	160	0-2560	-	137/4	410.61	80	20-10240	ns
	137/4	410.61	80	20-10240	-	CADÍ-6	447.34	320	40-1280	*
Sheep CAUBS n=54	NADL	70.37	40	0-640	-	Esp97	173.88	160	10-640	*
	NADL	70.37	40	0-640	-	CADÍ-6	167.22	160	10-640	*
	NADL	70.37	40	0-640	-	137/4	121.29	80	0-1280	*
	Esp97	173.88	160	10-640	-	CADÍ-6	167.22	160	10-640	ns
	Esp97	173.88	160	10-640	-	137/4	121.29	80	0-1280	*
	137/4	121.29	80	0-1280	-	CADÍ-6	167.22	160	10-640	*
Goats CAUBS n=32	NADL	177.5	80	20-1280	-	Esp97	160.62	120	20-640	ns
	NADL	177.5	80	20-1280	-	CADÍ-6	203.75	160	40-640	*
	NADL	177.5	80	20-1280	-	137/4	105.62	60	10-640	*
	Esp97	160.62	120	20-640	-	CADÍ-6	203.75	160	40-640	ns
	Esp97	160.62	120	20-640	-	137/4	105.62	60	10-640	*
	137/4	105.62	60	10-640	-	CADÍ-6	203.75	160	40-640	*
Cattle CAUBS n=33	NADL	542.12	160	10-5120	-	Esp97	128.48	40	0-1280	*
	NADL	542.12	160	10-5120	-	CADÍ-6	37.27	20	0-160	*
	NADL	542.12	160	10-5120	-	137/4	16.96	10	0-80	*
	Esp97	128.48	40	0-1280	-	CADÍ-6	37.27	20	0-160	ns
	Esp97	128.48	40	0-1280	-	137/4	16.96	10	0-80	*
	137/4	16.96	10	0-80	-	CADÍ-6	37.27	20	0-160	*

[a] *P-value in the Wilcoxon signed rank test <0.05;ns: non-significant.

Table 3: Antibody titres of 35 goats from VAPS against four pestivirus strains. Neutralizing antibody titres are expressed as the reciprocal of the highest dilution that neutralized 100 tissue culture infective doses (100 $TCID_{50}$) in all cultures.

BVDV-NADL	BDV-Esp97	BDV-Aran-1	BDV-137/4
640	160	10	10
640	80	40	10
0	20	160	20
0	20	320	10
640	640	320	160
80	10	10	10
20	0	0	0
20	0	0	0
10	10	0	0
80	0	0	0
20	0	0	0
640	320	320	160
20	0	0	0
20	0	0	0
20	10	0	20
80	160	640	40
20	0	20	0
80	320	320	80
20	0	0	0
320	20	10	20
160	40	10	0
40	10	0	0
20	0	0	0
80	20	10	0
10	10	0	0
20	20	0	0
10	20	10	0
80	20	0	0
0	160	80	80
80	80	1280	40
40	2560	20	20
40	0	0	10
0	40	10	0
20	40	40	20
10	40	40	40

Table 4: Antibody titres of positive red, fallow and roe deer and mouflon against four pestivirus strains. Neutralizing antibody titres are expressed as the reciprocal of the highest dilution that neutralized 100 tissue culture infective doses (100 $TCID_{50}$) in all cultures.

	Study area	BVDV NADL	BDV Esp97	BDV Aran/Cadí-6 [a]	BDV 137/4
Red deer no. 1	VAPS	0	80	20	40
Red deer no. 2	VAPS	10	20	80	20
Red deer no. 3	VAPS	320	20	320	0
Red deer no. 4	VAPS	10	0	320	0
Red deer no. 5	VAPS	1280	320	80	2560
Red deer no. 6	CAUBS	10	0	0	0
Red deer no. 7	CAUBS	10	0	0	0
Red deer no. 8	CAUBS	20	80	160	20
Red deer no. 9	CAUBS	0	160	160	160
Red deer no. 10	CAUBS	0	20	80	0
Roe deer no. 1	CAUBS	160	20	20	80
Fallow deer no. 1	VAPS	10	80	40	80
Fallow deer no. 2	VAPS	10	160	40	80
Mouflon no. 1	VAPS	640	160	160	2560

[a] Sera from VAPS were tested against BDV strain Aran, while sera from CAUBS were tested against BDV strain Cadí-6.

Virological results

Viral RNA was detected by RT-PCR in six (viroprevalence 5.26%; 95% CI: 2.43-11.00) apparently healthy hunted chamois from VAPS: a five-year-old male, an adult male, an old male and two females; the sex and age of the sixth animal was not reported. Viruses found in these animals appear in the phylogenetic tree in Figure 2 named as Aran-9, -10, -11, -12, -13 and -14. Sequences of 5'UTR from these viruses were deposited in the Gen Bank under accession numbers HE818617, HE818618, HE818619, HE818620, HE818621, and HE818622. No viruses were detected in chamois from CAUBS or in any of the other species in either study areas.

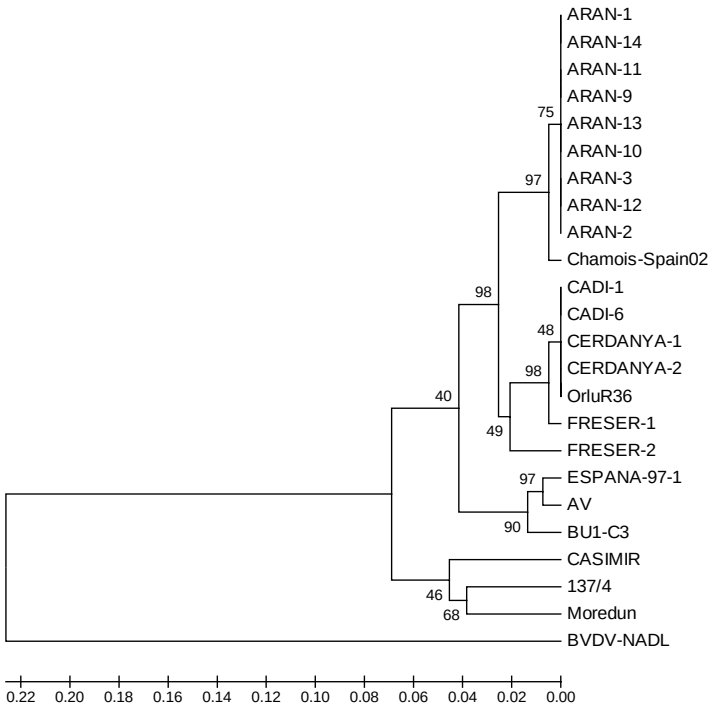

Figure 2: Unrooted neighbour-joining phylogenetic tree based on the 5'UTR sequence among pestiviruses. The strains detected in six chamois from VAPS appear as Aran-9, -10, -11.-12, -13, and -14. These strains cluster with other chamois viruses isolated in the bordering NHR of Alt Pallars-Aran (ARAN-1, -2, and -3). The numbers on the branches indicate the bootstrap values (in percentage; 1,000 replicates). Sequences of strains taken from Gen Bank with following accession numbers: ARAN-1 (AM765800), ARAN-14 (HE818622), Aran-11 (HE818619), ARAN-9 (HE818617), ARAN-13 (HE818621), ARAN-10 (HE818618), ARAN-3 (AM765802), ARAN-12 (HE818620), ARAN-2 (AM765801), Chamois-Spain02 (AY641529), CADI-1 (AM905918), CADI-6 (AM905923), CERDANYA-1 (AM905930), CERDANYA-2 (AM905931), ORLUR36 (DQ898294), FRESER 2 (FN691777), Espana-97 -1 (FR714860), AV (EF693984), BU1-C3 (DQ361068), CASIMIR (AB122085), 137/4 (U65052), Moredun (U65023), BVDV NADL (M31182). The sequence of strain FRESER-1 is not deposited in the Gen Bank.

Prevalence related factors

Seroprevalence was significantly higher in chamois from VAPS (OR $_{VAPS}$ = 2.46; 95% CI: 1.36-4.42) than in chamois from CAUBS. In CAUBS, the risk of being seropositive was significantly higher in chamois born before or during the outbreak (OR $_{Born\ before/during}$ = 10.12; 95% CI: 2.07-49.30) than in chamois born after the outbreak. However, no significant differences were observed between these groups in VAPS. No significant differences were detected in the prevalence between chamois sexes in either study area.

No differences were detected between the study areas in prevalence in either red or roe deer. In sheep and goats seroprevalence was significantly higher in CAUBS

(OR $_{\text{sheep CAUBS}}$ = 3.18; 95% CI: 2.18-4.64; OR $_{\text{goats CAUBS}}$ = 2.98; 95% CI: 1.72-5.17) than in VAPS. No differences were detected between the study areas in the prevalence in cattle.

Comparative VNT

Six pairwise combinations between viruses used in VNT were tested in each ruminant species analyzed. Table 5 shows the significant differences between pairwise comparisons. Pyrenean chamois from VAPS had significantly higher titres against the BDV strains; however, no significant differences were detected between the mean titres against the different BDV strains. The chamois from CAUBS had significantly higher titres against the strain of chamois origin, BDV-Cadí-6. In VAPS, the sheep had significantly higher titres against both BDV-Esp97 and BDV-Aran-1, the goats against both BVDV-NADL and both BDV-4 strains (BDV-Esp97 and BDV-Aran-1), and cattle against BVDV-NADL. One sheep showed null titres against all the strains and was considered as an ELISA false positive. In CAUBS, the comparison of means ranks determined that sheep had significantly higher antibody titres against both BDV-Esp97 and BDV-Cadí-6, goats against BVDV-NADL and both BDV-4 strains (BDV-Esp97 and BDV-Cadí-6), and cattle against BVDV-NADL.

DISCUSSION

This study focuses on the first two areas severely affected by outbreaks of BDV infection that were associated with high mortality in Pyrenean chamois. Unexpectedly, we found different scenarios in each population.

The prevalence of pestivirus antibodies in Pyrenean chamois was high in both study areas. However, prevalence was higher in VAPS than in CAUBS and remained stable during the post-outbreak years; by contrast, in CAUBS prevalence tended to decrease after the outbreak. The remarkable prevalence of antibodies in 2011 in VAPS (90.14%) is the highest prevalence ever described in a Pyrenean chamois population. These results suggest that high circulation of pestiviruses in Pyrenean chamois occurs in VAPS, which concurs with the results from the years immediately after the outbreak (2002–2004) (Marco *et al.,* 2008). Interestingly, when analysing the effect of year of birth, in VAPS we found the same prevalence in chamois born before and after the outbreak, which reinforces the idea that high circulation of BDV has existed in this area

since the disease outbreak in 2001 and 2002. However, in CAUBS, the lower prevalence in the chamois born after the outbreak suggests that the circulation of pestiviruses in this area has fallen since the high mortality recorded between 2005 and 2006.

The VNT results for chamois showed that these animals possessed specific BDV antibodies in both study areas. However, in VAPS no significant differences were found between the different border strains, while in CAUBS the chamois were found to have significantly higher titres against BDV-Cadí-6, the strain originating from the chamois. This result suggests that these animals were infected during the outbreak but then seroconverted and cleared the virus. It is interesting to note that all the chamois found to be clinically affected by the disease were antibody negative and died without seroconverting (Marco *et al.*, 2008).

In VAPS, the absence of significant differences between the different BDV strains could be due to serological cross-reactivity, which has been reported as occurring between all members of the genus Pestivirus (Schirrmeier *et al.*, 2004). Nevertheless, it is likely that this lack of significant differences is also related to the greater length of time elapsing since the outbreak in VAPS than in CAUBS (2001 and 2002 vs. 2005 and 2006). Even though antibodies against pestivirus are said to remain detectable for as long as an animal survives, it seems that with time it becomes more difficult to determine the pestivirus species that caused infection (OIE, 2008).

Antibodies against pestivirus were detected in all the studied wild ruminants: red, roe and fallow deer and mouflon. Interestingly, Marco *et al.* (2008) did not detect antibodies against pestivirus in these species (except red deer) during a study performed after the disease outbreak in the Alt Pallars-Aran NHR (part of the VAPS study area), most probably due to their small sample size.

In red deer the apparent prevalence was low (10.73% in VAPS and 15.15% in CAUBS). Although several studies have detected antibodies against pestivirus in other areas, generally associated with BVDV infection (Lillehaug *et al.*, 2003; Krametter *et al.*, 2004). Unexpectedly, VNT results showed that the majority (6/10) of the red deer (nº 1, 2, 4, 8, 9, 10) had specific antibodies against BDV strains. Red deer nº 10 had specific antibodies against both BDV-4 strains, suggesting that it had been infected with this BDV genotype. The finding of a red deer (nº 4) from CAUBS with a greater than two-fold higher titre against BDV-4-Cadí-6 is remarkable. This animal was probably infected during the disease outbreak that occurred in chamois between 2005 and 2006. Red deer nº 6 and 7 had specific BVDV antibodies, while nº 3 and 5 showed no differences

in the titres between BDV and BVDV. This could be due to cross-reactivity or the result of a past pestivirus infection and subsequent contact with a different pestivirus species.

The prevalence in roe deer from both study areas was very low (1.13% in VAPS and 3.58% in CAUBS). Although higher prevalences have previously been reported, studies in the Pyrenees and the Alps have failed to detect antibodies in this species (Lillehaug *et al*., 2003; Krametter *et al.*, 2004; Olde Riekerink *et al*., 2005; Gaffuri *et al*., 2006; Marco *et al.*, 2008, Fernández-Sirera *et al.*, 2012). The VNT performed on one roe deer showed no conclusive differences between the titres against BVDV and BDV and so it is not possible to determine which pestivirus species infected this animal.

Prevalence in fallow deer in VAPS was also low (8.60%). Other studies have failed to detect pestivirus antibodies in fallow deer (Nielsen *et al*., 2000; Krametter *et al.*, 2004). The VNT of two positive animals showed that they had BDV-specific antibodies, but no determinant differences were found between different BDV strains.

Deer species are more likely to have antibodies against BVDV (Vilcek and Nettleton, 2006) and indeed only BVDV has been isolated in red (Nettleton, 1990), roe (Romvary, 1965; Schellner, 1977; Frolich and Hofmann, 1995; Fischer *et al.*, 1998) and fallow deer (Neumann *et al*., 1980). The predominance of BDV-specific antibodies in cervids in our study is likely to be related to the high circulation of BDV in chamois populations during the outbreaks, which would have facilitated a spill-over of BDV from chamois to other species.

We detected an antibody-positive mouflon, but the sample size was too small to assess the infection status in this species. Marco *et al.* (2008) also found low seroprevalence in mouflons from the same area, although in other areas such as the Alps high seroprevalence has been reported (Martin *et al.*, 2011a).

The low prevalence of antibodies in wild ruminants (other than Pyrenean chamois) suggests that they do not play a key role in maintaining BDV in these populations. By contrast, the high circulation of BDV in Pyrenean chamois seems to lead to a spill-over of BDV to other wild ruminants, which then play the role of 'victims'.

The seroprevalence found in sheep from VAPS (23.12%) was lower than that previously reported from this area (64–69%) (Alba *et al.*, 2008; Marco *et al*., 2008). However, seroprevalence in CAUBS (49.49%) was significantly higher than in VAPS. BD is widespread in sheep from Spain, even though clinical disease and virus isolation have only been reported in recent years (Valdazo-González *et al., 2*004; García-Pérez *et*

al., 2008). Sheep from VAPS and CAUBS had similar titres against both BDV-Esp 97 and BDV originating from chamois, probably due to cross-reactivity, since both belong to the same genotype circulating in sheep flocks (Valdazo-González *et al., 2*004).

Prevalence in goats was significantly higher in CAUBS than in VAPS (32.65% vs. 13.47%) and both values are higher than previously described in the Pyrenees (7.3%) (Marco *et al*., 2008). It has been suggested that sheep and cattle are the main origin of pestivirus infection in goats (Krametter *et al*., 2010; Loken, 1995), which could explain why the goats showed no significant differences between the mean ranks of the titres against BDV and BVDV (due to the fact that some goats showed specific BDV antibodies while others had specific BVDV antibodies, see Table 3). Interestingly, we found three goats with specific antibodies against BDV-Aran-1 in VAPS, which suggests that not only sheep and cattle but also Pyrenean chamois can act as a source of infection for goats.

Although several studies have suggested that sheep could be a source of BDV infection for chamois (Krametter *et al*., 2010; Martin *et al*., 2011a) our results do not confirm this idea since seroprevalence in chamois is higher than in sheep and goats in VAPS and reaches similar levels in chamois, sheep and goats in CAUBS. Nevertheless, the seroprevalence in sheep and goats in both areas is moderate, suggesting that pestiviruses circulate in these species. Given that these animals cohabit with chamois, it is possible that pestiviruses exchanges occur. However, if we are to assess with greater accuracy the role of domestic ruminants in the epidemiology of BDV infection in chamois, more studies are needed to isolate the BDV strains circulating in sheep and goats in the Pyrenees.

The prevalence detected in cattle is high in both areas and specific titres are present against BVDV. However, vaccination in this species is a routine event, which could explain the high seroprevalence, as has previously been described (Marco *et al*., 2008). Despite the fact that vaccinating is commonplace (and usually unreported by veterinarians), our results suggest that BVDV circulates frequently, since in VNT we detected 11/35 individual goats with three-fold or more titres against BVDV-1-NADL.

Viral RNA was detected in six Pyrenean chamois, all from VAPS. Viral sequencing determined that they were infected with a BDV-4. This fact reinforces our previous hypothesis that BDV have circulated in VAPS ever since the severe outbreak of disease but may not circulate – or only at a low level – in CAUBS. These differences, together with the other differences mentioned above, reveal the existence of two

different epidemiological scenarios for BDV infection in the aftermath of the severe outbreaks of disease that decimated chamois populations.

It is difficult to determine the cause of the differences between the two studied areas. During the BDV epizooty, the highest mortality values in the whole of the Pyrenees were recorded in CAUBS (85.6%) (Marco *et al.*, 2009b). This extremely high mortality rate could have endangered the long-term maintenance of the BDV in the chamois population given the reduction in the number of animals susceptible to infection. Census data reveal that the recovery in this population after the outbreak was quick. However, our results suggest that there are an increasing proportion of naïve chamois and this situation could provoke the apparition of a second BDV outbreak in the future.

In VAPS, the chamois mortality described during the BDV outbreak was lower than in CAUBS (42%). However, in VAPS chamois numbers have not recovered as they have in CAUBS. This coincides with the isolation of BDV in chamois from this area and suggests that BDV infection does have a negative impact on chamois population dynamics. More studies are still needed if we are to assess the role of BDV in the decline of this chamois population, which could be associated with low survival and fecundity rates as occurs in pestivirus infection in domestic ruminants (Nettleton, 1990).

It is worth remarking that the chamois population from VAPS could be a source of BDV in other chamois populations. Indeed, a recent study has linked a BDV outbreak between 2009 and 2010 in Pyrenean chamois in Andorra with the arrival of BDV viruses from the VAPS population (Fernández-Sirera *et al*., in press). In addition, in 2011 clinical cases of BDV infection were described from the Spanish region of Aragon, which borders on VAPS to the west (unpublished data).

The lack of viral detection in domestic and wild ruminants other than Pyrenean chamois was expected. In domestic ruminants, the short viraemia in acute infections and the low prevalence of PI animals makes it very difficult to detect infected animals (Nettleton and Entrican, 1995), while in wild ruminants the low prevalence observed suggests very low virus circulation that hampers greatly the detection of viraemic animals.

Our study confirms that the BDV infection outbreaks in the Pyrenees only affected the Pyrenean chamois, although other wild ruminant species can occasionally be infected. After the severe BDV outbreaks, at least two different scenarios appeared in the Pyrenees: on the one hand, in some areas the disease has become endemic and

BDV circulates frequently in the chamois population, possibly having a negative impact on host population dynamics, while, on the other hand, in other areas BDV does not seem to circulate, and this fact seems to be related with the successful recovery of the chamois populations inhabiting these areas. Thus, management of chamois populations affected by these epizootics should be designed according to the epidemiological status and no generalizations should be made.

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7.2. STUDY II

BORDER DISEASE VIRUS IN A PYRENEAN CHAMOIS (*RUPICAPRA PYRENAICA PYRENAICA*) POPULATION IN THE EASTERN PYRENEES IS SELF-MAINTANED WITHOUT DEMOGRAPHIC EFFECTS

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ABSTRACT

Since 2001 a new disease associated with border disease virus (BDV) infection has caused serious declines in several Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) populations in the Pyrenees. A retrospective study detected a BDV-4 in two chamois sampled in the Freser-Setcases National Hunting Reserve (NHR) in 1996, five years before the first outbreak of the disease in the Pyrenees, along with a prevalence of pestivirus antibodies of 66%. Interestingly, this is the only population on the southern face of the Pyrenees that has not suffered a BDV outbreak. The aim of the present study was to assess whether, despite the lack of disease outbreaks, BDV persist in the Pyrenean chamois population in the Freser-Setcases NHR. Between 2003 and 2010, samples from 553 apparently healthy chamois and eight diseased chamois were studied. Also investigated were the presence of BDV in apparently healthy European mouflon, roe deer, sheep, goat and cattle sharing habitat with Pyrenean chamois. Pestivirus antibodies were detected in all the analyzed species except in roe deer and goats, with overall values for prevalences of 43.98% in chamois, 22.04% in mouflon, 9.15% in sheep and 71.50% in cattle. A compared virus neutralisation test using four BDV and one bovine viral diarrhoea virus (BVDV) strain showed that the chamois and mouflons had significantly higher titres against BDV-4 strains. Sheep showed no significant differences in the titres between BDV-4 and BVDV, while cattle had higher titres against the BVDV strain. Viral RNA was detected by RT-PCR in three chamois hunted in 2006 and 2009 and in one chamois with clinical signs consistent with BDV infection. Sequence analysis of two of these viruses determined that the chamois were infected with a BDV-4. The continued high seroprevalence in chamois, along with the detection of BDV in this species and the low seroprevalence in sheep, all suggest that BDV-4 is self-maintained in the Pyrenean chamois population in the Freser-Setcases NHR. The lack of mortality and the species' population dynamics suggest that this infection does not have a negative impact on this population. The circulation of BDV and the high seroprevalence in this area are likely to be important factors that hinder the dispersion of virulent BDV-4 strains and ensure that outbreaks of this disease are less liable to occur.

INTRODUCTION

Together with bovine viral diarrhoea virus of types 1 and 2 (BVDV-1 and BVDV-2) and classical swine fever virus (CSFV), border disease virus (BDV) belongs to the genus Pestivirus (Fam. Flaviviridae). Pestiviruses are distributed worldwide and are characterized by their high heterogeneity (including genetic properties), their wide spectrum of host species and their symptoms and virulence (Peterhans and Schweizer, 2010).

BDV mainly causes disease in sheep, but does also affect goats. Postnatal infection in sheep tends to be mild and is characterized by mild pyrexia and transient lymphopaenia, followed by the seroconversion of neutralizing antibodies (Nettleton *et al.,* 1998). However, interaction with the host species can at times be lethal (Peterhans and Schweizer, 2010). Severe outbreaks of disease with high mortality rates have occasionally been reported in relation with acute BDV infections in sheep (Chappuis *et al.,* 1984); as well, a mucosal disease syndrome has been described in persistently infected (PI) sheep (Monies *et al.,* 2004). Like all pestiviruses, BDV has the ability to penetrate the placenta and infect the foetus. When this occurs, the consequences vary: if infection occurs before day 60 of the gestation period (i.e. before foetal immunocompetence) and the foetus survives, the new-born will be PI, with specific immunotolerance against BDV and continuous shedding of the virus throughout its life. PI animals can be apparently normal but usually grow poorly and have lower life expectancy (Nettleton *et al.,* 1998). These animals have great epidemiological importance since they play a key role in maintaining BDV in the flock.

The Pyrenean chamois *(Rupicapra pyrenaica pyrenaica)* is a mountain ungulate belonging to the order Artiodactyla that is endemic to the Pyrenees (northern Spain, Andorra and southern France). Since 2001 several outbreaks of a previously unreported disease associated with BDV-4 infection have been described in chamois from the central Pyrenees and have decimated several Pyrenean chamois populations with mortalities ranging from 42% to 86% (Marco *et al.*, 2007; Marco *et al.*, 2009b),

A retrospective study performed by Marco *et al.* (2011) described the detection and isolation of a BDV-4 in two chamois sampled in the Freser-Setcases National Hunting Reserve (NHR) in 1996, five years before the first outbreak of this disease in the Pyrenees. These two chamois showed no clinical signs consistent with BDV infection. In addition, in the same NHR, a prevalence of pestivirus antibodies of 66% was described in chamois hunted during the 1990s (Marco *et al*., 2008).

Surprisingly, to date the Freser-Setcases NHR hosts the only chamois population on the southern face of the Pyrenees that has not yet been affected by a BDV-associated disease. The aim of this study was to assess whether, despite the lack of disease outbreaks, BDV persist in the Pyrenean chamois population in Freser-Setcases. We also investigated the presence of BDV in other domestic and wild sympatric ruminants such as cattle, sheep, goat, mouflon and roe deer.

MATERIALS AND METHODS

Study area

The study area consists of the Freser-Setcases NHR located in the eastern Pyrenees (Figure 1) (42° 23' N, 2° 9' E), a reserve characterized by communal pastures shared by different species of livestock (sheep, goats and cattle). High-level communal pasturing is a centuries-old farming practice that involves the pasturing of domestic ruminants from different farms on high-altitude grassland in the Pyrenees, generally from May/June until September. Pyrenean chamois share habitat with wild ruminants such as roe deer (*Capreolus capreolus*) and European mouflon (*Ovis aries*).

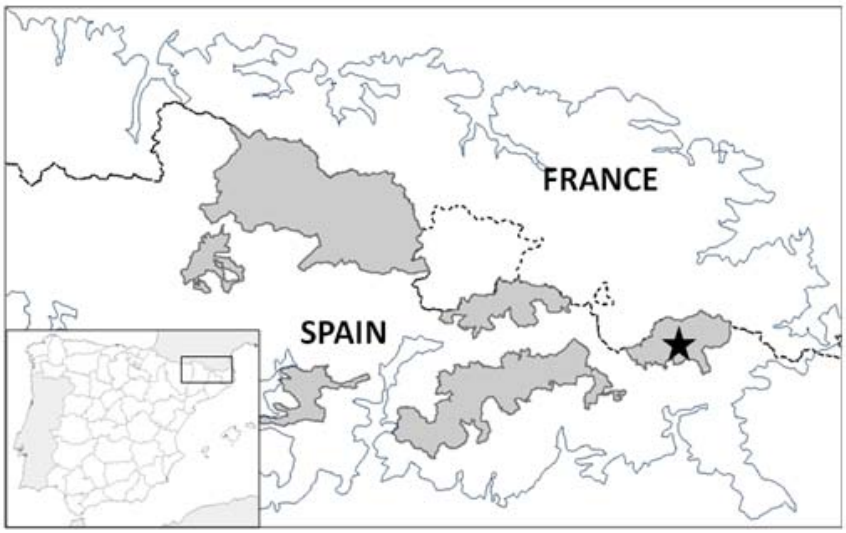

Figure 1: Map showing the study area in the Pyrenees, with the National Hunting Reserves (NHR) in grey and the Freser-Setcases NHR marked with a star.

Animals and samples

In total, we sampled 561 Pyrenean chamois (sera and/or spleen) between 2003 and 2010, and 92 European mouflon and 24 roe deer (sera and/or spleen) between 2007 and 2010. We also studied 159 sheep, 13 goats and 40 cattle sera collected between 2007 and 2011.

Specifically, chamois samples corresponded to 475 apparently healthy animals hunted during the hunting season, 78 apparently healthy chamois captured for scientific purposes and eight diseased or dead chamois. Necropsies were performed on all diseased chamois according to standard protocols. Samples from the rest of the wild ruminant species corresponded to apparently healthy animals hunted during the hunting season.

Blood samples from hunted and captured animals, obtained by intracardiac and jugular venepuncture, respectively, were placed in sterile serum separator tubes and centrifuged at 1,200 g for 15 minutes. Spleen samples from hunted or necropsied animals were also collected. Sera and spleen samples were stored at -20ºC until analyzed. Data from the animals were taken (sex, age, location). Sera from domestic ruminants were collected during the annual sanitary campaigns when livestock descended from their summer pastures.

Serological tests

Sera were tested for the presence of antibodies against pestivirus with a commercial blocking ELISA assay (BVD/MD/BD P80, Antibody Screening, Pourquier, Montpellier, France). This test has a minimum specificity of 99.2 % and an observed sensitivity of 100 % for testing BDV positive sheep sera.

To confirm the results of the ELISA test and to determine the specificity of the antibodies, VNT was performed on 215 chamois, 22 mouflon, 12 sheep and 16 cattle sera. The following strains used were: BVDV-1 strain NADL (Collett *et al.,* 1988; Gen Bank accession number M31182), BDV-1 strain 137/4 (Vilcek *et al.,* 1997; Gen Bank accession number U65052) and BDV-4 strain Esp97 (Vega *et al.,* 2002; Gen bank accession number FR714860). In addition, all sera from all species (except chamois), along with 144 chamois sera, were tested against BDV-4 strain Freser-4, a local strain isolated from a hunted chamois. Eighty-seven Pyrenean chamois sera were also tested with BDV-4 strain Aran-1 (Marco *et al.*, 2011; Gen Bank accession number AM765800) isolated from a diseased chamois found in 2001 in the Alt Pallars-Aran NHR.

VNT was performed according to 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 (1938). Titres of 1:10 and higher were considered positive. Viral replication was monitored by the Immuno-Peroxidase Monolayer Assay (IPMA) with polyclonal home-made pestivirus-specific serum.

Virus detection

Sera from domestic ruminants and from 393 Pyrenean chamois were tested for the presence of pestiviral antigen with a commercial sandwich ELISA assay (Synbiotics, Lyon, France) according to the manufacturer's procedure.

Reverse transcription-polymerase chain reaction (RT-PCR) was performed on spleen homogenates (or on sera when spleen was not available) for 436 Pyrenean chamois, all the roe deer and all the mouflon. RT-PCR was performed using described panpestivirus primers 324 and 326 (Vilcek *et al.,* 1994) and a commercial kit (One-Step PCR kit, Qiagen, Hilden, Germany). Before performing the RT-PCR, viral RNA was extracted using a commercial kit (Nucleospin Viral RNA Isolation, Macherey Nagel, Düren, Germany). RT-PCR was also performed on all domestic ruminant sera giving a positive or inconclusive result for the ELISA antigen detection test.

Sequence analysis

Sequence analyses of the 5'UTR region from RT-PCR positive samples were performed using primers 324 and 326 (Vilcek *et al.,* 1994). Purified amplicons (Minelute Gel Extraction Kit, Qiagen, Hilden, Germany) were analyzed with Big Dye Terminator v.3.1 Kit and the ABI 3130xl Genetic Analyzer (Applied Biosystems, Warrington, United Kingdom). The phylogenetic tree was constructed using the neighbour-joining method (Saitou and Nei, 1987) with automatic root location. To test tree-branch reliability, a bootstrap analysis of 1,000 replicates was performed with series of bootstrap samples.

Statistical analysis

For data analysis we used the functions for analysing epidemiological data in the library epiR (http://cran.r-project.org/web/packages/epiR/epiR.pdf/) and the free

statistical software R (http://www.r-project.org/). In this R package point estimates and confidence intervals are based on formulae provided by Rothman (2002). We calculated true prevalence and the confidence interval for single proportion calculations (exact binomial method) (Altman *et al.,* 2000). For Pyrenean chamois, we calculated seroprevalence by year, sex and age class (chamois were grouped into three classes: class 1 = <3 years old, class 2 = 3–10 years old, class 3 = >10 years).

We performed a Wilcoxon signed-rank test to assess whether the titres in the analyzed chamois, mouflon, sheep and cattle differed for the different viral strains. To test for differences in the mean ranks we compared pairwisely the antibody titres against each virus strain. The limit for statistic significance was defined as $P \leq 0.05$.

RESULTS

Animals

All the hunted and captured animals were apparently healthy when examined. Of the eight diseased or dead Pyrenean chamois, two were old chamois with no macroscopic lesions, while the remaining six animals had, respectively, infectious keratoconjunctivitis, multiple traumas, multiple spleen and liver abscesses (Vela *et al.*, 2011), caseous lymphadenitis, bacterial pneumonia and clinical signs consistent with BDV infection. The latter chamois was a four-year-old female with poor nutritional status, neurological symptoms, a marked limp and focal alopecia with associated skin hyperpigmentation in the cervical, scapular and dorsal regions.

Serological results

Pestivirus antibodies were detected in Pyrenean chamois, mouflon, sheep and cattle. Global prevalence in chamois was 43.98% (95% CI: 39.82-48.14). Figure 2 shows the evolution of the rate of prevalence in chamois during the study period. Seroprevalence of antibodies in mouflon was 22.04% (95% CI: 13.38-30.70), in sheep 9.15% (95% CI: 4.43-13.87) and in cattle 71.50% (95% CI: 57.24-85.77). Mean titres for Pyrenean chamois, mouflon, sheep and cattle against the different pestivirus strains and their median and range values are given in Table 1.

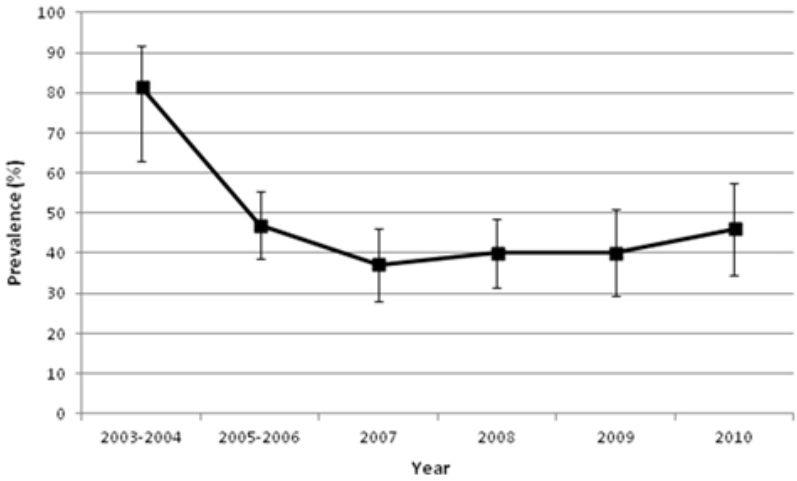

Figure 2: Annual evolution of the rate of prevalence in chamois originating from the Freser-Setcases NHR with 95% confidence interval (exact 95% binomial confidence intervals) for the pestivirus NS3 antibodies.

Table 1: Mean, median and range (min-max) for Pyrenean chamois, mouflon, sheep and cattle of antibody titres against five pestivirus strains and P-values obtained using the Wilcoxon signed rank test.

	Strain 1	Mean	Median	Range	vs	Strain 2	Mean	Median	Range	Wilcoxon signed rank test, P-value
	NADL	169.8	40	0-5120	-	ESP97	794.5	320	0-10240	1.10E-38[a]
	NADL	169.8	40	0-5120	-	ARAN-1	163.67	80	10-1280	5.11E-7
	NADL	169.8	40	0-5120	-	137/4	523.8	160	0-10240	1.73E-21
	NADL	169.8	40	0-5120	-	Freser-4	827.77	160	10-20480	8.99E-27
Pyrenean	ESP97	794.5	320	0-10240	-	ARAN-1	163.67	80	10-1280	3.04E-12
chamois	ESP97	794.5	320	0-10240	-	137/4	523.8	160	0-10240	5.25E-8
	ESP97	794.5	320	0-10240	-	Freser-4	827.77	160	10-20480	0.009
	137/4	523.8	160	0-10240	-	ARAN-1	163.67	80	10-1280	0.003
	ARAN-1	163.67	80	10-1280	-	Freser-4	827.77	160	10-20480	7.49E-7
	137/4	523.8	160	0-10240	-	Freser-4	827.77	160	10-20480	0.006
	NADL	232.8	80	0-2560	-	ESP97	535.23	320	40-5120	0.001
	NADL	232.8	80	0-2560	-	Freser-4	333.33	160	40-1280	0.008
European	NADL	232.8	80	0-2560	-	137/4	406.66	80	20-5120	0.130
mouflon	ESP97	535.23	320	40-5120	-	Freser-4	333.33	160	40-1280	0.429
	ESP97	535.23	320	40-5120	-	137/4	406.66	80	20-5120	0.019
	137/4	406.66	80	20-5120	-	Freser-4	333.33	160	40-1280	0.076
	NADL	91.66	20	0-640	-	ESP97	371.66	240	20-1280	0.004
	NADL	91.66	20	0-640	-	Freser-4	52.5	40	10-80	0.313
	NADL	91.66	20	0-640	-	137/4	135	100	20-320	0.082
Sheep	ESP97	371.66	240	20-1280	-	Freser-4	52.5	40	10-80	0.002
	ESP97	371.66	240	20-1280	-	137/4	135	100	20-320	0.068
	137/4	135	100	20-320	-	Freser-4	52.5	40	10-80	0.299
	NADL	504.37	320	0-2560	-	ESP97	97.5	60	0-640	0.02
	NADL	504.37	320	0-2560	-	Freser-4	80.62	80	0-160	0.019
Cattle	NADL	504.37	320	0-2560	-	137/4	29.37	25	0-80	0.003
	ESP97	97.5	60	0-640	-	Freser-4	80.62	80	0-160	0.742
	ESP97	97.5	60	0-640	-	137/4	29.37	25	0-80	0.118
	137/4	29.37	25	0-80	-	Freser-4	80.62	80	0-160	0.061

[a]E: represents *times ten raised to the power of* and is followed by the value of the exponent.

Virological results

The ELISA of antigen detection gave positive results in sera from 12 chamois, one sheep, one goat and one cow, as well as inconclusive results in ten sheep, 12 goats and two cows. RT-PCR results from these animals were positive for two chamois. In addition, viral RNA was detected by RT-PCR in spleen from three apparently healthy chamois and in the chamois with clinical symptoms consistent with BDV infection. Two of the viruses found in these animals were sequenced and named as Freser-1 (detected in a chamois hunted in 2006, and Freser-4 (detected in a chamois hunted in 2007). Both strains appear in Figure 3.

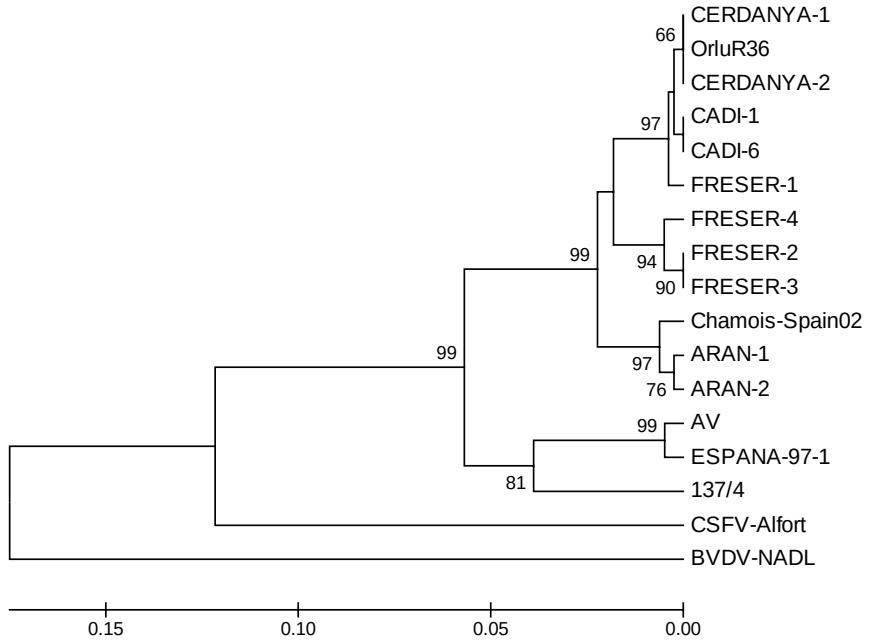

Figure 3: Unrooted neighbour-joining phylogenetic tree based on the 5'UTR sequence in pestiviruses. The numbers on the branches indicate the bootstrap values (in percentage; 1,000 replicates). Sequences of strains were taken from GenBank with the following accession numbers: CERDANYA-1 (AM905930), OrluR36 (DQ898294), CERDANYA-2 (AM905931), CADI-1 (AM905918), CADÍ-6 (AM905923), FRESER-2 (FN691777), FRESER-3 (FN691778), Chamois-Spain02 (AY641529), ARAN-1 (AM765800), ARAN-2 (AM765801), AV (EF693984), ESPANA-97-1 (FR714860), 137/4 (U65052), Alfort (X87939) and NADL (M31182). FRESER-1 and -4 are not deposited in the GenBank.

Prevalence related factors

Seroprevalence was significantly higher in females (OR $_{females}$ = 1.59; 95% CI: 1.11-2.28) than in males. The risk of being seropositive significantly increased in older

animals. In chamois of age class 3 seroprevalence was significantly higher than in chamois of age class 2 ($OR_{class\ 3}$ = 6.46; 95% CI: 3.47-12.04), while in chamois of age class 2 seroprevalence was higher ($OR_{class\ 2}$ = 3.3; 95% CI: 1.66-6.55) than in chamois of age class 1.

Comparative VNT

Ten combinations of viruses used in VNT were tested in the Pyrenean chamois. In the rest of the species six combinations were tested (Table 1). Comparison of mean ranks showed significant differences between antibody titres against the different strains (obtained P-values appear in Table 1). Chamois had significantly higher titres against BDV-ESP97, mouflon against BDV-ESP97 and BDV-Freser-4, sheep against BDV-ESP97, BDV-Freser-4 and BVDV-NADL, and cattle against BVDV-1-NADL.

DISCUSSION

BDV infection has been linked to the decimation of Pyrenean chamois populations since the first outbreak of the disease was described in 2001. Although the virus has been detected there, unexpectedly Freser-Setcases NHR has not yet been hit by the disease. Although factors such as genetic differences have been proposed to explain this singular epidemiological situation (Cavallero *et al.,* 2012), in this study we investigated epidemiological factors such as population immunity and viroprevalence.

The global prevalence of pestivirus antibodies found in the Pyrenean chamois of the Freser-Setcases NHR was high (43.98%) in comparison with prevalences described before the disease outbreaks in areas such as the Cadí NHR (estimated at 5%) (Marco *et al.,* 2009b). However, the seroprevalence observed at the beginning of this study in 2003-2004 was remarkably high (81.29%), which suggests that a high circulation of pestiviruses occurred during this period. Interestingly, although rangers noticed the presence of a few chamois with clinical signs consistent with BDV infection (e.g. difficulty in moving and alopecia) in 2001, no diseased animals could be captured and BDV infection could not be confirmed. A short-lived episode of mortality that had no effects on population dynamics seems to have occurred given that available census data from this NHR indicate a continuous increase in the total number of chamois (358 chamois in 1984, 1,339 in 1992, 2,607 in 1996, 3,077 in 2005 and 3,162 in 2011). In 2005 seroprevalence decreased to 46.87% but then remained constant until the end of

the study in 2010, indicating that pestiviruses may have been circulating in the chamois population during the whole study period, a finding that has been corroborated by virological results.

Higher seroprevalence in females and in old chamois has previously been reported (Pioz *et al*., 2007; Martin *et al.,* 2011). Males lead more solitary lives, whereas females form groups in which there is more social interaction that makes the transmission of pestiviruses more likely. The increasing proportion of seropositive chamois with age could be explained by the fact that older animals have had greater probabilities than younger animals of contacting the virus during their lifespans.

In the VNT of seropositive chamois, we unexpectedly found significantly higher titres against BDV-ESP97 strain than against BDV Freser-4 strain. This result is very interesting since BDV-ESP97 was isolated from a Spanish flock of sheep (Vega *et al*., 2002) and suggests either a close antigenic similarity between the two strains or that both strains are infecting this chamois population. However, significantly lower titres against chamois strains such as BDV-Aran-1, isolated from a diseased chamois during the first outbreak of disease in the central Pyrenees in 2001, were observed, indicating that this strain does not circulate in this chamois population.

Despite the presence of BDV-4 in the chamois population, the absence of outbreaks of disease in the Freser-Setcases NHR is probably related to, amongst other things, virological factors. The BDV-4 responsible for the disease outbreaks in other zones could be more infectious than the BDV-4 circulating in Freser-Setcases NHR, an hypothesis that could be related to the "avirulence theory", whereby some parasites (BDV in this case) with long-lasting evolutionary associations with their hosts come to have benign effects. This is based on the argument that parasites may endanger their own long-term survival if they are too virulent since they reduce their host population and thus run out of new hosts (Schmid-Hempel, 2011). In our study, this hypothesis is supported by the isolation of two BDV-4 in 1996 (Marco *et al.,* 2011) and the detection of a BDV in three apparently healthy chamois. The BDV-4 sequenced in a chamois in 2006 could have been a BDV originating from the epidemic in the nearby Cadí NHR (Marco *et al.,* 2009b) given that it forms a separate cluster with the Cadi and Cerdanya viruses, which are different from the two 1996 BDV and the other sequenced viruses from the Freser-Setcases NHR (Figure 3). The circulation of weakly virulent strains could have been the cause of the observed prevalence of antibodies, which could hinder the spread of more virulent strains within the chamois population. If as a result of seroconversion many individuals in a population are immunized by infection by

weakly virulent strains, any more virulent strain that subsequently invades the population will be blocked by immune individuals and therefore will not spread (Boots *et al.,* 2004). Indeed, since the detection of the first BDV in a healthy chamois in 2006 (which may have been related to the spread of the infection from the nearby Cadí NHR), to date only one diseased chamois has been found in Freser-Setcases NHR, no abnormal mortality has been observed and, as mentioned above, the population has tended to increase according to the census performed by the Reserve's rangers. Our hypothesis that prevalence is blocking the appearance of a disease outbreak in this population is supported by the findings of Cabezón *et al*. in 2011. In their study seven chamois from the Freser-Setcases NHR (five seronegative and two seropositive for BDV) were inoculated with a BDV isolated from a naturally infected diseased chamois. All the naïve chamois developed a long-lasting viraemia and nonsuppurative meningoencephalitis, which is seen in naturally infected chamois. These results show that the chamois in the Freser-Setcases NHR can develop the disease if they are infected with the strain that was involved in the disease outbreaks, and that cross-protection exists in seropositive animals.

The prevalence of antibodies in European mouflon (22%) was higher than described in a study performed in the central Pyrenees (3.5%) but lower than the values given for the southern French Alps (61.1%) (Marco *et al.*, 2008; Martin *et al.,* 2011). Mouflon had higher antibody titres against both BDV-ESP97 and BDV-Freser-4. This supports our hypothesis that there may be great antigenical similarity between these two strains.

The absence of antibodies in roe deer concurs with previous studies that only detected a null or very low prevalence in this species (Olde Riekerink *et al.,* 2005; Marco *et al.,* 2009a; Fernández-Sirera *et al.*, 2012). Seronegativity in roe deer could be explained by different habitat selection and the lack of social behaviour in this species, which leads a more solitary life than chamois (Wilso and Mittermeier, 2011e).

The prevalence detected in sheep in this study is very low (9.15%) if compared with values from previous studies performed in other parts from the Pyrenees (64–69%) (Alba *et al*., 2008; Marco *et al.,* 2009a). It is remarkable that sheep had higher antibody titres against both BDV-ESP97 and BDV-Freser-4 but also against BVDV-1-NADL. This absence of significant differences between the titres against BDV-4 and BVDV-1 is probably related to the infection of sheep by both bovine and ovine pestiviruses. The absence of antibodies in goats agrees with the low prevalence detected in sheep since sheep are considered to be pestivirus suppliers for goats.

However, our sample size was too small and this result must be interpreted with caution. The prevalence detected in cattle is high in both areas, with specific titres against BVDV. Vaccination in this species is a routine event, which as previously described could explain this high seroprevalence (Marco *et al*., 2009a).

Although previous studies have suggested that sheep could be a source of BDV for Pyrenean chamois (Marco *et al.*, 2009a), our results lead us to rule out this species as a source of BDV for Pyrenean chamois today in Freser-Setcases. Nevertheless, it is possible that in the past the BDV-4 once spilled over from sheep to chamois. This hypothesis is supported by the similar titres in chamois to the BDV-ESP97, of ovine origin, and the BDV-Freser-4. The very low seroprevalence in sheep in this study in comparison with the high and constant prevalence in chamois suggests that BDV may be self-maintained in this chamois population. The fact that the chamois is the only species with viral detection strongly supports this hypothesis. However, we still need to elucidate the mechanism that maintains BDV in the chamois populations. PI animals are needed for maintaining pestiviruses in domestic ruminant flocks (Peterhans *et al.,* 2010) since they eliminate the virus in their secretions and excretions almost constantly and are the single most important source of infection in other animals (Barlow *et al.*, 1980). The existence of PI chamois has not yet been proved, although if we assume that BDV is self-maintained in this chamois population, then it is likely that PI chamois do exist. After a decade studying BDV disease outbreaks in Pyrenean chamois, we firmly believe that it is crucial that the main objective of future studies is the detection of PI chamois. However, these studies will be costly since they will require the use of capture-recapture techniques as a means of detecting the long-lasting viraemias that characterize PI animals.

In conclusion, BDV-4 is self-maintained in the Pyrenean chamois population in the Freser-Setcases NHR but apparently has no negative effects on population dynamics. The circulation of BDV, possibly only weakly virulent, and the high seroprevalence in this area are probably two of the most important factors that hinder the dispersion of virulent BDV-4 strains and make the occurrence of disease outbreaks less likely.

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7.3. STUDY III

SURVEILLANCE OF BORDER DISEASE IN WILD UNGULATES AND THE FIRST OUTBREAK OF DISEASE IN PYRENEAN CHAMOIS (*RUPICAPRA PYRENAICA PYRENAICA*) IN ANDORRA

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ABSTRACT

The Principality of Andorra is surrounded by areas in which Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) populations were severely affected by outbreaks of border disease virus (BDV) infection between 2001 and 2009. Nevertheless, Andorran chamois populations were not affected by the disease during this period. In light of the severe impact of BDV on several of these neighboring Pyrenean chamois populations, we monitored local Andorran populations in an effort to detect pestivirus antibodies and BDV in wild ungulates. In addition, an episode of mortality detected between 2009 and 2010 in chamois was investigated. We analyzed samples (spleen and/or serum) from 175 Pyrenean chamois, 284 European mouflon (*Ovis aries)*, and 13 roe deer (*Capreolus capreolus*). With the exception of three dead chamois found between 2009 and 2010 during an episode of mortality, all samples came from healthy animals hunted during the hunting season. A commercial blocking ELISA assay was used to test sera for the presence of antibodies against pestivirus. Positive sera were subsequently tested with a comparative virus neutralization test using three BDV strains and a bovine viral diarrhea virus strain. Reverse transcription-polymerase chain reaction (RT-PCR) was performed on all sera and spleen homogenates. Antibodies against pestivirus were detected by ELISA in four of the 69 chamois (4.84%; 95%CI: 1.29-13.11). The results of the VNT determined that three of these chamois had been infected with a BDV. Viral RNA was detected by RT-PCR in three chamois – one apparently healthy animal hunted in 2009 and in two dead animals. The resulting sequences of the detected viruses showed that the three chamois were infected with a BDV-4, the same genotype as was involved in previous episodes of mortality in the Pyrenees. In conclusion, until 2009 Pyrenean chamois from Andorra had had little contact with the pestiviruses, but in this year the BDV was detected and associated with a severe outbreak of disease.

INTRODUCTION

Pestiviruses (Family *Flaviviridae*) are enveloped spherical viruses approximately 40–60 nm in diameter. Four species of pestiviruses are officially accepted by the International Committee on Taxonomy of Viruses (ICTV): Bovine viral diarrhea virus 1

(BVDV-1) and 2 (BVDV-2) affecting cattle, border disease virus (BDV) infecting sheep and goat, and classical swine fever virus (CSFV) affecting swine (Thiel *et al.*, 2005).

Ruminant pestiviruses are not strictly host-specific and antibodies against these viruses have been reported in several domestic and wild *Artiodactyla* species (Loken, 1995; Nettleton and Entrican, 1995; Nettleton *et al.*, 1998a). However, no cases of disease caused by pestivirus in free-ranging wild ruminants other than Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) have ever been reported in Europe. In this species, a BDV genotype-4 (Arnal *et al.*, 2004) has been shown to be the etiological agent of the disease that appeared for the first time in 2001 in the Alt Pallars-Aran National Hunting Reserve (NHR). After this outbreak, the population was found to have decreased by about 41%, most probably due to the disease (Marco *et al.*, 2007). After this first outbreak several epizooties took place in other chamois populations in the Pyrenees. During 2005 a disease outbreak led to collapse the chamois population in the Cerdanya-Alt Urgell NHR, with an estimated cumulative rate of decrease of 85.6%. In June 2005, the disease spread to the nearby Cadí NHR and private hunting areas, causing an estimated cumulative rate of decrease of 63% (Marco *et al.,* 2009b). Interestingly, a retrospective study performed by Marco *et al*. in 2011 revealed that BDV infection had been present in chamois populations since at least 1990, 11 years before the first disease outbreak.

The Pyrenean chamois is a small mountain ruminant. It is one of the most characteristic animals of the Pyrenees and is also a highly important game species that generates substantial economic benefits for local communities. The disease associated to BDV-4 had a great social and economic impact on the Pyrenees. The Pyrenean chamois affected by this disease were depressed and weak, and experienced difficulties when moving. Some animals presented abnormal behavior and varying degrees of alopecia and skin hyperpigmentation as well as hematological and biochemical alterations. At necropsy, cachexia and secondary infectious processes were described in all animals (Marco *et al*., 2007).

The Principality of Andorra is a country in the Pyrenees that lies between France and Spain. It is surrounded by areas in which chamois populations were severely affected by the disease between 2001 and 2009. Unexpectedly, however, Andorran chamois populations were not affected by the disease during this period. Bearing in mind that BDV infection had been related to severe falls in chamois populations in several areas of the Pyrenees, the aim of this study was to survey the

presence of pestivirus antibodies and BDV in wild ungulates in this country. In addition, an episode of mortality detected between 2009 and 2010 was investigated.

MATERIALS AND METHODS

Study area

The study area consisted of the Principality of Andorra, a 468-km² country situated in the Pyrenees bordering on France and Spain (Figure 1). It is a mountainous country with 65 peaks over 2,500 m. The country's hunting areas are divided into three different types: there are two small reserves in which hunting is banned and the Enclar Hunting Reserve (HR) where hunting is managed by the Andorran Government; in the rest of the country chamois hunting is allowed and hunters apply great pressure to this species. Consequently, chamois in the hunting reserves are relatively isolated and reach moderate to high densities (18 chamois/100 ha in the Enclar HR) while in the rest of the territory densities are low (2 chamois/100 ha).

Animals

We analyzed samples from 175 Pyrenean chamois, 284 European mouflon (*Ovis aries*), and 13 roe deer (*Capreolus capreolus*). All samples came from healthy animals hunted during the hunting season (Table 1). At the beginning of 2010, chamois hunting was banned in Andorra due to the decline in its population and no more samples could be obtained from this source.

Samples were also obtained from three chamois carcasses found between 2009 and 2010: spleen samples were taken from a 1-year-old male chamois found dead with cachexia and pneumonia and also from a very scavenged and decomposed chamois carcass; the third sample was taken from the brain of a decomposed and scavenged chamois carcass.

Blood samples were obtained by intracardiac venipuncture from hunted animals. Spleen was obtained after hunting and at necropsy from two of the three carcasses. In one case only the head (brain) was available. Blood samples were placed in sterile serum separator tubes (Eurotubo, Rubí, Spain) and centrifuged at 1,200 *g* for 15 minutes. Sera, spleen samples, and the brain sample, were stored at -20ºC until analyzed.

Table 1: Samples from hunted ungulates by species, hunting season and type of sample.

Species	2004/2005	2005/2006	2006/2007	2007/2008	2008/2009	2009/2010	Total n
Chamois	n=13 13 sera	n=15 15 sera	n=1 2 sera	n=45 1 serum 44 spleen	n=48 19 sera 44 spleen	n=53 19 sera 48 spleen	175
Mouflon	n=46 46 sera	n=110 110 sera	n=68 68 sera	n=60 60 sera	- -	- -	284
Roe deer	n=1 1 sera	n=6 6 sera	n=3 3 sera	n=3 3 sera	- -	- -	13

Serological tests

Sera were tested for the presence of antibodies against pestivirus with a commercial blocking ELISA assay (BVD/MD/BD P80, Antibody Screening, Pourquier, Montpellier, France). This test has a minimum specificity of 99.2 % and an observed sensitivity of 100 % for testing BDV positive sheep sera. In order to confirm the results of the ELISA test and to determine the specificity of the antibodies, positive sera by ELISA assay were subsequently tested with a comparative virus neutralization test (VNT) using BVDV-1 strain NADL (Collett *et al*., 1988; Gen Bank accession number M31182), BDV-1 strain 137/4 (Vilcek *et al,*. 1997; Gen Bank accession number U65052), BDV-4 strain Esp97 (Vega *et al.*, 2002; Gen bank accession number FR714860) and BDV-4 strain Aran-1 (Marco *et al.*, 2008); Gen Bank accession number AM765800). This final strain was isolated from a diseased chamois found in 2001 in the Spanish NHR of Alt Pallars-Aran, which borders Andorra to the west.

VNT 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 titers 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 (1938). Titers of 1:10 and higher were considered positive. Viral replication was monitored by

the Immuno-Peroxidase Monolayer Assay (IPMA) with home-made polyclonal pestivirus-specific serum.

Virus detection

Reverse transcription-polymerase chain reaction (RT-PCR) was performed on all sera, spleen homogenates and on one brain homogenate, using previously described panpestivirus primers 324 and 326 (Vilcek *et al*., 1994) and a commercial kit (One-Step PCR kit, Qiagen, Hilden, Germany). Before the RT-PCR, viral RNA was extracted using a commercial kit (Nucleospin Viral RNA Isolation, Macherey Nagel, Düren, Germany).

Sequence analysis

Sequence analyses of the 243 bp fragment of the 5'UTR region of the RT-PCR positive samples were performed using primers 324 and 326 (Vilcek *et al*., 1994). Purified amplicons (Minelute Gel Extraction Kit, Qiagen, Hilden, Germany) were analyzed with Big Dye Terminator v.3.1 Kit and the ABI 3130xl Genetic Analyzer (Applied Biosystems, Warrington, United Kingdom). The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) using automatic root location. A bootstrap analysis of 1,000 replicates was performed by creating series of bootstrap samples to test tree branch reliability (Figure 2).

Statistical analysis

We calculated true prevalence and its 95% confidence interval by using the functions for analyzing epidemiological data included in the library epiR (http://cran.r-project.org/web/packages/epiR/epiR.pdf/) and the free statistical software R (http://www.r-project.org/).

RESULTS

Surveillance program

Antibodies against pestivirus were detected by ELISA in four (one animal hunted in 2005, another in 2006 and two in 2009) of the 69 chamois (4.84%; 95%CI: 1.29-13.11). No antibodies were found in the other species. The neutralizing antibody titers of the positive chamois (named *a, b, c* and *d*) are shown in Table 2. The OIE has established that a three-fold difference or more between end-points of two titrations

should be considered decisive for an infection by the virus species yielding the highest titer (OIE, 2008). However, no rule has been established for different subgroups of the same pestivirus species. Three chamois (*a*, *c* and *d*) had BDV specific antibodies but differences between titers against the different BDV strains were too small to determine the BDV infective strain. The fourth chamois (*b*) had less than three-fold differences between the titers against BVDV and BDV.

Viral RNA was detected by RT-PCR in one apparently healthy chamois hunted in December of 2009 (hereafter chamois 1) and genetic typing revealed that the genotype in this case was BDV-4. The virus appears in the phylogenetic tree as ANDORRA-1 (Figure 2). Information about this viropositive chamois appears in Table 3.

Table 2: Virus neutralization titers against four pestivirus strains in the four seropositive chamois. Neutralizing antibody titers are expressed as the reciprocal of the highest dilution that neutralized 100 tissue culture infective doses (100 $TCID_{50}$) in all cultures. BVDV: bovine viral diarrhea virus, BDV: border disease virus.

Chamois	BVDV-1 NADL	BDV- 1 137/4	BDV-4 ESP-97	BDV-4 Aran 1
a.	40	320	320	160
b.	320	1280	320	160
c.	10	80	80	320
d.	80	80	320	640

Table 3: Summary of information available about the three viropositive chamois.

Chamois no.	Cause of death	Collection date	Age and sex	Necropsy findings	Available samples	Virus name and accession number
1	hunted	December 2009	6 year old male	Apparently healthy	Spleen Serum	ANDORRA-1 HE615083
2	unknown (scavenged)	October 2009	NA[a]	Cachexia	Brain	ANDORRA-2 HE615084
3	unknown (scavenged)	January 2010	1 year old male	Cachexia, pneumonia	Spleen	ANDORRA-3 HE615085

[a] NA: Not available

Description of outbreak

In October 2009 during the monitoring program, a chamois carcass was found in the west of the Enclar HR, near the border with the Spanish Alt Pallars-Aran NHR. In the same area, two more chamois carcasses were detected between November and December; subsequently, an unusually high number of carcasses was found there in January. In all, between October 2009 and March 2010, 79 carcasses were found. All the carcasses were found in the Enclar HR, the only exceptions being a carcass found in the north of the country in January 2010 and another in the east in March 2010 (Figure 1). All but six carcasses consisted of only skin and bones and necropsy could only be performed on three of them. Viral RNA was detected by RT-PCR in two (hereafter chamois 2 and 3) (Table 3). The genetic typing of these viruses revealed that these chamois were infected with BDV-4 and were named as ANDORRA-2 and ANDORRA-3 (Figure 2).

Although the total chamois mortality could not be assessed accurately due to the remoteness of the area involved, the census undertaken by the Andorran Government provides an indirect indication of mortality by determining population decrease. In the 2009 census, just before the episode of mortality, the chamois population in Andorra was 939 chamois and in 2010 it dropped to 555, an overall decrease of 41%. In the Enclar HR, the decline of the population was higher (a fall from 415 to 173 chamois, a decrease of 58%) than for the whole of Andorra. Game keepers observed the final presumably BDV- infected clinical case at the beginning of 2011, when a chamois with alopecia was observed. However, it was not possible to capture this animal and subsequently no more clinical cases or dead chamois were found or reported.

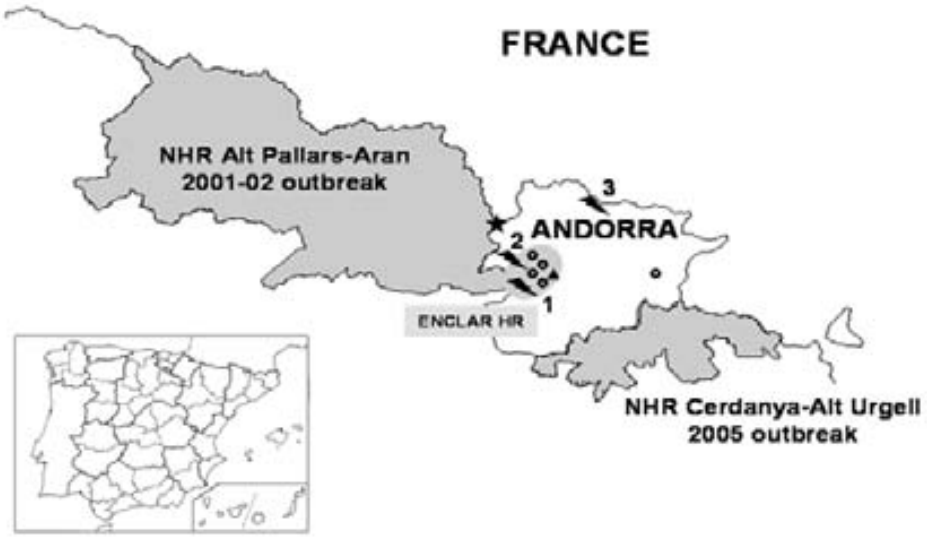

Figure 1: Map showing the location of the Principality of Andorra (left) and its borders with two NHR in Spain where important disease outbreaks have occurred in recent years (right). In the right-hand map appear the most relevant findings regarding the BDV infection outbreak.

RT-PCR positive cases (chamois 1, 2 and 3). Chamois 1 was hunted and was an apparently healthy animal; chamois 2 and 3 consisted of carcasses with clinical signs consistent with pestivirus infection.

Carcass with lesions consistent with pestivirus infection unconfirmed by RT-PCR

Carcasses with lesions consistent with pestivirus infection not analyzed by RT-PCR

RT-PCR positive chamois found in Andorra in 2002 (Arnal *et al*., 2004)

NHR: National Hunting Reserve

HR: Hunting Reserve

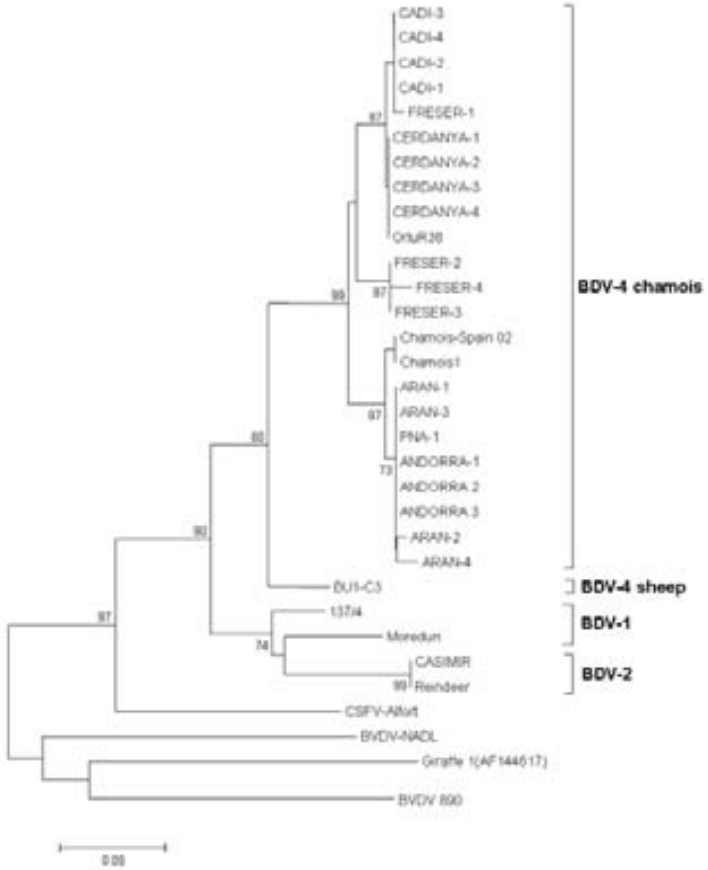

Figure 2: Unrooted neighbor-joining phylogenetic tree based on the 5'UTR sequence among pestiviruses. Chamois strains appear enclosed in a differentiated group into border disease virus 4 (BDV-4). The strains detected in Chamois No. 1, 2 and 3 appear named as ANDORRA-1, -2 and -3, respectively. These strains cluster with other chamois viruses isolated in the bordering NHR of Alt Pallars-Aran (ARAN-1, -2, -3 and -4). The numbers on the branches indicate the bootstrasp values (in percent; 1000 replicates). Sequences of strains taken from Gen Bank with following accession numbers: Chamois-Spain02 (AY641529), ARAN-1 (AM765800), CADI-1 (AM905918), CADI-2 (AM905919), CADI-3 (AM905920), CADI-4 (AM905921), CERDANYA-1 (AM905930), CERDANYA-2 (AM905931), CERDANYA-3 (AM905932), CERDANYA-4 (AM905933), ORLUR36 (DQ898294), FRESER 2 (FN691777), FRESER 3 (FN691778), ARAN-1 (AM765800), ARAN-2 (AM765801), ARAN-3 (AM765802), ARAN-4 (AM765803), AND-1 (HE615083), AND-2 (HE615084), AND-3 (HE615085), BU1-C3 (DQ361068), 137/4 (U65052), Moredun (U65023), CASIMIR (AB122085), Reindeer (AF144618), Alfort (X87939), BVDV 890 (U18059) and NADL (M31182). Sequences of strains FRESER-1, FRESER-4 and PNA-1 are not deposited in the Gen Bank.

DISCUSSION

The overall apparent prevalence of antibodies against pestivirus found in Pyrenean chamois in Andorra (4.8%) between 2001 and 2009 was low when compared with other areas of the Pyrenees, where seroprevalence ranged from 48.6% to 70.3% (Pioz *et al*., 2007, Marco *et al*., 2011). These results suggest that, despite being surrounded by severely infected populations, pestiviruses were not circulating before the outbreak of disease in Andorra. A single case of a chamois affected by the disease was described in 2002 (Arnal *et al*., 2004) near the border with Spain but was most probably related to the BDV outbreak that occurred between 2001 and 2002 in the Alt Pallars-Aran NHR (Marco *et al.*, 2007). In 2005, a second affected chamois was detected (unpublished data), most probably linked to the 2005 outbreak in the NHR of Cerdanya-Alt Urgell (Marco *et al*., 2009b). Sheep grazing, which has been associated with BDV transmission to Pyrenean chamois and which may be responsible for a high seroprevalence in some areas (Martin *et al.*, 2011a), is not common in Andorra. Thus, the low prevalence of antibodies against pestivirus detected in Andorra suggests that Andorran chamois were highly susceptible to epizooty in the case of contact with BDV infected chamois.

The absence of antibodies in roe deer and mouflon from Andorra agrees with the low circulation of pestiviruses in chamois. BDV infection in roe deer is rare (Olde Riekerink *et al.*, 2005; Marco *et al.*, 2008) and previous studies performed in the neighboring Alt Pallars-Aran NHR also indicate a very low seroprevalence in mouflon (Marco *et al*., 2008). However, in this area BDV were circulating extensively in Pyrenean chamois. Martin *et al.* (2011) described a prevalence of antibodies against pestivirus of 61.1% in mouflons from the southern French Alps. This high seroprevalence could be associated with greater susceptibility in mouflon to BDV-6 (the BDV type circulating in this part of the French Alps) infection rather than to BDV-4, or with high infection pressure from domestic animals.

The results of VNT from three positive chamois suggest that these animals came into contact with a BDV, although in one chamois no differences between titers against BDV and BVDV were detected. The absence of greater differences between the different BDV strains could be due to serological cross reactivity, which has been reported as occurring between all members of the genus Pestivirus (Schirrmeier *et al.*, 2004). Two of the positive chamois were hunted in 2005 and 2006. Given that they were hunted in the west of Andorra near the Spanish border, these animals could have

been infected with a BDV from chamois from the Alt Pallars-Aran NHR. However, infection with a BDV originating from sheep cannot be ruled out. The antibody positive chamois detected in autumn 2009 were hunted at the same time as the first chamois carcasses were observed and so could be related with the onset of the disease outbreak. Indeed, during the same period, we detected a BDV in a hunted healthy chamois collected during the surveillance program (chamois 1).

The detection of a BDV in the hunted chamois and in two of the three analyzed carcasses, together with the field observations and decrease in the population, suggest that the high chamois mortality detected in Andorra between 2009 and 2010 was related to an outbreak of BDV infection. The resulting sequences of viruses isolated in the three viropositive chamois, showed that these animals were infected with the BDV-4 genotype, the same BDV type identified as the etiological agent for the BDV associated disease in chamois (Cabezón *et al*., 2011). The lack of viral detection in the third carcass could have been caused by viral RNA degradation, since the carcass was highly decomposed. Despite being surrounded by severely affected areas since 2001, no clinical cases consistent with BDV infection or any significant mortality were observed in Andorra in its Pyrenean chamois population until the end of 2009. A number of different factors including behavior have probably played a part in this delay, For example, Pyrenean chamois tend to form spatial clusters and mixing between different groups rarely occurs. This could reduce pathogenic contamination between animals of different groups, as described in the case of the infectious keratoconjunctivitis (Crampe *et al*., 2007a, 2007b). In addition, chamois in Andorra are mainly concentrated in reserves and this type of distribution, could have delayed virus and disease transmission.

The total number of carcasses found between 2009 and 2010 in the Enclar HR was exceptional when compared with the number of dead chamois that are usually found each year (3–5 animals). The finding of the first RT-PCR positive chamois in this area and the clustering of the three isolated viruses with those isolated in diseased chamois from the Alt Pallars-Aran NHR (Chamois-Spain-02, Chamois 1, ARAN-1, ARAN-2, ARAN-3 and ARAN-4, see Figure 2) suggest that the virus/infection entered from this reserve bordering on the west of Andorra in autumn of 2009. The chronology of the carcasses found and the presumptive clinical cases observed from that moment onwards would seem to indicate that the virus spread through the chamois population in a NE direction until it reached the east of the country in spring 2010. The overall estimated mortality in Andorra was 41%, while the estimated mortality in the Enclar

HR was 58%, higher than in the rest of Andorra. This difference is probably related to the higher chamois density in Enclar, which would have facilitated virus transmission.

This study reveals that the question of pestivirus infections in chamois is not limited to the Spanish Pyrenees and that it could potentially expand to neighboring populations. We conclude that the severe decline in the chamois population in Andorra observed in 2010 was associated with an outbreak of disease due to BDV infection, and that the source of infection was the neighboring chamois population of Alt Pallars-Aran NHR in Spain.

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7.4. STUDY IV

SURVEY OF PESTIVIRUS INFECTION IN WILD AND DOMESTIC UNGULATES FROM SOUTH-WESTERN ITALIAN ALPS

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ABSTRACT

The transmission of pestiviruses between domestic and wild ruminants has not been documented in communal alpine pastures shared between wildlife and livestock. The aim of this study was to investigate the role of domestic and wild ungulates species from Varaita Valley (SW Italian Alps) in the epidemiology of pestivirus infections. Sera from free-ranging Alpine chamois (*Rupicapra rupicapra*) and roe deer (*Capreolus capreolus)* were collected from 1994 to 2009 and 2001 to 2009, respectively. Also, sera from cattle and sheep sampled in 2009 were studied. Sera were tested for the presence of antibodies against pestivirus with an ELISA assay. Sera from positive animals were subsequently tested with a comparative virus neutralisation test using the BVDV-NADL and BDV-137/4 strains. Sera were tested for the presence of pestiviral antigen and the presence of viral RNA with a commercial ELISA assay and RT-PCR. Antibodies against pestivirus were detected in 132 out of 312 (42%) chamois, in 30 out of 175 (17%) cattle and 6 out of 24 (25%) sheep. No antibodies were found in roe deer. No pestivirus antigen or RNA was detected in any of the samples. Results indicate circulation of pestiviruses among the studied chamois, cattle and sheep populations. However the role of wild ungulates in the dynamics of pestivirus infection is still unknown and monitoring the presence of these viruses in wild ungulates would be of importance, especially in the chamois population, where pestiviruses seem to circulate extensively.

INTRODUCTION

Pestiviruses (family Flaviviridae) are single-stranded, positive-sense RNA viruses, which have the ability to cross species barriers and to infect a wide range of artiodactyls. Thus, bovine viral diarrhoea virus (BVDV) and border disease virus (BDV) are not strictly host specific and antibodies against these viruses have been reported in several species of domestic and wild ruminants (Nettleton and Entrican, 1995; Loken, 1995; Nettleton *et al.,* 1998). Pestiviruses have also been isolated from different artiodactyls such as camelids (Evermann, 2006), cervids (Frolich and Hofmann, 1995) and in different Bovidae species (Vilcek and Nettleton, 2006).

In Pyrenean chamois, important disease outbreaks with high mortality rates have been described, associated with a BDV-4 strain (Marco *et al.,* 2007). Pestiviruses

cause in livestock a wide range of reproductive clinical manifestations entailing severe economic losses worldwide. Acute BVDV infection in cattle produces enteric disease consisted in diarrhoea, pyrexia and mild depression with high morbidity and low mortality, and, in some cases, acute fatal haemorrhagic syndrome has been described (Carman *et al.,* 1998).

BDV infection among sheep causes acute infections characterized by short-period viraemia (Nettleton *et al.,* 1998). However some outbreaks of fatal disease associated to BDV have been described in sheep (Chappuis *et al.* 1984). After acute BVDV and BDV infections, neutralising antibodies appear in serum. However, the success of pestiviruses is based in their capacity to be transmitted congenitally to foetus. This congenital infection can lead to the birth of persistently infected (PI) animals characterized by the immunotolerance to the virus and continuous replication and excretion of it.

BVDV and BDV infections in free-ranging wild ruminants have been relatively poorly studied in Europe. There is a lack of knowledge about the epidemiology of pestivirus infection between livestock and wildlife. Pestivirus investigations in domestic and wild ruminants from Alps have been performed in Switzerland (Holzwarth *et al.,* 2011), Austria (Krametter-Froetscher *et al.,* 2010), France (Martin *et al.,* 2011) and Italy (Olde Riekerink *et al.,* 2005). Also, studies on pestivirus epidemiology in alpine areas have been reported (Krametter- Froetscher *et al.,* 2007) describing a transmission of pathogens between domestic livestock and wildlife, especially during the sharing of communal alpine pastures along the summer.

Varaita Valley is an alpine valley located in the Piedmont region in Italy. This region recently has started a BVDV pilot eradication programme in cattle. In Varaita Valley, use of communal alpine pastures during the summer is common, but pestivirus infections in wild ungulates have not been studied in this area. Because of this, data about the transmission of pestiviruses between domestic and wild ruminants from this valley are unknown. The aim of this study was to investigate pestivirus infections in ungulates from Varaita Valley and to assess the role of domestic and wild ungulates species in the epidemiology of pestivirus infections.

MATERIALS AND METHODS

Study area

The Varaita Valley (44°49'0'' N, 7°36'0'' E) is an alpine valley of south-western Piedmont (NW Italy, Figure 1). The valley is approximately 60 km long, from the city of Verzuolo to the 2,748-m-high Colle dell'Agnello, which connects the valley to the French Vallée du Guil. Several wild Artiodactyla species inhabit this area such as Alpine ibex (*Capra ibex*), Alpine chamois (*Rupicapra rupicapra*), roe deer (*Capreolus capreolus*), and red deer (*Cervus elaphus*). Domestic livestock (cattle, sheep and goats) share the pastures with wild ungulates during the summer. Approximately 8,550 cattle and 4,000 sheep and goats ascend each year from the neighbouring regions to the alpine meadows of Valle Varaita.

Figure 1: Map of Italy, showing Varaita Valley in the South-Western Alps**.**

Animals and samples

Blood samples from free-ranging Alpine chamois (n=312) and roe deer (n=213) were collected from 1994 to 2009 and 2001 to 2009, respectively (Table 1). These ungulates are major game species in Varaita Valley, and samples were collected during the hunting season (September–November) following the established hunting programmes. Blood samples were collected whenever possible after being shot by hunters themselves. Sex, age and location of shot were given. The age of the animals

varied between 0 and 16 years old in the chamois and between 0 and 8 years old in the roe deer.

Also, sera from cattle (n=175) and sheep (n=24) sampled in 2009 were studied (Table 1). Cattle and sheep came from different small herds (16 and 5, respectively) breed in the valley and that spend the summer months in the alpine pastures. Sampling of these animals was performed in autumn, at the returning from the summer pastures. The sampling of the cattle was designed and balanced in order to cover all the high mountain summer grazing area of the valley. In addition the cattle came from non-BVD-vaccinated herds. Blood samples from wild ungulates were obtained by intracardiac venipuncture from dead animals after being hunted. Blood samples from domestic ungulates were obtained by venipuncture of the tail vein in cattle and of the jugular vein in sheep. Samples were placed into sterile serum separator tubes and centrifuged at 1,200×g for 15 min. Sera were stored at −20°C until processed.

Serological tests

Sera were tested for the presence of pestivirus-specific antibodies against NS3 protein using a commercial blocking ELISA assay (Pourquier, Montpellier, France). Sera from positive animals were subsequently tested with a comparative virus neutralisation test (VNT) for neutralising antibodies against BVDV-1 strain NADL (Collett *et al.*, 1988; Gen Bank accession number M31182) and BDV-1 strain 137/4 (Vilček *et al.,* 1997; Gen Bank accession number U65052). Since there is no information about which pestivirus strains are circulating in the study area, we choose two reference strains to perform the VNT test. Our results should allow us to differentiate if the animals were infected with a BVDV or with a BDV. VNT was performed under procedure described in the "Manual of diagnostic tests and vaccines for terrestrial animals" (OIE, 2008) using Madin–Darby bovine kidney cells. Neutralising antibody titres were expressed as the reciprocal of the highest dilution that neutralised 100 tissue culture infective doses (100 TCID50) in all cultures, calculated according to the method of Reed and Muench (1938). Titres of 1:10 and higher were considered positive. Viral replication was monitored by the immuno-peroxidase monolayer assay with polyclonal homemade pestivirus-specific serum. We considered that the antibodies were specific of a pestivirus strain if the titre against this strain was at least three times higher when compared to the titre against the other strain (OIE, 2008).

Virus detection

Chamois, cattle and sheep sera were tested for the presence of pestiviral antigen with a commercial sandwich ELISA assay (Synbiotics, Lyon, France) according to the manufacturer's procedure. Reverse transcription polymerase chain reaction (RT-PCR) was performed in sera with a positive or inconclusive result in the antigen ELISA test. Total viral RNA was extracted directly from 150 µl of sera by a commercial kit (Macherey Nagel Nucleospin Viral RNA Isolation; Düren, Germany) according to the manufacturer's procedure. The RT-PCR was performed using previously described panpestivirus primers 324 (5'-ATGCCCWTAGTAGGACTAGCA–3'; W = A or T) and 326 (5'-TCAACTCCATGTGCCATGTAC-3', Vilcek *et al.* 1994) and a commercial kit (One-Step PCR kit; Qiagen Inc., Hilden Germany).

Statistical analysis

For data analysis we used the functions for analysing epidemiological data included in the library epiR (http://cran. r-project.org/web/packages/epiR/epiR.pdf/) by using the free statistical software R (http://www.r-project.org/). In order to test for differences in the observed proportions of positive animals by sex, we computed a chi-squared test stratified by age and reported the odds ratio values with their confidence interval. The same test was computed to test for differences by class of age (animals were separated in two groups: animals under or with 2 years and animals over 2 years). Within this R package point estimates and confidence intervals are based on formulae provided by Rothman (2002). A p value for the chi-squared statistic below 0.05 was interpreted as a lack of homogeneity between the proportions and therefore as a statistical significant difference between groups. Confidence intervals for the proportion of positive animals by year were calculated by using the confidence interval for a single proportion calculation (exact binomial method) implemented in the epiR package (Altman *et al.,* 2000). An analysis of variance was applied to test for differences between virus titres.

RESULTS

Serological results are reported in Table 1. Antibodies against pestivirus were detected by ELISA in 132 out of 312 (42%) chamois, in 30 out of 175 (17%) cattle and in 6 out of 24 (25%) sheep. No antibodies were found in the 213 samples of roe deer. The

VNT confirmed the ELISA results in all positive animals except 11 chamois that had negative titres against both pestivirus strains. Seroprevalence was significantly higher in chamois over 2 years (OR>2 years=2.98; 95%CI: 1.78-4.99). The antibody prevalence was significantly higher in females than in males in the chamois over 2 years (OR, female=2.13; 95% CI: 1.22-3.73), but no significant differences were found in the prevalence between males and females in the ≤2 years old group. The VNT results indicate that 24 chamois were infected with a BDV (titre range 10–2,560). However, seven chamois had significant higher titres against the BVDV strain (titre range 20–10,240). In 101 chamois, the specificity of the antibodies could not be determined. No significant differences were found between the titres against BD-137/4 and BVD-NADL in the chamois and in the sheep group. Cattle had significant higher antibody titres against the BVDV-NADL strain. Twelve chamois had positive results in the ELISA of antigen detection test, and 13 presented inconclusive results. They were all negative to RT-PCR.

Table 1: Annual and species repartition of sera samples, positive samples for pestivirus antibodies and geometric mean of titres obtained for each virus strain.

Species	Year	No. samples	Seropositive	Estimated prevalence and 95% Confidence interval	**Geometric mean of virus neutralisation titres**	
					BVDV-NADL	*BDV-137/4*
Alpine chamois	1994	41	25	0.61 (0.45-0.74)		
(***R. rupicapra***)	1995	38	22	0.57 (0.42-0.72)		
	2000	16	5	0.32 (0.14-0.55)		
	2001	29	15	0.51 (0.34-0.68)		
	2002	43	14	0.32 (0.20-0.47)		
	2003	28	9	0.32 (0.17-0.50)		
	2004	17	9	0.52 (0.31-0.73)		
	2005	33	14	0.42 (0.27-0.59)		
	2006	20	10	0.50 (0.29-0.70)		
	2007	15	3	0.20 (0.07-0.45)		
	2008	5	1	0.20 (0.03-0.62)		
	2009	27	5	0.18 (0.08-0.36)		
	Total	312	132	0.42 (0.37-0.47)	**71.68**[a]	**74.41**[a]
Roe deer	2001	10	0	0.00 (0.00-0.27)		
(***C. capreolus***)	2002	35	0	0.00 (0.00-0.09)		
	2003	31	0	0.00 (0.00-0.11)		
	2004	25	0	0.00 (0.00-0.13)		
	2005	25	0	0.00 (0.00-0.13)		
	2006	25	0	0.00 (0.00-0.13)		
	2007	25	0	0.00 (0.00-0.13)		
	2008	10	0	0.00 (0.00-0.27)		
	2009	27	0	0.00 (0.00-0.12)		
	Total	213	0	0.00 (0.00-0.01)	**ND**[b]	**ND**[b]
Cattle	2009	175	30	0.17 (0.12-0.23)	**163.73**[c]	**41.04**[c]
Sheep	2009	24	6	0.25 (0.12-0.44)	**56.57**[d]	**63.50**[d]

[a] 132 samples tested; [b] ND: Not done; [c] 30 samples tested; [d] 6 samples tested

DISCUSSION

The results of the present study indicate high exposure of Alpine chamois to pestiviruses in the Varaita Valley. Serosurveys of antibodies to pestivirus previously performed in Alpine chamois populations also described high prevalence, ranging from 25.5% to 45.9% (Olde Riekerink *et al.,* 2005; Martin *et al.,* 2011a). These studies associated the high seroprevalence with the transmission of the infection from domestic ruminants to chamois in alpine pasture areas. The global prevalence (42% CI:

37-47) detected in chamois in the present study is similar to the one described by Martin *et al*. (2011a) in 2005 in the French South Alps.

During our study period, the prevalence in chamois populations showed fluctuations. Interestingly, high rates of antibodies were found in samples from 1994 and 1995 (apparent prevalence of 61% CI: 45-74 and 57% CI: 42-72, respectively). This is the highest seroprevalence against pestivirus described in an Alpine chamois population. Moreover, the antibody titres during this period were higher compared to other years, suggesting that it was an epidemic phase of the infection. However, no reduction has been described in the chamois population during this period. In addition, although pestivirus has demonstrated to cause disease in Pyrenean chamois (Marco *et al.,* 2009b), detection of abortions or deaths of adult animals due to pestivirus infection has not been described in Italian Alpine chamois populations. The reproduction rate (kids/females) showed fluctuations during the study period, but it did not show significant decreases (Dematteis, unpublished data).

The statistically significant differences observed in the seroprevalence between sexes (higher seroprevalence in females than in males) may be explained by the different social behaviour of both sexes: the males lead a more solitary life while the females form groups, where there are more social interactions between animals and the transmission of pestiviruses is more probable. In addition, no sex differences were observed in the group of ≤2 years old animals. This can be explained by the fact that the young males and females live together with the adult females, and both sexes of this group have the same probability of becoming infected with the virus. Other studies have described these sex differences in pestivirus antibody rates (Martin *et al.*, 2011a; Olde Riekerink *et al.,* 2005).

Also, the results of the present study showed that the oldness increases the risk of seroconversion in chamois, as described by other authors (Martin *et al*., 2011a). The increasing proportion of seropositive chamois with age could be explained by the fact that older animals had more probabilities of contacting the virus during their life when compared to younger animals.

The VNT results of chamois sera indicate that 24 chamois were infected with a BDV. However, seven chamois had higher titres against the BVDV strain, suggesting that they probably were infected with a virus from bovine origin. Nevertheless, in a high number of chamois (101), the specificity of the antibodies could not be determined, and no significant differences were found when the titres between BD-137/4 and BVDV-NADL of the chamois group were compared. Different hypotheses

could explain these results. The most probable is that the chamois became infected with a BDV, since the mean antibody titre of the chamois was higher against the BDV strain when compared to the BVDV strain. The lack of major differences between the titres against BDV-137/4 and BVDV-NADL could be explained by the fact that chamois were infected with a BDV strain other than BDV-137/4. Due to the BD strain used in the VNT test was the BD-137/4, a strain of type 1, there is the possibility that a different type of BDV was circulating in these chamois populations. Cross-reactivity of antibodies to pestivirus is high, and this would have contributed to make difficult the differentiation between the BVDV and BDV strains. The extent of crossreactivity depends on the strain of pestivirus involved and the interval between infection and time of sampling (Wensvoort *et al.,* 1989; OIE, 2008). A less likely hypothesis could be the infection with both BDV and BVDV in most of the chamois, preventing us from establishing differences between the titres against the two pestivirus species (Olde Riekerink *et al*., 2005).

There are two possible explanations for the chamois that had negative results in the VNT but were positive to ELISA antibody test. They could have a low antibody titre to a different pestivirus strain, or they could be false positives in the ELISA test. We consider that the first explanation is more probable. We have used extensively this ELISA kit, and it has a high sensitivity, since it detects very low titres of antibodies (<1/10). In our laboratory we have tested ovine sera with negative titres against several pestivirus strains and a very low titre against the circulating strain in the area of origin.

Although the prevalence rate among chamois suggests a high circulation of pestiviruses, no viruses were found in the analysed samples. Pestiviruses are most easily detected in PI animals, but the existence of PI in chamois has not been proved. If they existed, they would have a poor survival rate. In this study only 20 of the 312 sampled chamois were under 1 year old. This could have decreased the chance of detecting possible PI animals and therefore the virus. Twelve chamois showed positive antigen ELISA results, but they were negative to RT-PCR.

There are two possible explanations for these ELISA-positive but RT-PCR negative animals. The first one is that sensitivity and specificity of the commercial test used are known for domestic animals only. In addition, sera samples of this study were collected in dead animals and then specificity and sensitivity values are lower than in live animals (Olde Riekerink *et al*., 2005). Actually, the presence of false positives using this ELISA commercial kit in chamois samples has already been described (Marco *et al.,*

2009a), suggesting a low specificity of this ELISA kit when used in sera from hunted animals. This emphasizes the problems of commercial kits' use in species and conditions different from those recommended by the manufacturer. The second explanation is that these animals are not false positive but real positives, and that degradation of viral RNA during storage at −20°C has caused failure to detect pestiviral RNA by RT-PCR. We consider this second explanation less probable, since in retrospective studies performed in our laboratory, we have detected pestiviral RNA in samples stored at −20°C during 15 years.

The negative results observed in roe deer concur with other studies performed in roe deer (Olde Riekerink *et al.,* 2005; Marco *et al.,* 2009a). Seronegativity in roe deer could be explained by the different habitat and different social behaviour in this species compared to chamois. Roe deer are leading a more solitary life and prefer a more bush containing habitat. Because of fewer inter-animal contacts, infectious diseases like BD or BVD may be expected to be less prevalent.

Summer communal pasturing is a common practice in Varaita Valley. This farming system frequently has been associated with the transmission of pathogens from domestic to wild ruminants (Loken, 1995). The low seroprevalence of antibodies detected in cattle does not suggest a high circulation of pestiviruses among this species during the sampling period (2009), but there is a lack of information relating the frequency of antibodies in cattle in previous years. Unfortunately a very little number of sheep could be sampled, but the proportion of positive sheep was similar to the proportion of positive chamois in the same year. However, due to this lack of samples, these results should be interpreted with caution.

Regarding VNT results from livestock, cattle showed higher specific titres against the BVDV-NADL strain. Taking into account that sampled cattle came from non-vaccinated herds, the results of the present study indicate that BVDV are circulating in the herds from the valley. However, no differences were found in the titres between the two pestivirus strains in sheep. This strongly suggests the circulation of a different type of BDV, since sheep are more likely to be infected with BDV rather than with BVDV.

Although seroprevalence and VNT results from sheep and chamois from our study suggest that pestiviruses are shared between these two species, we cannot dismiss the possibility of specific pestivirus cycle into chamois populations. The higher proportion of seropositive females supports this hypothesis since it indicates that pestiviruses are transmitted between chamois in the group of females, and the

maintenance of the virus in the population could be possible. However this study has some limitations like the relatively small sample size. Therefore, more studies with a bigger sample size should be performed.

The occurrence of BVDV-specific antibodies and isolation of virus from wild animals has lead to the speculation that free-living ungulates may be a reservoir of virus for transmission to cattle and sheep. This would be especially important in countries which are applying BVD eradication programmes (Vilcek and Nettleton, 2006). In Italy voluntary BVDV control programmes for cattle have been described (Ferrari *et al*., 1999). The Region Piedmont, where Varaita Valley is located, started recently an eradication pilot programme for BVDV (Conterbia *et al.*, 2010). Interestingly, results of our study indicate high exposure to pestiviruses in the chamois population of this valley, suggesting that this species could act as a pestivirus reservoir. But our findings do not allow us to dismiss the possibility that the chamois were infected by domestic ruminants, playing a "victim" role in the dynamics of pestivirus infection. For this reason, monitoring the presence of these viruses in wild ungulates would be of importance and this surveillance would be crucial in the chamois population, where pestiviruses seem to circulate extensively.

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7.5. STUDY V

INVESTIGATIONS OF PESTIVIRUS INFECTIONS IN WILD CAPRINAE IN EUROPE

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ABSTRACT

The Alpine ibex (*Capra ibex*) and Iberian ibex (*Capra pyrenaica*) are not considered threatened by the International Union for Conservation of Nature, but there is concern regarding genetic diversity, the founder effect and minimum viable populations. Therefore, information on reproductive disorders could be of importance for the conservation and management of this species. The objectives of this study were to determine the prevalence of antibodies to pestiviruses and to investigate the presence of pestiviruses in Alpine ibexes from the Italian Alps and Iberian ibexes from Spain. Sera from 223 Alpine ibexes and 328 Iberian ibexes were analysed. Sera were tested for the presence of antibodies to pestivirus with a commercial blocking ELISA. Sera from positive ibexes were subsequently tested with a comparative virus neutralisation test (VNT) for antibodies to bovine viral diarrhoea virus (BVDV)-NADL and border disease virus (BDV)-137/4 strains for Alpine ibexes, and BVDV-NADL and BDV-Moredun strains for Iberian ibexes. RT-PCR was performed in all sera. Antibodies to pestiviruses were found in 16 (7.17 %) of 223 Alpine ibexes and in two (0.6%) of 328 Iberian ibexes. The VNT confirmed the ELISA results in 15 Alpine ibexes and in both Iberian ibexes. One Alpine ibex had negative titres against both pestivirus strains studied. All VNT-confirmed animals had higher antibody titres against the BDV strain, except one Alpine ibex that had higher titres against the BVDV strain and three Alpine ibexes that had the same titres against both strains. RT-PCR was negative in all animals. Exposure to pestiviruses in Alpine and Iberian ibex populations in the Italian Alps and Spain, respectively, is low. The results indicate that Alpine ibex and Iberian ibex may not play a role in the spread of pestiviruses among other species and suggest low exposure to these viruses in the studied populations.

INTRODUCTION

Pestiviruses (family Flaviviridae) are single-stranded, positive-sense RNA viruses that are traditionally classified in four species: bovine viral diarrhoea virus type 1 (BVDV-1) and type 2 (BVDV-2), which affect cattle, border disease virus (BDV), which infects small ruminants and classical swine fever virus, which affects pigs. BVDV and BDV are not strictly host-specific and antibodies to these viruses have been reported in several species of wild ruminants (Vilcek and Nettleton, 2006). Pestiviruses can cause a

wide range of reproductive clinical manifestations in livestock, and recently, BDV infection has been associated with severe outbreaks of disease in the Pyrenean chamois (*Rupicapra pyrenaica*), leading to severe reductions in its populations (Marco *et al.*, 2007; 2009b).

The Alpine ibex (*Capra ibex*) and Iberian ibex (*Capra pyrenaica*) are mountain ruminants belonging to the subfamily Caprinae (family Bovidae). Although these species are not considered threatened by the International Union for Conservation of Nature, there is concern regarding their genetic diversity, the founder effect and minimum viable populations (Schakleton, 1997). Therefore, information about reproductive disorders could be of importance for the conservation and management of these species.

The objectives of the present study were to determine the prevalence of antibodies to pestiviruses and to investigate the presence of pestiviruses in Alpine ibexes from the Italian Alps and in Iberian ibexes from Spain.

MATERIALS AND METHODS

Serum samples from 223 Alpine ibexes and 328 Iberian ibexes were analysed. The Alpine ibexes comprised 107 animals from the Gran Paradiso National Park (45°30′10″N, 7°18′36″E), 60 from the Maritime Alps Natural Park (44°13′53″N, 7°10′36″E), 48 from the Lanzo Valley (45°17′58″N, 7°25′07″E) and eight from the Varaita Valley (44°49′0″N, 7°36′0″E), all in the Italian Alps. The Iberian ibexes surveyed comprised 315 animals from the Ports de Tortosa i Beseit Natural Hunting Reserve in north-east Spain (40°48′N, 0°19′E) and 31 from the Sierra Nevada National Park in south-east Spain (37°09′N, 3°16′W). No data regarding the sex or age of the animals were available.

The samples from Alpine ibexes were collected from healthy, chemically immobilised animals captured between 1994 and 2004. The samples from Iberian ibexes were collected from healthy, chemically immobilised animals captured in 2005 at the Sierra Nevada National Park, and from healthy animals collected during the hunting season from the Ports de Tortosa i Beseit Natural Hunting Reserve between 1995 and 2006. Blood samples from chemically immobilised ibexes were collected from the jugular vein and those from hunted ibexes were collected by cardiac

venepuncture. Samples were placed into sterile serum separator tubes and centrifuged at 400 *g* for 15 minutes. Sera were stored at –20°C until processed.

Sera were tested for the presence of antibodies to pestiviruses with a commercial blocking ELISA assay (BVD/MD/BD P80, Antibody Screening; Pourquier). Sera from positive ibexes were subsequently tested with a comparative virus neutralisation test (VNT) for neutralising antibodies to the BVDV-NADL and BDV-137/4 strains for Alpine ibexes, and BVDV-NADL and BDV-Moredun strains for the Iberian ibexes. The VNT was performed following a previously described procedure (OIE, 2004). Neutralising antibody titres were expressed as the reciprocal of the highest dilution that neutralised 100 tissue culture infective doses (100 $TCID_{50}$ per cent) in all cultures. Titres of 1:10 and higher were considered positive. Viral replication was monitored by the immunoperoxidase monolayer assay with polyclonal home-made pestivirus-specific serum. RT-PCR was performed on all sera, using the previously described pan-pestivirus primers 324 and 326 (Vilcek *et al.,* 1994) and a commercial kit (One-Step PCR kit; Qiagen). For the RT-PCR, viral RNA was extracted from the sera using a commercial kit (Nucleospin Viral RNA Isolation; Macherey Nagel).

RESULTS

Antibodies to pestiviruses were found in 16 (7.17%) of 223 Alpine ibexes and in two (0.6%) of 328 Iberian ibexes. The VNT confirmed the ELISA results in 15 Alpine ibexes and in both of the Iberian ibexes (Table 1). One Alpine ibex had negative titres against both pestivirus strains studied. All VNT-confirmed animals had higher antibody titres against the BDV strain compared with the BVDV strain, except for one Alpine ibex that had higher titres against the BVDV strain and three Alpine ibexes that had the same titre against both strains. RT-PCR was negative in all animals.

Table 1: Virus neutralization titres of 15 Alpine ibexes and two Iberian ibexes against three different pestivirus strains. ND; not done.

	BVDV-NADL	BD-Moredum	BD-137/4
Alpine ibex 1	160	ND	640
Alpine ibex 2	160	ND	160
Alpine ibex 3	640	ND	640
Alpine ibex 4	160	ND	160
Alpine ibex 5	0	ND	10
Alpine ibex 6	40	ND	320
Alpine ibex 7	20	ND	160
Alpine ibex 8	0	ND	40
Alpine ibex 9	80	ND	160
Alpine ibex 10	640	ND	40
Alpine ibex 11	40	ND	160
Alpine ibex 12	40	ND	160
Alpine ibex 13	80	ND	160
Alpine ibex 14	80	ND	160
Alpine ibex 15	320	ND	640
Iberian ibex 1	0	40	ND
Iberian ibex 2	20	160	ND

DISCUSSION

The prevalence of antibodies to pestiviruses has been reported in several wild ungulate species (Vilcek and Nettleton, 2006). However, to the authors' knowledge, this is the first serosurvey of pestivirus antibodies in the Alpine ibex and the second in the Iberian ibex. The overall seroprevalence found in the Alpine ibexes in the present study is low, and is similar to the seroprevalence described in other wild ruminants from the Italian Alps, such as roe deer (*Capreolus capreolus)* (0%) and red deer (*Cervus elaphus*) (5.9%) (Olde Riekerink *et al.*, 2005) However, a high seroprevalence (25.5%) has been described in Alpine chamois (*Rupicapra rupicapra*) in the same area, and recently, in the French Alps, an even higher seroprevalence has been reported (45.9%) (Martin *et al.*, 2011a). In both studies, this high seroprevalence was associated with the transmission of the infection from domestic ruminants in Alpine pasture areas, where these species share the habitat with wild ruminants. In the areas from which animals were sampled in the present study, the few cases of seropositive animals detected could also have been associated with transmission of the infection from domestic ruminants, suggesting that the Alpine ibex may not play a role in the spread of BDV among other species. The VNT confirmed the ELISA results in all animals except one Alpine ibex. The higher titres against the BDV strain suggest that the Alpine ibexes had been infected with this pestivirus species, most probably of ovine origin. However,

one Alpine ibex had significantly higher titres against the BVDV strain, and could have been infected with a strain of bovine origin.

The low seroprevalence found in the Iberian ibexes in the present study agrees with that previously reported (Santiago-Moreno *et al.,* 2010). The two ELISA-positive serum samples from Iberian ibexes showed higher titres of neutralising antibodies to the BDV strain, which also suggests that these animals were infected with an ovine strain and that Iberian ibexes may not be important in the maintenance of pestivirus infections.

The low seroprevalence found in both the Alpine and Iberian ibexes is in agreement with published data showing that Border disease in domestic goats is rare (Nettleton *et al.*, 1998). However, monitoring the presence of pestiviruses in these ibex species is important, since the populations of another member of the subfamily Caprinae, the Pyrenean chamois, have been severely reduced by several outbreaks associated with BDV infection (Marco *et al*., 2007; 2009). In conclusion, the results of the present study indicate low exposure to pestiviruses in Alpine and Iberian ibex populations from the Italian Alps and Spain, respectively, and suggest that pestiviruses may not play a significant role in the epidemiology of infections or have a significant effect on the health of these ruminant species.

7.6. STUDY VI

SPECIFICITY OF PESTIVIRUS ANTIBODIES IN WILD RUMINANTS FROM SWITZERLAND

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ABSTRACT

A previous study carried out in the Switzerland detected antibodies against pestivirus in several wild ungulates. The present study has the aim to specify the antibodies detected in nine Alpine chamois (*Rupicapra rupicapra*), four Alpine ibex (*Capra ibex*) and four red deer (*Cervus elaphus*) by using a comparative serum neutralization test with five different pestivirus strains: two BVDV (bovine viral diarrhea virus) strains, one of type 1 and 2 each, and three BDV (border disease virus) strains (type 3, 4 and Swiss). Six chamois showed higher titers against the BDV strains. A high cross-reactivity was observed between the different BDV strains. One chamois showed an exceptionally high antibody titer against the BVDV-1 strain. In two chamois the titers were generally very low and no determinant differences between the titers against BDV and BVDV were detected. Two ibexes showed clear higher titer against BDV. However in two ibexes the differences between the titers against BVDV and BDV were not meaningful. Three of the four analyzed deer, had higher antibody titers against the BVDV strains, and in two deer the titers against BVDV-1 were significantly higher. In only one deer the titers were similarly high against BVDV and BDV. The Alpine chamois, Alpine ibex and red deer populations in Switzerland can be infected with different pestivirus species, not only BVDV but also BDV. Although the majority of the chamois showed higher titers against the BDV strains, the finding of a chamois with an anti-BVDV titer comparable to the one in cattle carrying PI animals is notable.

INTRODUCTION

Several countries have started bovine viral diarrhea (BVD) eradication programs due to the high economical losses caused by this viral infection of cattle. Switzerland started a BVD national eradication campaign in 2008 (Zimmerli *et al.*, 2009). However, cattle rearing in Switzerland have some peculiarities which could interfere with this campaign. This country covers about 20% of the Alps and the shared use of Alpine pastures by multiple cattle herds during the summer grazing season is a widespread practice (involving one third of the cattle population). This common use of communal summer pastures is thought to play an important role in maintaining and transmitting BVD in Switzerland (Braun *et al*., 1998). Due to the mixing of animals from different herds, cattle of uninfected herds can enter in contact with BVDV infected animals and

subsequently introduce the virus in their naïve herd of origin. Furthermore, cattle grazing on communal pastures can also share the grazing grounds with other ungulate species, mainly sheep and goats but also wild ungulates (Casaubon, 2012).

BVDV belongs together with classical swine fever virus (CSFV) of pigs and border disease virus (BDV) of sheep to the genus Pestivirus. Pestiviruses are not strictly host-specific (Vilcek and Nettleton, 2006) and therefore interspecific virus transmission is possible. BVDV and BDV infections have been described in a wide spectrum of domestic and wild ruminants (Becher *et al.*, 1997; Vilcek and Nettleton, 2006). Thus, wild ungulates could act as a source of infection for cattle, and this may have two consequences. Firstly, if cattle are infected during the days 40-120 of gestation, the virus can infect the fetus and lead to birth of a persistently infected (PI) animal that sheds the virus throughout its life. Secondly, even if a non-pregnant animal is infected, an acute infection occurs and the resulting seroconversion could interfere with serological surveillance of BVDV freedom in cattle.

In order to determine if wild ruminants may play a role in pestivirus epidemiology in Switzerland, a virological and serological survey was carried out in 2009-2011 with the aim to determine whether wild ruminants may be a reservoir of pestivirus. Although results revealed very low prevalences, several Alpine chamois (*Rupicapra rupicapra*), Alpine ibex (*Capra ibex*) and red deer (*Cervus elaphus*) were found antibody positive by ELISA (prevalences of 2.6%, 1.8% and 2.7% respectively) and a serum neutralization test (SNT) using a BVDV-1 strain. Moreover, in the same study a BVDV was detectedin a chamois (Casaubon, 2012). However, cross-reactivity between pestiviruses is known to be high (OIE, 2008) so that further investigations are needed in order to discriminate between antibodies against BVDV and BDV. The determination of the specificity of pestivirus antibodies, i.e., the identification of the pestivirus species against which they are directed, is crucial to understand pestivirus epidemiology and assess the occurrence of interspecific transmission on Alpine pastures. Therefore, the present study has the aim to specify the antibodies detected in red deer, alpine chamois and ibexes by using a comparative SNT.

MATERIALS AND METHODS

Sera from nine Alpine chamois, four Alpine ibex and four red deer were included in the present study. All these animals were hunted in 2009 in the Swiss Alps,

namely in the cantons of Valais, St Gall, Glarus, Nidwald, Grisons, Appenzell and Obwald. Blood samples were collected post-mortem from the heart or body cavities into serum separator tubes and shipped by post mail to the laboratory. Upon reception, blood samples were centrifuged, serum was aliquoted into eppendorf tubes and stored frozen until analysis (Casaubon, 2012). All 17 samples analysed by SNT were previously tested positive in an inhouse BVDV antibody detection ELISA described by (Canal *et al*., 1998) and except of two alpine chamois and one ibex also confirmed by SNT against a BVDV-1 strain.

The SNT was performed using five different pestivirus strains: two BVDV strains, one of type 1 and 2 each, and three BDV strains. The BVDV-1h virus 04-01b was isolated in 2004 at the Institute of Veterinary Virology from serum of a Swiss PI cow (Bachofen *et al*., 2008). BVDV-1h viruses are abundant in cattle in Switzerland (Stalder *et al.,* 2005; Bachofen *et al*., 2008) and is the only strain to have ever been isolated from an Alpine chamois (Casaubon, 2012). The BVDV-2a strain CS8644 (Wolfmeyer *et al*., 1997) was kindly provided by G. Wolf (Institute of Medical Microbiology, Infectious and Epidemic Diseases, Munich, Germany). The BDV Swiss strain CH-BD4 originates from leukocytes of a Swiss PI sheep and belongs to a subgenotype of BD viruses preliminary named BDV Swiss described by Reichert in 2009. The BDV-3 virus used was the first BDV isolated in Switzerland and is described as CH-BD1 (Stalder *et al.*, 2005). The BDV-4 strain H2121/1 was isolated from tissue of a Pyrenean chamois from Andorra (Arnal *et al.*, 2004) and was kindly provided by Peter Nettleton. The BVDV-1h, the BD-Swiss and the BD-3 strains are widespread in Switzerland, while the other two have never been never been found in this country.

SNT was performed using primary bovine turbinate cells for the BVD viruses and primary lamb synovial membrane cells for the BD viruses. A commercial cell culture medium (Earle's minimal essential medium (MEM, Seromed Biochrom, Munich, Germany) was used. Foetal calf serum (FCS, Sigma or Oxoid GmbH, Wesel, Germany) was free of BVDV and antibody to BVDV as tested by virus isolation and SNT, respectively. Since the volumes available for SNT were very limited, sera were pre-diluted 1:16 in MEM and inactivated at 56ºC for 30 minutes. Virus stock was diluted in MEM to give a working suspension with a concentration of 2,000 $TCID_{50}$/ ml. Sera were consequently diluted in 2-fold dilution steps. Of each antibody dilution, 0.5 ml was mixed with 0.5 ml of the virus working suspension and incubated for 1 h at 37 °C and 5% CO_2. The final serum dilutions ranged from 1:32 to 1:65’536. Following incubation, each virus-antiserum mixture was distributed to eight wells (100 µl each) of a 96 well

microtiter plate seeded with cells, yielding an approximate virus concentration of 100 $TCID_{50}$ per well. After 5 days of incubation at 37 °C and 5% CO_2, cells were fixed and stained by the Immuno-Peroxidase Monolayer Assay (IPMA) (OIE, 2008).The serum neutralization titer is expressed as the reciprocal of the serum dilution capable to inhibit infection in 50% of the wells. For being able to calculate even low titers, we made the assumption that if any well of the 1:32 dilution was positive, the underlying 1:8 and 1:16 dilutions (that were not actually done due to limited amount of serum) were positive, too. Titers between 23.8 (one single well positive in the 1:32 dilution) and 45.3 thus represent only the highest possible titer and may in reality be lower but not negative. Titers were calculated according to the method of Reed and Muench (1938). In order for the SNT to be valid, the titration of the working suspension had to yield a virus concentration of 600–6,000 $TCID_{50}$/ml to result in 30–300 $TCID_{50}$ per well.

RESULTS

Neutralizing antibody titers against the different pestivirus strains are shown in Table 1.

Table 1: Neutralizing antibody titers of the Alpine chamois, Alpine ibex and red deer sera against five pestivirus strains.

	BVDV-1h	**BVDV-2a**	**BDV-Swiss**	**BDV-3**	**BDV-4**
chamois 1	14000	406	891	3440	955
chamois 2	689	448	446	1450	3170
chamois 3	80,6	<23.8	190	589	1150
chamois 4	448	556	2720	2430	2900
chamois 5	294	119	549	1024	1450
chamois 6	156	<23.8	588	1280	838
chamois 7	90,5	<23.8	36,8	446	362
chamois 8	25,4	32	<23.8	25,4	<23.8
chamois 9	<23.8	40,3	<23.8	<23.8	<23.8
ibex 1	340	156	1570	1540	2500
ibex 2	105	223	1024	771	955
ibex 3	0	0	64	36,8	40,3
ibex 4	401	119	223	680	588
red deer 1	32	25,4	0	0	0
red deer 2	137	55,7	105	55,7	50,8
red deer 3	236	27,9	32	23,8	0
red deer 4	85	0	25,4	0	0

DISCUSSION

Serological cross-reactivity between different pestiviruses has to be taken in consideration when interpreting these results. The extent of cross-reactivity depends on the strain of ruminant pestivirus involved and the interval between infection and time of sampling (Wensvoort *et al.*, 1989). The OIE has established that a three-fold difference or more between end-points of two titrations should be considered decisive for an infection by the virus species yielding the highest titer (OIE, 2008). However, when comparing different subgroups of the same pestivirus species, this rule may be too stringent.

The more than three-fold higher titer of chamois no. 1 against the BVDV-1h strain is congruent with the results from virus isolation in this animal, which had shown that it was infected with a BVDV strain from this genotype (Casaubon, 2012). However, most of the chamois (no. 2, 3, 4, 5 and 6) had significantly (>3 fold) higher titers against the BDV strains. This coincides with the results described in other serological studies performed in Alpine chamois, which also documented a BDV specificity of pestivirus antibodies in most of the positive chamois (Olde Riekerink *et al.,* 2005). Chamois no. 2, 3 and 7 had significantly higher titers against BDV-3 and BDV-4 than against BDV Swiss. However, the differences between BDV-3 and -4 titers were too small for allowing us to clearly determine the specificity within the BDV strains. Since BDV-4 has never been detected in Switzerland, it is likely that these animals had contact with BDV-3 viruses. Chamois no. 4, 5 and 6 had similarly high titers against BDV-3, BDV-4 and BDV-Swiss strains. It is interesting to note that there seems to be a rather high cross-reactivity between the BDV viruses from Switzerland, particularly the BDV-3 strain and BDV-4, which was associated to severe outbreaks of disease in chamois in the Pyrenees (Arnal *et al*., 2004). This result suggests an antigenic similarity of these viruses. In chamois no. 8 and 9, titers were generally very low and no meaningful differences between the titers against BDV and BVDV were detected. Cross-reactivity between pestiviruses usually is high, and it increases with time after infection (OIE, 2008). Although antibodies against pestiviruses are said to remain detectable livelong, it seems that, over time, it gets more and more difficult to determine the pestivirus species that caused the infection. This could explain the observations in chamois no. 8 and 9. Therefore, the finding of a low titer against the BVDV-2a strain in chamois no. 9 should be interpreted with caution. Infections with this strain have been occasionally reported in Germany, Italy, France, Belgium and Austria but have so far not been detected in

Switzerland (Wolfmeyer *et al.*, 1997; Pratelli *et al.*, 2001; Couvreur *et al.*, 2002; Vilcek *et al.*, 2003).

The ibex no. 1 and 2 showed significantly higher titers against the BDV than against the two BVDV strains. This result coincides with the results of a previous study which determined BDV specificity of antibodies in seropositive Iberian and Alpine ibexes (Fernandez-Sirera *et al.*, 2011a). Ibex no. 3 had generally low titers and no clear antibody determination was possible. Interestingly, the titers of ibex no. 4 were very similar for all five viruses though not being noticeably low. This may be due to an infection with another pestivirus such as CSFV that cross-reacts with both BVDV and BDV, or to a contact with both BVDV and BDV. The first is very unlikely as CSFV is eradicated in Switzerland and wild boar as presumable reservoir hosts (Köppel *et al*., 2007) live in a completely different habitat than ibex. The second option may be possible as on some Alpine pastures cattle are brought up to altitudes of 2,500 meter above sea level, where they could meet ibex. However, interactions involving ibex in Switzerland are more common with sheep than with cattle (Ryser-Degiorgis *et al*., 2002).

The antibody titers in red deer were generally low and thus a clear determination of the antibody specificity was difficult. However, of the four tested deer, three (no. 1, 3 and 4) had higher antibody titers against the BVDV strains, and in deer no. 3 and 4 titers against BVDV-1h were significantly higher compared to the other viruses. This strongly suggests that they had been infected with this virus strain. In only one case (red deer no. 2) the titers were similarly high against BVDV and BDV.

Although our sample size was low, it is interesting to note that our results concur with previous studies, which documented that ibex and Alpine chamois mostly have antibodies against BDV (Olde Riekerink *et al.*, 2005; Fernández-Sirera *et al.*, 2011a; 2012), while deer are usually infected with BVDV (Krametter *et al.,* 2004; Duncan *et al.*, 2008). This could be due to a higher susceptibility of cervids to BVDV infection. Indeed, BVDV have been isolated from different cervid species including red deer (Vilcek and Nettleton, 2006). More likely, however, the difference in natural habitat determines which pestivirus species wild animals are most likely to become infected with. Red deer live at lower altitudes than chamois and ibex and are therefore more likely to have contact with BVDV PI cattle all year round (Casaubon, 2012). On summer pastures, sheep and goats are grazing often on higher and steeper pastures than cattle and are usually left roaming unattended for several weeks. Thus, it seems

more likely for chamois and ibex to have contact to sheep and therefore BD virus. Moreover contacts between sheep and ibex are common (Ryser-Degiorgis *et al.*, 2002).

Pestivirus seroprevalence in sheep and goats in Switzerland have been previously reported, with values of 16.1% and 25.4%, respectively (Danuser *et al.*, 2009). Interestingly, in contrast to sheep in which we find BDV-3 and BDV-Swiss with similar frequency (unpublished data, C. Bachofen), chamois may not be infected very frequently by BDV-Swiss viruses, as seroprevalence against pestivirus is generally very low in Swiss chamois (Casaubon, 2012) and in nearly all cases investigated here the titers were higher against BDV-3 and BDV-4 than against BDV-Swiss. However, data of the present study indicate a high cross-reactivity between the three BDV strains used, which suggest an antigenic similarity between these strains. Alternatively, the observed reactions may be due to cross-reactions with yet unidentified BDV strains circulating in wild ruminants in Switzerland.

In conclusion, Alpine chamois, Alpine ibex and red deer populations in Switzerland can be infected with different pestivirus species, not only BVDV but also BDV. The altitude of their natural habitat and the contact rates with domestic livestock may influence the likelihood of either infection.

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8. GENERAL DISCUSSION

8. GENERAL DISCUSSION

Epidemiology of pestivirus infection in ruminants from the Pyrenees

In recent years several outbreaks of disease associated with BDV-4 infection have caused serious declines in a number of Pyrenean chamois populations to the extent that this disease is nowadays the greatest threat facing this characteristic Pyrenean species (Hurtado *et al.,* 2004; Marco *et al.,* 2009).

The study of Pyrenean chamois populations severely affected by disease outbreaks in the past has revealed the existence of two different scenarios: in some populations BDV did not seem to circulate at all or only to circulate at a very low rate (Cerdanya, Alt Urgell, Berguedà and Solsonès regions: CAUBS). The apparently disappearance of BDV in CAUBS may have been due to the extremely high mortality rate in the chamois population during the disease outbreak, which could have endangered the long-term maintenance of the BDV in the population by reducing the number of animals susceptible to infection. Yet, census data revealed that this population recovered quickly. Nevertheless, we should bear in mind that low BDV circulation implies an increase in the proportion of naïve chamois, which could facilitate the appearance of a second disease outbreak if BDV comes into contact with the same population again. This situation could potentially be very dangerous since a second wave of mortality could cause another bottleneck effect.

A different chamois population (Val d'Aran, Pallars Sobirà regions, VAPS), in which BDV still circulated even ten years after the first disease outbreak in 2001, represented the opposite case. During the course of the study, BDV was regularly detected in hunted chamois from this population and isolated cases of disease were reported. Moreover, the sequence analysis of the 5'UTR showed that the detected viruses clustered with viruses isolated in 2001 from diseased chamois. The continuous circulation of BDV helped maintain a relatively constant prevalence of pestivirus antibodies. Interestingly, census data from VAPS revealed that this chamois population had recovery poorly, suggesting that BDV had a negative effect on population dynamics in the Pyrenean chamois in this area. More studies are needed if we want to assess the role of BDV in the decline of this chamois population, which – as occurs in pestivirus infection in domestic ruminants (Nettleton, 1998) – is possibly associated with poor survival of kids and low fecundity rates. A further highly important issue to be taken into consideration is the fact that the chamois population in VAPS may act as

a source of BDV for other chamois populations since the appearance of BDV in Andorra was linked to the arrival of BDV from the VAPS population.

The Principality of Andorra represented an altogether different scenario. This country is surrounded by areas in which Pyrenean chamois populations were severely affected by outbreaks of BDV infection between 2001 and 2009. Nevertheless, Andorran chamois populations were not affected by the disease during this period. In light of the severe impact of BDV on several Pyrenean chamois populations in areas abutting onto Andorra, we monitored the Andorran populations in an attempt to detect pestivirus antibodies and BDV in wild ungulates. Our study detected a very low prevalence of antibodies (4.8%) in chamois hunted between 2004 and 2009. This result indicated that this chamois population had had little contact with pestiviruses before 2009 and suggested that it was highly susceptible to a disease outbreak when BDV-4 arrived. Indeed, during the study period (between 2009 and 2010) an episode of mortality in chamois was detected that gave rise to an estimated 42% overall decrease in chamois numbers in Andorra. The detection of BDV-4 in two carcasses and in one healthy hunted chamois, together with field observations of chamois with clinical signs consistent with BDV infection, suggested that the high chamois mortality detected in Andorra between 2009 and 2010 was related to an outbreak of BDV infection. Moreover, the detected BDV-4 formed a separate cluster with other viruses from VAPS. This fact, along with the observation of the first cases of the disease in the western part of Andorra bordering on VAPS, strongly suggested that the virus arrived from this area. A number of different factors including behaviour had probably played a part in delaying the appearance of the disease in Andorra. The distribution of chamois in Andorra, mainly concentrated in reserves, could also have delayed virus and disease transmission.

The Eastern Pyrenees and, specifically, the Pyrenean chamois population of the Freser-Setcases NHR completed this interesting and diverse epidemiological panorama. This is the only chamois population on the south face of the Pyrenees that has not yet been affected by a BDV disease outbreak. Interestingly, Marco *et al.* (2011) detected two BDV-4 in two hunted chamois from this NHR in 1996. The research performed in this thesis with chamois samples collected between 2003 and 2010 showed that BDV-4 circulated regularly in this chamois population. This hypothesis was supported by the high constant levels of seroprevalence and the detection of a BDV in four chamois during the study period. However, no mortality episode was observed during this study and to date only one diseased chamois has been found in 2007.

Moreover, the census data reflected a continuous increase in the total number of chamois during all the study period. The high seroprevalence in this chamois population and the long-term circulation of possible low virulent strains may be important factors that hindered the dispersion of virulent BDV-4 strains and prevented disease outbreaks from occurring. Other factors such as population genetics had also been suggested as explanations for the absence of severe mortality in this area (Cavallero *et al.*, 2012).

Antibodies against pestivirus were detected in all the studied wild ruminants from the Pyrenees, although seroprevalence was always low. In deer species we detected low prevalences: 10.73–15.15% in red deer, 1.13–3.58% in roe deer and 8.60% in fallow deer. Deer are more likely to possess antibodies against BVDV (Vilcek and Nettleton, 2006), which have been isolated in red (Nettleton, 1990), roe (Romvary, 1965; Schellner, 1977; Fischer *et al*., 1998) and fallow (Neumann *et al.,* 1980) deer. Interestingly, the predominance of BDV-specific antibodies in cervids from the Pyrenees was likely to be related to the exceptionally high circulation of BDV in chamois populations during disease outbreaks, which would have facilitated spill-over of BDV from chamois to other species.

In mouflon we detected different prevalences depending on the geographical area (5% in the Central Pyrenees, 0% in Andorra and 22% in the Eastern Pyrenees). However, all these values were lower than those described from the southern French Alps, which had been related to high infection pressure from domestic ruminants (Martin *et al.,* 2011). VNT determined that mouflons mostly had BDV antibodies, which was to be expected since mouflons are closely related to sheep. However, infection in this species with BDV originating from chamois could not be discarded.

The low prevalence of antibodies detected in wild ruminants other than Pyrenean chamois suggested that these animals do not play an important role in maintaining BDV in wild ruminant populations. On the contrary, the high circulation of BDV in Pyrenean chamois seemed to cause a spill-over of BDV to other wild ruminants in the Pyrenees, who then play the role of “victims”. The absence of virus detection in wild ruminants agreed with the observed low prevalences and suggested that virus circulation is low; consequently, the detection of viraemic animals is a difficult task.

Several studies have suggested that sheep could be the source of BDV infection in chamois (Marco *et al.,* 2009a; Martin *et al.*, 2011a). Seroprevalence in sheep and goats from all the analyzed areas varied between zones (ranging between 9% and 49%) and was always lower than or similar to seroprevalence in chamois; thus could discard

high infection pressure from small domestic ruminants. The lowest seroprevalence was recorded in sheep sharing pastures with Pyrenean chamois in the Freser-Setcases NHR. This is a strong indication that BDV-4 is self-maintained in this chamois population and ruled out sheep as a source of pestiviruses in Pyrenean chamois in this area. Nevertheless, the moderate seroprevalence in sheep and goats from other areas suggested that elsewhere pestiviruses circulate in these species. Given that they cohabit with chamois, we cannot totally rule out the possibility that these species are a source of infection in chamois. However, an accurate assessment of the role of domestic ruminants in the epidemiology of BDV infection in chamois requires further studies that may be able to isolate the BDV strains circulating in sheep and goats in the Pyrenees. In the VNT, sheep had significantly higher titres against the strain BDV-Esp97 and the BDV originating from chamois. This is probably due to cross-reactivity, since both belong to the same genotype as circulates in sheep flocks (Valdazo-González *et al.,* 2007).

Seroprevalence in goats was variable (ranging from 0% to 32%). As in sheep, the lowest value was detected in goats from the Eastern Pyrenees. It has been suggested that sheep and cattle are the main origin of pestivirus infection in goats (Loken, 1995; Krametter-Froetscher *et al.*, 2010). Indeed, we detected a few goats with higher antibody titres against the BDV-4 originating from chamois. Thus, bearing this in mind, we can affirm that Pyrenean chamois in the Pyrenees can also act as a pestivirus supplier for goats.

The overall prevalence detected in cattle in the Pyrenees was high in all areas and specific titres against BVDV exist. However, vaccination in these animals is a routine event that could explain their high seroprevalence, as has already been described (Marco *et al.,* 2009a). Despite the fact that vaccinating is common (and usually unreported by veterinarians), our results suggested that BVDV circulates occasionally since in VNT we detected several goats with specific BVDV titres and it seems likely that they were infected by cattle.

No viruses were detected in any domestic ruminant species. In these ruminants, the short viraemia in acute infections and the low prevalence of PI animals probably hampered the detection of viruses (Nettleton and Entrican, 1995).

Pestivirus infection in other mountain areas and in other wild ruminant species

High-level communal pasturing is a very common practice throughout the Alps and the pressure of domestic ruminants in high mountain pastures is usually higher

than in the Pyrenees. In a study performed in the south-west Italian Alps a high seroprevalence of pestivirus antibodies in Alpine chamois was detected (42%); likewise, high antibody prevalences have been described in other studies performed in the Italian and French Alps (Olde Riekerink *et al.,* 2005; Martin *et al.,* 2011a). However, in the Swiss Alps low seroprevalence was found to exist (2.6%, Casaubon, 2012). This would seem to indicate that pestiviruses circulate extensively in Alpine chamois in certain geographical areas and that members of the genus *Rupicapra* are likely to be infected with pestiviruses. VNT showed no significant differences in the titres between BVDV and BDV in the Alpine chamois from the south-west Italian Alps, probably because we did not include the local circulating strain in the test. No antibodies were detected in roe deer, although a large number of animals were sampled. This result concurred with previous studies from the Italian Alps (Olde Riekerink *et al.*, 2005). Seronegativity in roe deer could be explained by the differences between this species' habitat choices and social behaviour and those of the chamois. Roe deer lead more solitary lives and prefer habitats containing more bushes (Wilso and Mittermeier, 2011e). Due to the fewer inter-animal contacts that occur, infectious diseases such as BD or BVD will probably be less prevalent. The prevalence of antibodies in cattle and sheep was 17% and 25%, respectively. Since only herds from non-vaccinated cattle were sampled, these results suggested that pestiviruses circulate in both sheep and cattle. Unfortunately, no viruses were detected in any of the analyzed species.

The study of the specificity of pestivirus antibodies in wild ungulates from the Swiss Alps is of importance since all the positive wild ruminants detected in 2009 in Switzerland are included in this study. We should bear in mind that this country is currently undertaking an expensive BVDV eradication programme in cattle, which reinforces the need to study pestivirus epidemiology. The majority of the studied Alpine chamois had BDV antibodies, while the red deer had BVDV antibodies. However, one Alpine chamois had an exceptionally high antibody titre against BVDV-1, which is the strain that is most frequently isolated in cattle from Switzerland. This result showed that wild ungulates from this country can be infected with different pestivirus species, that is, with both BVDV and BDV. However, Casaubon (2012) described a very low prevalence of pestivirus antibodies in wild ungulates. By contrast, seroprevalence in sheep and goats in Switzerland has been previously reported with values of 16.1% and 25.4%, respectively (Danuser *et al.,* 2009). Thus, it seems that the scenario whereby wild ruminants in the Swiss Alps are infected by domestic ruminants is more likely to occur than the opposite case.

The recent appearance of the BDV-associated disease in Pyrenean chamois raised concerns that other vulnerable wild ungulate species such as the Iberian and Alpine ibex might be affected by this infection. Therefore, we examined the role of pestiviruses in these species by investigating the presence of pestiviruses in Alpine ibex from the Italian Alps and in Iberian Ibex in Spain from the Ports de Tortosa i Beseit National Hunting Reserve and the Sierra Nevada National Park, both protected areas of Spain.

The low seroprevalence found in both the Alpine and Iberian ibex (7.17% and 0.6%, respectively) agreed both with published data showing that BD in domestic goats is rare (Nettleton *et al.*, 1998) and with results previously reported for Iberian Ibex by Santiago-Moreno *et al.* in 2010. Most positive Alpine ibexes had BDV antibodies and were probably infected by ovine viruses. Interestingly, one Alpine ibex had significantly higher titres against the BVDV strain and could have been infected by a strain of bovine origin. It is remarkable that the Alpine ibex sera from the Swiss Alps also clearly showed higher titres against the BDV strains. In the same fashion, positive Iberian ibex had higher titres of neutralising antibodies against the BDV strain. These results suggested that, like chamois, ibexes are more likely to be infected with BDV viruses. It seems probable that the difference in natural habitat determines which pestivirus species are likely to infect wild animals. On the summer pastures, sheep and goats often graze on higher and steeper alpine pastures than cattle and are usually left to roam freely for several weeks at a time. Thus, it seems more likely that ibex came into contact with sheep and thus were infected by BDV.

9. CONCLUSIONS

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1. Border disease virus infection outbreaks in the Pyrenees have only affected the Pyrenean chamois, although other wild ruminant species were occasionally infected showing a low seroprevalence.

2. Deer species from the Pyrenees with pestivirus antibodies showed higher titres against the BDV strains, and this is probably related to the high circulation of BDV in the Pyrenean chamois population.

3. The seroprevalence in sheep and goats was always lower or similar than the seroprevalence in Pyrenean chamois and this discards a high BDV infection pressure from domestic livestock to chamois.

4. After the severe BD outbreaks in Pyrenean chamois, two different scenarios have appeared: in some areas, the disease has become endemic and BDV circulates frequently in the population, possibly having a negative impact on host population dynamics, while, in other areas, BDV does not seem to circulate. Thus, management of these chamois populations should be designed according to the epidemiological status and no generalizations should be made.

5. Border disease virus is self-maintained in the Pyrenean chamois population from Freser-Setcases NHR, apparently without causing negative population dynamic effects.

6. In this area of the Eastern Pyrenees, the circulation of BDV, possibly low virulent, and the high seroprevalence, may be important factors that hinder the dispersion of virulent BDV strains and the appearance of disease outbreaks.

7. The infection with BDV-4 in Pyrenean chamois is not limited to the Spanish Pyrenees and it can potentially expand to neighboring populations. The severe decline in the chamois population in Andorra observed in 2010 was associated with an outbreak of disease due to BDV infection, and the source of infection was the neighboring chamois population of Alt Pallars-Aran NHR in Spain.

8. In the south-west of the Italian Alps there is a high exposure to pestiviruses in the Alpine chamois population, suggesting that this species could act as a Pestivirus reservoir. However, we cannot dismiss the possibility that the chamois were infected by domestic ruminants, playing a “victim” role in the dynamics of pestivirus infection.

9. Exposure to pestiviruses in Alpine and Iberian ibex populations from the Italian Alps and Spain, respectively, is very low and suggests that pestiviruses may not play a significant role in the epidemiology of infections or have a significant effect on the health of these ruminant species.

10. In Switzerland, the higher antibody titers to BVDV in red deer and in one chamois, and to BDV in the Alpine ibexes and rest of chamois, indicate that wild ruminants from this country can be infected with both pestivirus species.

10. REFERENCES

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