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Infectious keratoconjunctivitis at the wildlife-livestock interface from mountain systems

Dynamics, functional roles and host-mycoplasma interactions

Xavier Fernández Aguilar
PHD Thesis

Infectious keratoconjunctivitis at the wildlife-livestock interface from mountain systems: dynamics, functional roles and host-mycoplasma interactions

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Els doctors **Jorge Ramón López Olvera**, professor titular d'universitat de l'Àrea de coneixement de Medicina i Cirurgia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona i **Óscar Cabezón Ponsoda**, investigador associat al Servei d'Ecopatologia de Fauna Salvatge i al Centre de Recerca en Sanitat Animal (CRESA – IRTA),

Informen:

Que la memòria titulada "**Infectious keratoconjunctivitis at the wildlife-livestock interface from mountain systems: dynamics, functional roles and host-mycoplasma interactions**", presentada per **Xavier Fernández Aguilar** per a la obtenció del grau de Doctor 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,3 de Maig de 2017.

Óscar Cabezón Ponsoda   **Jorge Ramón López Olvera**

Director   **Director**

Xavier Fernández Aguilar

Doctorand

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Llegados a éste punto, escribir los agradecimientos de la tesis me obligan a ejercer un acto reflexivo, retrospectivo, sobre los últimos años de mi vida. Des de que empecé con el doctorado ha pasado bastante tiempo y son muchas las personas que me han acompañado, apoyado y influido en este tiempo, tanto en el ámbito personal como profesional. Han sido muchos muestreos, trabajo de campo y de laboratorio en los que mucha gente ha desempañado un papel muy importante y necesario para esta tesis, y estoy profundamente agradecido. Si no te menciono en estos agradecimientos debido a esta mala memoria que tengo, por favor, date por aludido. Porque si me conoces, sabrás que estas palabras surgen de la más absoluta sinceridad.

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TABLE OF CONTENTS

1. SUMMARY OF THE THESIS

1.1 Summary

Infectious keratoconjunctivitis (IKC) is an ocular disease that affects wild and domestic Caprinae species. Epidemiology of IKC may rely in complex multi-host systems at the wildlife-livestock interface of alpine areas, where contrasting hypotheses of reservoir hosts have been proposed based on limited data. The main objective of this thesis is to provide comprehensive epidemiological data in order to infer the functional roles of domestic and wild host populations from mountain systems.

In the **Study I** and **Study II**, the epidemiological situation of *Mycoplasma conjunctivae* in domestic small ruminants from mountain areas in two distant regions (Northern Spain and Pakistan), seasonally sympatric with wild host populations, was studied in order to assess the potential source risk they suppose for their sympatric wild counterparts. *Mycoplasma conjunctivae* was commonly detected in the Pyrenees and the Karakoram mountain range, and more rarely in the Cantabrian Mountains. The relatively high prevalence of mostly asymptomatic infections observed suggests that sheep (*Ovis aries*) is a good host for the endemic maintenance of *M. conjunctivae* with a low host-fitness cost (i.e. clinical signs and body condition). *Chlamydiaceae* was also endemic in the eyes of sheep and goats (*Capra hircus*) from Pakistan and could be a predisposing factor for IKC that needs of further research (**Study II**).

The long-term dynamics of *M. conjunctivae* and its molecular epidemiology in the whole community of wild and domestic hosts from the Pyrenees and Cantabrian Mountains was assessed in the **Study III**. Results showed that independent *M. conjunctivae* sylvatic and domestic cycles occurred at the wildlife-livestock interface in the Pyrenees. Specific *M. conjunctivae* strain clusters were maintained in some Pyrenean chamois (*Rupicapra p. pyrenaica*) populations without the substantial contribution of other hosts. Furthermore, host population characteristics and *M. conjunctivae* strains resulted in different epidemiological scenarios in chamois, ranging from the fading out and the epidemic persistence of the mycoplasma in lower density populations and the endemic persistence in a higher density population. Nevertheless, incidental

cross-species transmission of *M. conjunctivae* occurred between chamois and mouflon and between chamois and sheep. Despite most of the IKC cases in free-ranging chamois were not associated with concurrent sheep strains, domestic sheep may have been historically important for the introduction of *M. conjunctivae* in wild hosts. This is suggested in the **Studies II and III** by the consistent epidemiological scenario observed in sympatric sheep and chamois from the Cantabrian Mountains and the Pyrenees.

In the **Study IV**, the transition from epizootic IKC to high prevalence of asymptomatic *M. conjunctivae* infections is described in a captive Iberian ibex (*Capra pyrenaica*) population, in which the host-mycoplasma interaction evolved to decreased clinical signs, lower mycoplasma loads and probably longer persistence in the eyes (Study IV). This study also provides evidences of the potential role of wild hosts in *M. conjunctivae* maintenance, associated to different and dynamic epidemiological scenarios.

Finally, in the **Study V**, the detection of *M. conjunctivae* in other locations apart from the eyes was explored in different host species in relation to transmission routes and within-host persistence. Transmission of *Mycoplasma conjunctivae* has been reasserted to be possible through direct contact from ocular and nose secretions and indirectly by eye-frequenting flies (*Musca* sp.). *Mycoplasma conjunctivae* was also detected in the ear canals, which may have important epidemiological implications.

Overall, the results obtained highlight the capacity of *M. conjunctivae* to establish diverse interactions with its hosts and persist in the population, also with different transmission patterns. Host populations and species can act either as maintenance hosts or as spill-over hosts depending on host- and *M. conjunctivae*-related factors. Population characteristics may also shape host-mycoplasma interaction through connectivity among individuals, *M. conjunctivae* transmission, and ultimately may determine its persistence and host functionalities in the system.

1.2 Resumen

La queratoconjuntivitis infecciosa (QCI) es una enfermedad ocular que afecta a ovinos y caprinos, tanto domésticos como salvajes. Su epidemiología puede ser compleja e involucrar diversas especies de hospedadores en la interfaz domestico-salvaje de zonas alpinas. En este contexto, hipótesis contrarias sobre especies reservorio se han formulado mediante datos epidemiológicos poco concluyentes. El objetivo principal de esta tesis es proporcionar información epidemiológica completa a fin de inferir la funcionalidad de los distintos hospedadores presentes en los sistemas montañosos.

En el **Estudio I y II**, la situación epidemiológica sobre *Mycoplasma conjunctivae* se evaluó en ovinos y caprinos domésticos de tres zonas montañosas, Pirineo y Cordillera cantábrica (Norte Español) y la Cordillera del Karakorum (Himalaya, Pakistán), a fin de evaluar el riesgo que éstos pueden suponer como fuente de QCI para la fauna. *Mycoplasma conjunctivae* fue detectado frecuentemente en los rebaños de ovejas y cabras que pastan en las zonas alpinas de los Pirineos y del Karakorum, y de forma más esporádica en la Cordillera Cantábrica. La alta prevalencia encontrada, junto con la práctica ausencia de enfermedad ocular, sugiere que la oveja doméstica (*Ovis aries*) es un buen hospedador para el mantenimiento endémico de *M. conjunctivae*. Además, *Chlamydiaceae* se encontró presente en los ojos de rumiantes de Pakistán y podría ser un factor predisponente para el desarrollo clínico de la QCI (**Estudio II**).

En el **Estudio III**, la dinámica de *M. conjunctivae* y su epidemiología molecular fue estudiada en la comunidad de hospedadores domésticos y salvajes del Pirineo y la Cordillera Cantábrica. Los resultados mostraron ciclos epidemiológicos selváticos y domésticos separados. Se observaron distintos escenarios epidemiológicos en las poblaciones de rebeco (*Rupicapra p. pyrenaica*), que fueron de la desaparición de *M. conjunctivae* a la persistencia endémica y epizoótica del micoplasma. La transmisión interespecífica de *M. conjunctivae* fue puntualmente constatada entre rebeco, muflón y oveja. A pesar de que la mayoría de casos de QCI encontrados en le rebeco no fueron originados por cepas de rebaños domésticos, las ovejas posiblemente han

tenido un papel histórico importante en la introducción de *M. conjunctivae* en las poblaciones de fauna salvaje. Esto es sugerido en los **Estudio II** y **III** debido al consistente escenario epidemiológico encontrado entre ovejas y rebecos simpátricos.

En el **Estudio IV**, el curso de una epizootía de QCI fue estudiada a largo plazo en una población cautiva de cabra montés (*Capra pyrenaica*), en la que la interacción micoplasma-hospedador evolucionó hacia signos clínicos más leves, menor carga bacteriana y posiblemente una mayor persistencia ocular del micoplasma. Este estudio además proporciona evidencias sobre el potencial rol que pueden jugar los hospedadores salvajes en el mantenimiento de *M. conjunctivae* en relación a distintas situaciones epidemiológicas.

Finalmente, en el **Estudio V**, se exploró la presencia de *M. conjunctivae* en localizaciones distintas a los ojos en relación con su transmisión y persistencia individual. La transmisión de *M. conjunctivae* es posible a través de contacto directo con secreciones oculares y nasales y de forma indirecta mediante vectores (*Musca* sp.). *Mycoplasma conjunctivae* además se detectó en el conducto auditivo, lo cual puede tener implicaciones epidemiológicas importantes.

En conclusión, los resultados de esta tesis resaltan la capacidad de *M. conjunctivae* para establecer distintas interacciones con sus hospedadores y persistir en las poblaciones mediante patrones de transmisión distintos. El papel de reservorio o hospedador accidental está determinado tanto por factores relacionados con el propio hospedador como con el micoplasma. Las características de las poblaciones susceptibles posiblemente también modulan la interacción micoplasma-hospedador mediante la conectividad entre individuos, transmisión de *M. conjunctivae* y finalmente determinar su persistencia y el papel de los distintos hospedadores en el sistema.

1.3 Resum

La queratoconjuntivitis infecciosa (QCI) és una malaltia ocular que afecta a ovins i caprins, tant domèstics com salvatges. La seva epidemiologia pot arribar a ser complexa i involucrar diverses espècies d'hostes a la interfase domèstic-salvatge de zones alpines. En aquest context, hipòtesis contràries sobre espècies reservori s'han formulat mitjançant dades epidemiològiques poc concloents. L'Objectiu principal d'aquesta tesi és proporcionar informació epidemiològica complerta per tal de inferir la funcionalitat de les diferents poblacions d'hostes presents als sistemes muntanyosos.

En l'**Estudi I** i **II**, la situació epidemiològica de *Mycoplasma conjunctivae* es va avaluar en els ovins i caprins domèstics de tres zones muntanyoses, els Pirineus, la Serralada Cantàbrica (Nord Espanya) i la serralada del Karakorum (Himàlaia, Pakistan) per a tal d'avaluar quin risc poden suposar com a font de QCI per a la fauna salvatge simpàtrica. L'elevada prevalença trobada, junt amb la pràctica absència de signes clínics, suggereix que l'ovella (*Ovis aries*) és un bon hoste per al manteniment endèmic de *M. conjunctivae*. A més, Chlamydiaceae es va trobar de forma endèmica als ulls dels remugants domèstics de Pakistan i podria ser un factor predisposant per al desenvolupament clínica de QCI (**Estudi II**).

A l'**Estudi III**, la dinàmica de *M. conjunctivae* i la seva epidemiologia molecular es va estudiar en les comunitats d'hostes domèstics i salvatges del Pirineu i la Serralada Cantàbrica. Els resultats van mostrar cicles epidemiològics selvàtics i domèstics separats al Pirineu. Clústers específics de soques de *M. conjunctivae* van ser mantinguts en algunes poblacions d'isards pirinencs (*Rupicapra p. pyrenaica*) sense la contribució substancial d'altres hostes. A més, es van observar diferents escenaris epidemiològics en les poblacions d'isard que van anar de la desaparició de *M. conjunctivae* a la persistència epidèmica i endèmica del micoplasma. La transmissió interespecífica de *M. conjunctivae* va ser puntualment constatada entre isard, mufló i ovella. Tot i que la gran majoria de casos de QCI trobats a l'isard no van ser originats per soques d'origen domèstic, les ovelles possiblement han tingut un paper històric important en la introducció de *M. conjunctivae* en les poblacions de fauna

salvatge. Això és suggerit als **Estudis II** i **III** degut al consistent escenari epidemiològic trobat entre ovelles i isards simpàtrics.

En l'**Estudi IV**, el curs d'una epizoòtia de QCI va ser estudiat en una població captiva de cabra salvatge (*Capra pyrenaica*), en la que la interacció micoplasma-hoste va evolucionar cap a signes clínics més lleus, menor càrrega bacteriana i possiblement una major persistència ocular del micoplasma. A més, aquest estudi proporciona evidències sobre el potencial rol que poden jugar els hostes salvatges en el manteniment de *M. conjuntivae* en diferents situacions epidemiològiques.

Finalment, a l'**Estudi V**, es va explorar la presència de *M. conjuntivae* en localitzacions diferents als ulls i en relació amb la transmissió i persistència individual. La transmissió de *M. conjuntivae* és possible a través del contacte directe amb secrecions oculars i nasals i de forma indirecta mitjançant vectors (*Musca* sp.). A més, *Mycoplasma conjunctivae* es va detectar al conducte auditiu de diferents espècies d'hostes, fet que pot tenir implicacions epidemiològiques importants.

En conclusió, els resultats d'aquesta tesi ressalten el capacitat de *M. conjuntivae* per a establir diferents interaccions amb els seus hostes i persistir a les poblacions mitjançant patrons de transmissió diferents. El paper de reservori o d'hospedador accidental està determinat tant per factors relacions amb el propi hospedador com amb el micoplasma. Les característiques de les poblacions d'hostes possiblement també modulen la interacció micoplasma-hoste mitjançant la connectivitat entre individus, la transmissió de *M. conjuntivae* i finalment determinar la seva persistència i el paper dels diferents hostes en el sistema.

2. INTRODUCTION

2.1 Mountain ungulates of Europe

Bovidae is the most diverse and successful family of artiodactyls (even-toed hoofed mammals from the order Ungulata), and can be found in a wide range of ecosystems, from deserts to wetlands and from sea level to the top of the mountains (Wilson and Mittermeier, 2011). Among the bovines, the Caprinae subfamily mostly assembles medium-sized ungulates that are specifically adapted to mountain and steep habitats. Representative species of this subfamily occur in most of the mountain ranges of the world and are often referred to as mountain ungulates. The geographic expansion and isolation of suitable habitats in recurrent glacial and interglacial periods during the Pleistocene (2,588,000 - 11,700 years ago) has driven the evolutionary divergence of endemic Caprinae species in the European continent (Rodríguez et al., 2010). Two species of the genus *Rupicapra* (Northern chamois, *Rupicapra rupicapra*; Southern chamois, *Rupicapra pyrenaica*) and two from the genus *Capra* (Alpine ibex, *Capra ibex*; Iberian ibex, *Capra pyrenaica*) can be exclusively found in the European and Near East mountain ranges. The origins of the species and subspecies from the genus *Ovis* present in Europe are controversial (see section 2.1.6). Wild mountain ungulate species are phylogenetically related with domestic livestock and more specifically with sheep and goats (Gentry et al., 2004). This fact has a number of implications regarding cross specific interactions and transmission of infectious diseases that will be discussed in next sections.

Direct human persecution has caused severe declines of mountain ungulate populations, pushing them to the edge of extinction by the end of the XIX century (Apollonio et al., 2010). Conservation efforts and reintroductions have yielded successful recoveries of their populations in most of their historical range and mountain ungulates occur nowadays in nearly all of the European summits. Historic collapses of the populations had however caused severe genetic bottleneck and several populations suffer from poor genetic diversity (Amills et al., 2004; Stüwe and Scribner, 1989), or have had genetic introgression from distant relatives along with reintroductions (Crestanello et al., 2009). Interbreeding is also a concern for some small and isolated populations (Aulagnier et al., 2008). Low genetic variability results in a lower resilience of the population to stochastic factors, such as the emergence of severe infectious

diseases. In fact, outstanding die-offs because of disease outbreaks in Iberian ibex and Southern chamois are believed to be in part influenced by an original low genetic variability (León-Vizcaíno et al., 1994; Marco et al., 2009b).

In this thesis, mountain ungulates will be referred to Caprinae species that are specifically adapted to mountain ecosystems. Other highly adaptable ungulates, such as red deer (*Cervus elaphus*), can also opportunistically occupy ecological niches in alpine areas, but are not going to be considered in the former definition. Next are summarized common characteristics of mountain ungulates, followed by specific information of subject species, to provide an ecological and biological background to the research studies performed in this thesis.

2.1.1 General features of wild mountain ungulates

Mountain ungulates are highly gregarious species, which is beneficial to visually detect threats in open areas such as the habitats above the timberline. Group-living also increases foraging efficiency by reducing the time devoted to alert behavior (Berger, 1978; Risenhoover and Bailey, 1985). The size of the group is density-dependent (Pépin and Gerard, 2008), but may affect differently between males and females (Vander Wal et al., 2013). Solitary females are rare and occasional in some specific moments (i.e. female parturition) but males roam alone more frequently (Lovari and Cosentino, 1986). Social structure generally segregates females with young animals from males during most part of the year, with the exception of the rut and mating period when mixed groups occur (Catusse et al., 1996). Social behavior and group size can however be influenced by many factors such as the population density, the allocation of resources or the season (Lovari and Cosentino, 1986; Pepin et al., 1996; Pépin and Gerard, 2008).

Food availability and environmental constraints are highly seasonal in alpine ecosystems, which determine altitude migrations of mountain-dwelling ungulates and most of their biological aspects (Crampe et al., 2007). In the colder months, vegetative growth stops and snow cover hinder movements especially in the higher parts of the mountains that force ungulates to move to lower altitudes (Catusse et al., 1996). Altitude displacements depend on the

accumulative snowfall and environmental conditions (Grignolio et al., 2004), but also to the elevation of the summer pastures selected, suggesting that different spatial use strategies may occur within a population (Lovari et al., 2006). Species-specific sensitivity to extreme temperatures can also influence different winter altitude among mountain ungulates (Darmon et al., 2014).
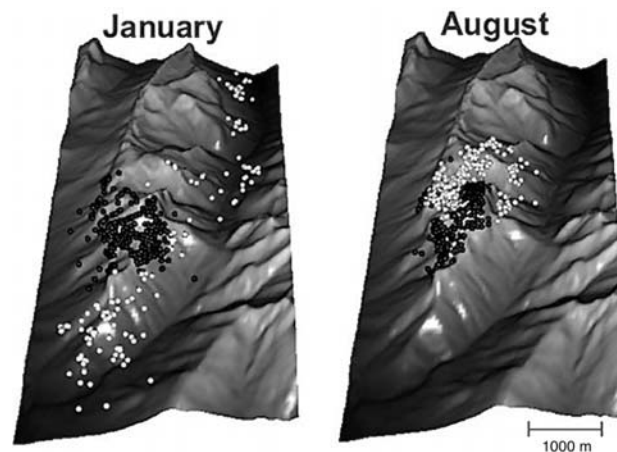
Figure 2.1 Seasonal movements of two wild mountain ungulate species collared with GPS (Alpine chamois black dots; European mouflon white dots) in the massif of Bauges (Pre-Alps, France). Displacements to lower altitudes occur with snow cover in winter, also with evident differences of altitude locations in winter between both species (Image adapted from (Darmon et al., 2014).

Biological cycles are also influenced by seasonality. The rut and mating season generally occur in late autumn – beginning of winter, with a gestation that generally takes around five-six months. The birthing peak is in mid-late spring and coincides with the best forage quality (Wilson and Mittermeier, 2011).

Horns are secondary sexual characteristics displayed in mountain ungulates by both sexes with the exception of the European mouflon (Catusse et al., 1996). The development of the horns is continuous throughout the animal life and shows an intermittent growth pattern, which is primarily modulated by photoperiod in a species-dependent manner (Toledano-Díaz et al., 2012). The horn growth is interrupted during the colder months and cause a subsidence

around the horn, which can be used for aging Caprinae individuals easily and accurately (Corlatti et al., 2015b; Fandos, 1995).
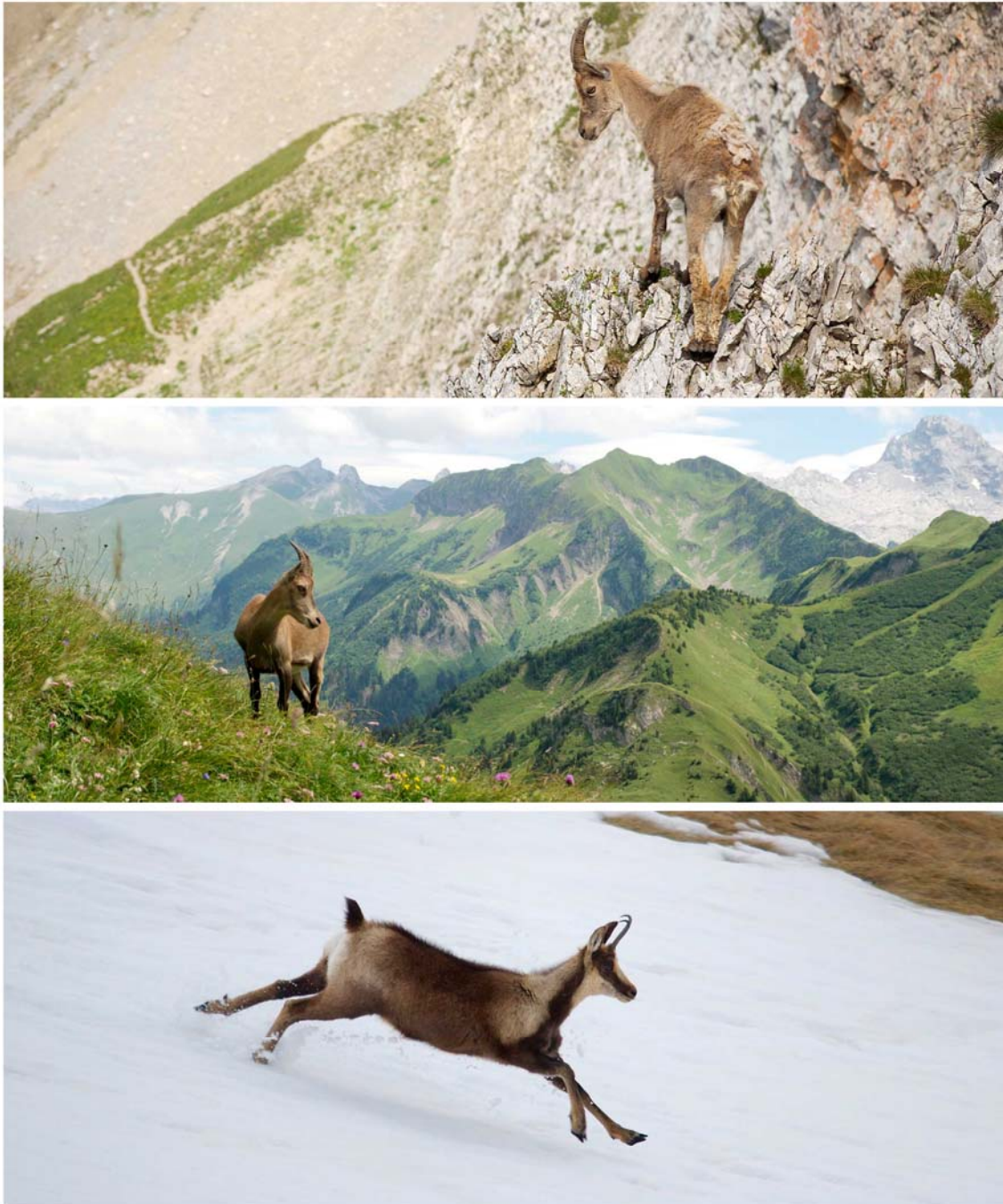
Figure 2.2 Wild Caprinae on its natural environment in the French Alps (Alpine ibex, Massif des Bauges, top and middle images) and Catalan Pyrenees (Southern chamois, Freser-Setcases, bottom). Two highly adaptable hoofs with prehensile capacity provide Caprine higher climbing abilities than the rest of bovines and a good running performance in treacherous terrain.

2.1.2 Factors driving population dynamics of wild mountain ungulates

Living on the heights has some advantages beyond the privileged views. Abundant and good quality primary production occurs in late spring and summer, which is precise for breeding and assures good nutrition in the most energy-demanding period (Garel et al., 2011). Altitude and steepness also supply refuge against predators, and climbing is a good escape route for well-adapted species. Mortality associated to predation is in general lower in mountain-dwelling ungulates than for forest ungulates (Meriggi et al., 1996; Molinari-Jobin et al., 2002). However, alpine ecosystems are highly seasonal environments, with harsh weather and severe shortage of food supplies during winter, which can have a strong influence on its survival (Gonzalez and Crampe, 2001). Infectious diseases can also exert a strong influence on wild populations by causing important disease outbreaks (Arnal et al., 2013a; Marco et al., 2009b) or by affecting the recruitment of the population (Rudolph et al., 2007). Population dynamics of mountain ungulates are therefore mainly driven by the natural factors of infectious diseases and climatic conditions (Jonas et al., 2008; Rughetti et al., 2011; Serrano et al., 2015), and by human hunting/poaching pressure (Aulagnier et al., 2008).

2.1.3 The natural and human value of mountain ungulates

Mountain ungulates are among the largest mammals in alpine ecosystems and exert a strong influence on other animal communities, especially insects, birds and small rodents by changing the relative abundance and composition of vegetation (Suominen and Danell, 2006). Since mountains are the last strongholds of large carnivores in most of the developed countries, mountain ungulates also represent part of the natural prey for top predators in these areas, which are key species for proper ecosystem functionality (Llaneza et al., 2000; Meriggi et al., 1996; Molinari-Jobin et al., 2002). Mountain ungulates therefore play an important role in ecological dynamics of alpine and subalpine ecosystems through several direct and indirect interactions (Augustine and McNaughton, 1998).

From an anthropogenic perspective, mountain ungulates are also iconic species of the mountain wilderness, appreciated for tourism, and exploited as a hunting resource. Yet trophy hunting of mountain ungulates is highly appreciated and hunting revenues can be an important income in rural areas. Abundant populations of Caprinae are managed through hunting plans in most of their occurrence (Apollonio et al., 2010), which has significant implications. On the one hand, it requires knowledge of population status and dynamics to implement proper management actions. On the other hand, hunting activities provide the opportunity to collect high quality individual data and biological samples to perform surveillance of potential threats and assess the output of population management (Ryser-Degiorgis, 2013).

2.1.4 Chamois *Rupicapra* spp.

Catalan: isard; French: chamois/isard; Italian: camoscio; Spanish: rebeco/sarrio/gamuza.

Chamois species belong to the tribe Rupicaprini from the Caprinae subfamily. Modern taxonomic revisions consider two distinct species: Northern chamois (*Rupicapra rupicapra*) with seven subspecies, and Southern chamois (*Rupicapra pyrenaica*) with three subspecies (Figure 2.3). This taxonomic classification is not free of discussion and may be reassessed if new genetic studies arise (for a extent discussion see (Corlatti et al., 2011)). Chamois are the most common mountain ungulate of Europe, with a total estimated population of 484.000 Northern chamois distributed in twenty-one countries, and 76.000 Southern chamois distributed in three countries (Apollonio et al., 2010) (Figure 2.3). It has been also introduced in Argentina and New Zealand for hunting purposes. Despite its general abundance, three subspecies are scarce: *R. r. tatrica* is listed as Critically Endangered and *R. r. cartusiana* and *R. p. ornata* as Vulnerable by the IUCN (Aulagnier et al., 2008; Herrero et al., 2008).

Populations of chamois in the Iberian Peninsula are distributed in the Cantabrian Mountains and the Pyrenees, where Cantabrian chamois (*R. p. parva*) and the Pyrenean chamois (*R. p. pyrenaica*) inhabit respectively (Figure 2.3). Around 53,000 Pyrenean chamois (estimated in 2000) occupy most of the optimal habitats in the Pyrenean mountain range (Herrero et al., 2004), and that 17,500 Cantabrian chamois (estimated in 2008) can be found in two connected population nuclei (Pérez-Barbería et al., 2009). However, these total estimations are not updated and crashes and expansions of several metapopulations have occurred after these estimations (López-Martín et al., 2004). It is also remarkable the impact of a novel disease originated by a border disease virus in the Catalan Pyrenees since 2001 (Arnal et al., 2004; Hurtado et al., 2004), which has caused different outbreaks with outstanding mortalities that reached up to 87% of the population (Marco et al., 2009b).
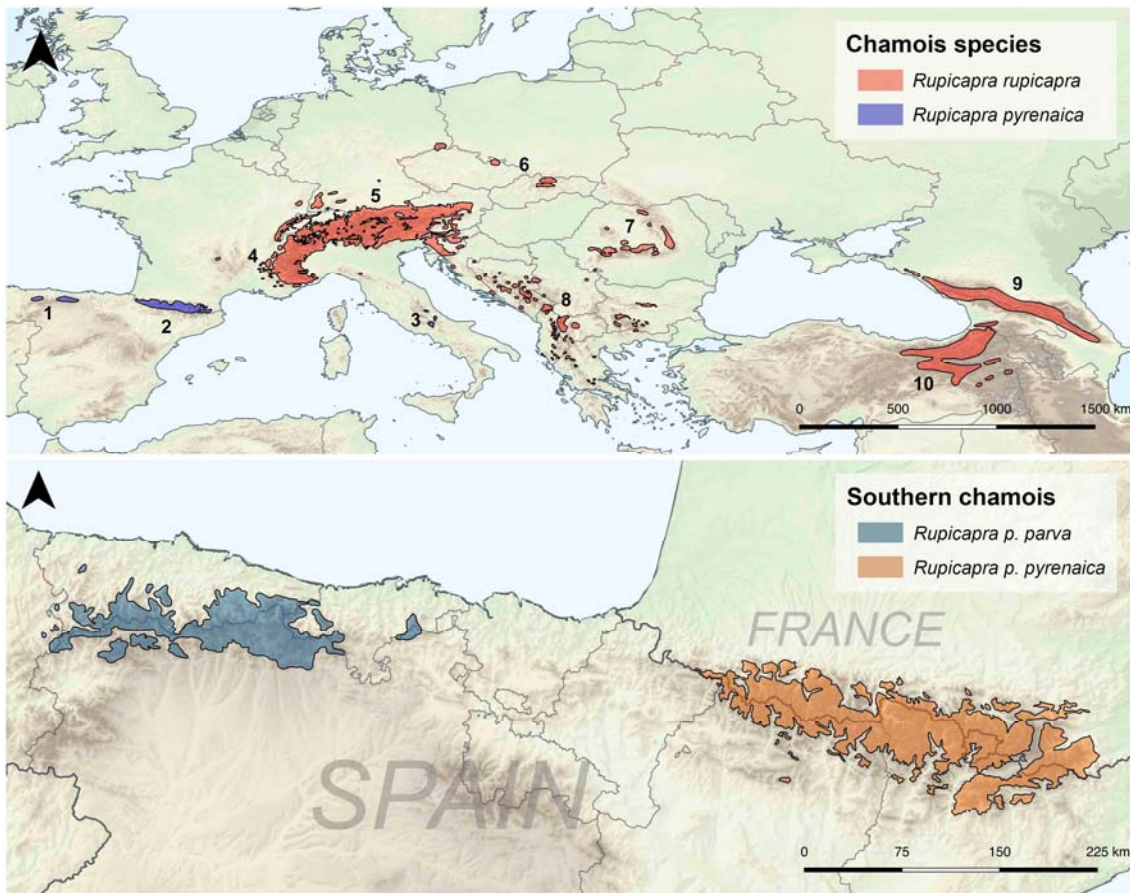
Figure 2.3 Top: current distribution of *Rupicapra* spp. and all the subspecies, distributed in different mountain ranges of Europe and Near East. *Rupicapra pyrenaica*: (1) *pyrenaica* in the Pyrenees*;* (2) *parva* in the Cantabrian Mountains; and (3) *ornata* in the Abruzzo. *Rupicapra rupicapra*: (4) *cartusiana* in the Chartreuse massif; (5) *rupicapra* in the Alpine arc; (6) *tatrica* in the Tatra mountains; (7) *carpatica* in the Carpathians; (8) *balcanica* in the Balkan mountains, (9) *caucasica* in the Caucasus; and (10) *asiatica* in the Pontic mountains and Armenian highlands.

Bottom: distribution of both Southern chamois subspecies present in the Iberian Peninsula.

The natural history of chamois is closely linked to mountain ecosystems and it is highly adapted to alpine and subalpine habitats. Chamois better tolerate the environmental harshness of the mountain than other mountain-dwelling ungulates (Darmon et al., 2014), and can be located between 400 and 2,800 meters high in the Iberian Peninsula (Herrero et al., 2008). It selects grassland and sub-alpine forest habitats ranging from 1,600 to 2,200 meters, females showing preference for higher altitude pastures (Dalmau et al., 2013; García-

33

Gonzalez and Hidalgo, 1989). Seasonal altitudinal migrations of chamois range approximately between 300 and 800 meters (Crampe et al., 2007; Lovari et al., 2006).

The studies of this thesis have been developed only in Southern chamois (*R. p. pyrenaica* and *R.p.parva*) and, despite general information are common for all chamois species, biometrics and more specific information provided in this section belongs to *R. pyrenaica*.

Sexual dimorphism is subtle in chamois, both in body size (5% of skeletal size difference) and horn size (Bassano et al., 2003; Sáez de Buruaga et al., 1991) (Figure 2.4). However, there is a seasonal changing pattern of sexual size dimorphism since males can be 40% heavier than females in autumn (rut) by accumulating body resources but only 4% heavier in spring (Rughetti and Festa-Bianchet, 2011).Total body length is of 100-110 cm, shoulder height 69-75 cm, and weight ranges 20-32 Kg for females and 25-40 Kg for males (*R. p. pyrenaica*) (Weber, 2001). Horns are relatively smaller than other Caprinae members, rising almost straight from the head and ending in a hook directed backwards (Figure 2.4 and 2.5). The angle of the hook is more acute in males than females, whose horns are also generally thinner, shorter and with a rounder section than males (Bassano et al., 2003; Sáez de Buruaga et al., 1991). Chamois have two different coats adapted to the wide temperature variation of the alpine ecosystems. Summer coat is composed by short brown-reddish hair with darker-brown bands in both sides of the head and a darker mid-dorsal stripe. Winter coat has longer hair with six lighter patches in Southern chamois and four in Northern chamois, whose colors are contrasted from cream-colored to dark brown/black (Masini and Lovari, 1988). In winter, males have also a marked dorsal ridge of longer hair (Figure 2.4).

Figure 2.4 Southern chamois subspecies in the Iberian Peninsula A) Adult male of Pyrenean chamois with winter coat; B) Adult female of Pyrenean chamois with winter coat; C) Adult male of Cantabrian chamois with summer coat; D) Adult female and kid of Cantabrian chamois; E) Yearling of Pyrenean chamois; F) Kid of Cantabrian chamois.
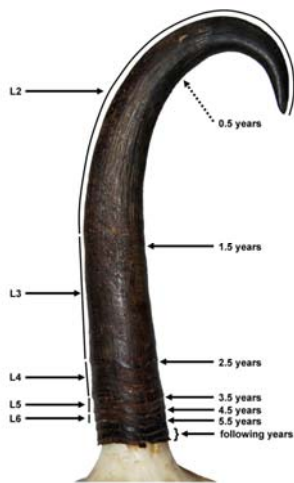
Figure 2.5 Horn growth pattern of alpine chamois (male). The length of each segment (L2-L6) is represented on the left side, and the corresponding age years are shown on the right. Horn growth is interrupted in wintertime (November-March) and is visible as sharp boundaries between length segments, which can be used for accurately dating the age of chamois (Corlatti et al., 2015b).

The diet of chamois is quite diverse and its composition largely depends on the availability of grasses (i.e. graminoids) and forbs, which are selectively foraged (Pérez-Barbería et al., 1997). However, in mountain ecosystems grasses and forbs are scarce in winter period and chamois shift diet composition to more woody plants in this period (Rayé et al., 2011). This intermediate feeding strategy between grazer and browser cause died overlaps with other mountain-dwelling ungulates like red deer, mainly in winter (Bertolino et al., 2009), and mouflon, Alpine ibex or domestic sheep in summer diet (La Morgia and Bassano, 2009; Redjadj et al., 2014). The niche breadth and feeding habits of chamois can be affected by direct competition with livestock that can relegate chamois to lower food availability areas (Chirichella et al., 2013; La Morgia and Bassano, 2009). Conversely, fine-scale spatial mechanisms and differential activity patterns among wild mountain ungulates may minimize competition for forage and feeding resources favouring coexistence (Darmon et al., 2014, 2012; Schweiger et al., 2015). Despite females show more exploratory movements than males, phylopatric behaviour is higher in females than males, which show greater tendency to disperse before reproduction (Crampe et al., 2007; Loison et al., 1999).

Social behaviour of chamois determines population structure from solitary individuals (females without kids, mean ± SD = 1.62 ± 1.00; solitary males, 1.73 ± 1.78 individuals) to groups of diverse size (females with kids, 5.59 ± 5.42

individuals). Males tend to be more solitary or form smaller groups than females, and sexual segregation takes place during almost all the year with the exception of the rut season when mixed groups occur (female groups with males, 8.91 ± 7.91 individuals) (Lovari and Cosentino, 1986; Pérez-Barbería and Nores, 1994). Group size can however vary both within a population and among populations (ranging from two to more than 120 individuals) because of several factors such as population density (Pépin and Gerard, 2008), type of habitat (forest vs open habitats) (Dalmau et al., 2013; García-González et al., 1992; Pepin et al., 1997), season and concentration of food resources (Bruno and Lovari, 1989; Pepin et al., 1997) or even the hour of the day (Pepin et al., 1996). The rut takes place from October-November to the beginning of December, when adult males show temporal territoriality and may defend aggressively a small area with female groups. Other non-territorial behaviours have also been described as alternative mating strategies (Corlatti et al., 2013). The concurrence of these alternative mating tactics with the limited sexual dimorphism, compensatory growth of the horn (Rughetti and Festa-Bianchet, 2010) and non sex-biased and high survival rates (Bocci et al., 2010), suggest a conservative male mating strategy to maximize survival (Corlatti et al., 2015a), which is singular among polygynous species such the rest of mountain ungulates.

Major threats for chamois depend on the specific population, but he most common are poaching and competition with domestic livestock, particularly in small and isolated populations (Aulagnier et al., 2008; Herrero et al., 2008). Infectious diseases, such as sarcoptic mange in the Cantabrian mountains and some parts of the European Alps (Fernández-Morán et al., 1997; Rossi et al., 2007, 1995) and the novel border disease virus in the Pyrenees (Arnal et al., 2004; Hurtado et al., 2004), are also an important threat for the populations affected. Resilience of Pyrenean chamois to the circulation of this pestivirus is highly variable depending on the populations (Fernández-Sirera et al., 2012), but the steady decrease of total chamois numbers in some Catalan and French populations is of concern. This disease has reduced chamois density in several of the Eastern Pyrenees areas and is spreading towards central and Western Pyrenees (Arnal et al., 2013b).

2.1.5 Iberian ibex *Capra pyrenaica*

Catalan: cabra salvatge; French: bouquetin des Pyrénées; Italian: stambecco della Spagna; Spanish: cabra montés.

The Iberian ibex is a caprine endemic to the Iberian Peninsula and closely related with the Alpine ibex and other ibexes of the world (Herrero and Pérez, 2008). Up to four subspecies were described in the beginning of the XXth century based on morphological differences: *Capra pyrenaica pyrenaica* in the Pyrenees, *C. p. victoriae* in Central areas of Spain, *C. p. lusitanica* in the North of Portugal and Southern Galicia, and *C. p. hispanica* in the South and East of the Iberian peninsula (Cabrera, 1911). Despite it has been discussed whether these morphological differences are conclusive traits for taxonomy classification, genetic differences among the subspecies suggest that they can be considered as significant evolutionary units (Manceau et al., 1999). Two of these subspecies are currently extinct because of overhunting and direct human persecution, being the disappearance of *C.p.pyrenaica* (bucardo) in 2000 one of the more recent extinction and sad episode of wildlife conservation in Europe (García-González and Herrero, 1999). For many decades the Iberian ibex distribution area had been restricted to Spain, which had also given to the vernacular name of Spanish ibex or Spanish wild goat (Moço et al., 2008; Sarasa et al., 2012).

Iberian ibex populations have successfully recovered after the dramatic low numbers at the beginning of XXth century. It is estimated that about 70,000 Iberian ibex occur nowadays in most of the Eastern and central mountains of Spain (Apollonio et al., 2010; Pérez et al., 2002), Northern Portugal (Moço et al., 2006), and more recently Southern France (Lafitte et al., 2016) (Figure 2.6). Despite the total population number ensures the species survival, some of the populations origin from few individual founders and suffer from low genetic diversity (Amills et al., 2004). The high mortality (95%)

consequences of this were evidenced in Cazorla Segura y las Villas Natural Park (South Spain) where 95% of the ibex population died because of a

sarcoptic mange (*Sarcoptes scabiei*) outbreak between 1988-1991 (León-Vizcaíno et al., 1994). To prevent genetic losses derived from stochastic factors such as disease outbreaks, several enclosures were built in the nineties by the Spanish administration to maintain in captivity genetically representative Iberian ibex stock-reservoir populations (Consejería de Agricultura pesca y medio ambiente, n.d.).

Despite the Iberian ibex have a preference for mountain ecosystems as other *Capra* species (Escós and Alados, 1992; Granados et al., 2001), altitude is not a limiting factor and it can be located from sea level to up to 2,500 meters, as long as the orography is steep and hilly (Granados et al., 2003). Iberian ibex can occur from the alpine habitats of Sierra Nevada (Southern Spain), to the arid mountains of Almería (Sierra Cabrera, Sierra de Alhamilla) or the rainy and humid mountains of Peneda-Gerês National Park of Portugal (Acevedo and Cassinello, 2009) (Figure 2.6).
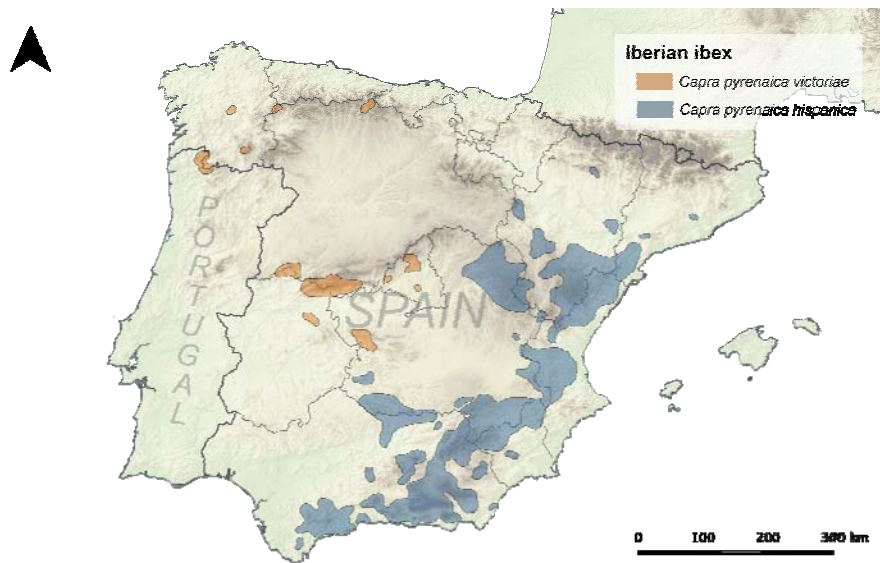
Figure 2.6 Distribution of the two existent subspecies of Iberian ibex. *C. p. hispanica* range extends throughout the Mediterranean mountains from Eastern Spain, Sierra Morena and part of the Iberian system. *C. p. victoriae* occupies the central mountains of Spain, some small populations in the Cantabrian Mountains and Galicia, the Peneda-Gerês National Park from Portugal. More recently Iberian ibex have been reintroduced in the Pyrénées National Park from France and the Catalan Pyrenees.

The Iberian ibex has a great sexual dimorphism as all the species of the *Capra* genus (Granados et al., 2001) (Figure 2.7). Total length is 130 cm females and 140 cm males, shoulder height 75 cm females and 90 cm males, and weight ranges 35-46 Kg for females and 60-80 Kg for males (Alados and Escós, 2012; Granados et al., 1997). Horns in males grow backwards and partially twisted upwards and can be longer than 90 cm. Conversely, horns in females are thinner and smaller and rarely exceed 15 cm of length (Fandos, 1991; Granados et al., 1997) (Figure 2.8). The winter coat is brown colour-schemed with clearer ventral parts contrasted with black patches, which are greater in older males (Figure 2.7). A black line in the mid back and a beard are also exhibited by adult males. The summer coat is shorter and lighter, with more subtle black-contrasted parts (Fandos, 1991) (Figure 2.7).
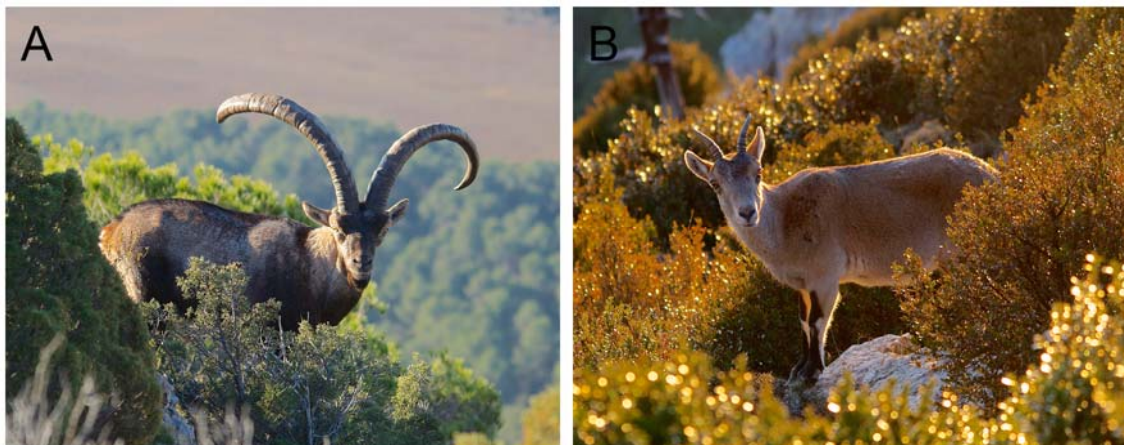
Figure 2.7 A) Adult male of Iberian ibex with winter coat B) Adult female of Iberian ibex with winter coat.

Figure 2.8 Horns of the Iberian ibex are the biggest among the Caprinae in the Iberian Peninsula and are highly appreciated for trophy-hunting. Yearly segments (*medrones*) are clearly marked in the horns.

The Iberian ibex is an intermediate feeder that selectively feeds on grasses and forbs depending on its availability (Acevedo and Cassinello, 2009). It has however a great feeding

plasticity, being also able to browse on more woody plants if digestible vegetation is scarce (García-González and Cuartas, 1992). Iberian ibex diet therefore shows seasonal differences (Moço et al., 2013), but also differences related to age and sex following a diet selection pattern depending on body size (Martínez et al., 2010). Ibexes prefer pasture-scrub and non-cultivated lands (Granados et al., 2003). However, competence with extensive domestic goats can displace Iberian ibex to suboptimal areas of poor sparse vegetation, cultivated lands and forests (Acevedo et al., 2007a; Moço et al., 2014). Habitat overlapping with non-native wild ruminants, such as the Aoudad (*Ammotragus lervia*) or fallow deer (*Dama dama*), has been also suggested to cause adverse effects to Iberian ibex populations (Acevedo et al., 2007b; Escós and Alados, 1992).

Ibexes are highly gregarious species and isolated individuals are not common, representing around 3% of the individuals of the population (Granados et al., 2001). Group size and composition changes around the year and mean group size can range from two to more than 30 individuals (Pérez et al., 1994). Sexual segregation of females with kids and young animals occurs during most of the year with the exception of the mating season in November-December, when bigger mixed groups of females and males take place. Mean group size is five for adult male groups, three for females, four for females with kids and seven for mixed groups (Granados et al., 2001). Apart from the seasonal variation, group size and sex-segregation is also influenced by population density and the concentration of feeding sources, so it may differ among Iberian ibex populations (Alados, 1986; Fandos, 1991).

There are not major threats for Iberian ibex. It has been however identified interspecific competition with domestic livestock and non-native ruminants as a potential future conservation problem. Infectious diseases, and specially sarcoptic mange, have also caused the crash of local populations (Herrero and Pérez, 2008).

2.1.6 The European mouflon *Ovis aries musimon*

Catalan: mufló, French: mouflon, Italian: muflone, Spanish: muflón.

The taxonomy of European mouflon (*Ovis aries musimon*) has been controversial and several scientific names can be found in the literature (i.e. *Ovis musimon*, *Ovis gmelini musimon*, *Ovis ammon musimon* and *Ovis orientalis musimon*). Recent genetic studies showed that mouflon shares common ancestors with domestic sheep and it is now considered a pre-domesticated wild sheep from the Neolithic that remained free-ranging in Corsica, Sardinia and Cyprus (Chessa et al., 2009; Hiendleder et al., 2002; Rezaei et al., 2010). It has been introduced in continental Europe since the XVIIIth century for hunting purposes and now free-ranging populations occur in at least 20 European countries, with an overall estimated population of more than 140.000 individuals. It is the second wild bovine species more abundant in Europe after Alpine chamois (Apollonio et al., 2010) (Figure 2.9). European mouflon populations are no longer assessed by the International Union for the Conservation of the Nature (IUCN) since it is not considered a wild species.
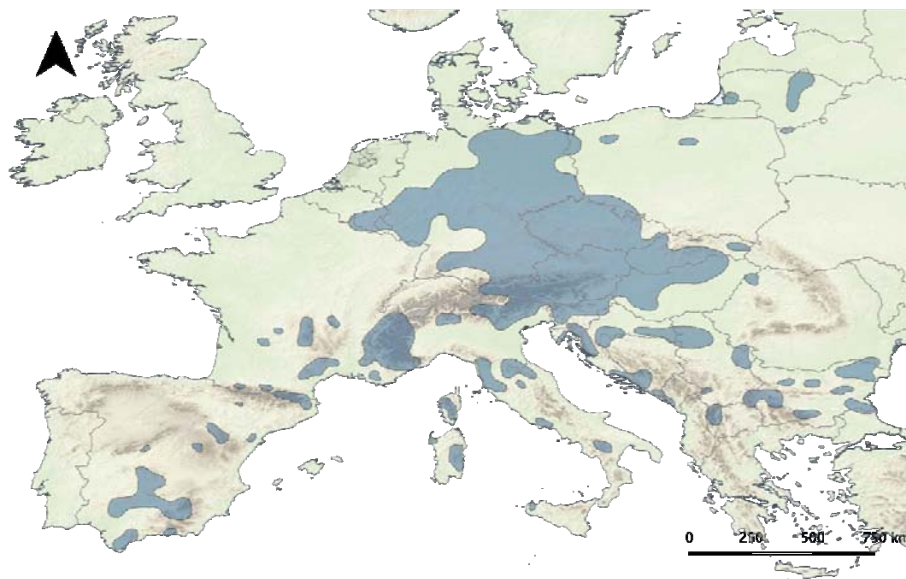
Figure 2.9 Distribution of the European mouflon showed in blue-grey shaded areas.

The mouflon is more a generalist ungulate rather than a mountain ungulate and can be found in several ecosystems (Santiago-Moreno et al., 2004). However, the high adaptability of mouflon has enabled this species to successfully establish in mountain or steep habitats. In the Pyrenees (Andorra, France, Spain), the Central Massif (France) or the Alps (France, Italy, Switzerland and Austria), European mouflon is restricted to some valleys and massifs, but the populations are continuously increasing (Apollonio et al., 2010).

The European mouflon is the smallest extant wild (if considered so) ovine, with a total length of 120-140 cm, a shoulder height of 65-75 cm in females and 70-80 in males, and a weight of 25-35 Kg for females and 35-55 Kg for males (Santiago-Moreno et al., 2004). Apart from the difference in size, sexual dimorphism is also present in the horns, which are curved and round-concentric in the males (up to 80-90 cm) and generally absent or exceptionally smaller and rudimentary in females (Figure 2.10) (Catusse et al., 1996). The coat is composed by short brown-reddish hair that turns slightly darker, longer and thicker in winter. The ventral parts of the body (belly and below the knee and hock) and the snout are white or cream-colored. Adult males have also a white-colored saddle patch in the flanks and a long black beard along the neck (Figure 2.10) (Rodríguez-Luengo et al., 2007).
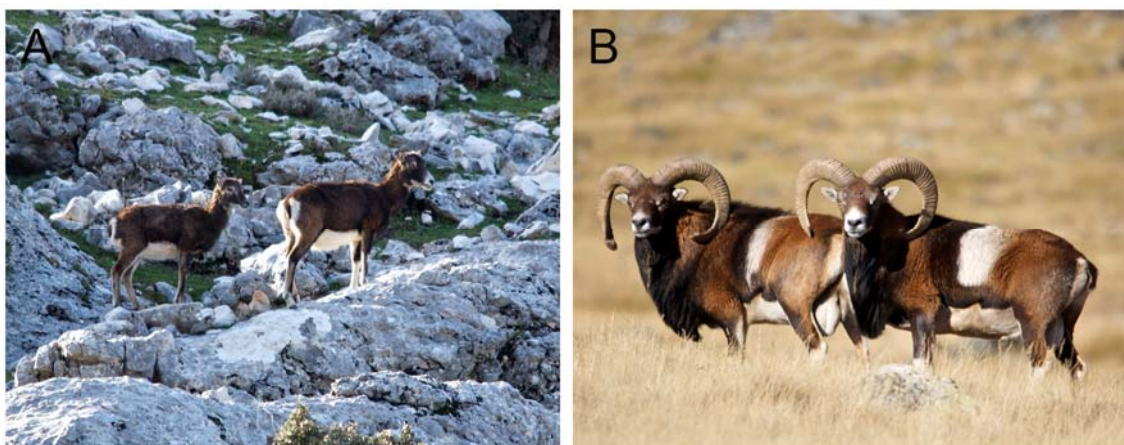
Figure 2.10 A) Adult female and lamb of European mouflon with winter coat. B) Adult males of European mouflon with winter coat.

The mouflon is an herbivore with a generalist grazing strategy, considered to be a grazer but being able to feed on a large variety of plants (García-González and Cuartas, 1989; Marchand et al., 2013). It has more eclectic feeding habits than other wild ruminants, such as chamois or red deer, whose diet can widely overlap with mouflon (Bertolino et al., 2009; Redjadj et al., 2014).

Mouflon live in mixed small or medium-size groups (13.6 ± 1.41 females, 8.11 ± 0.72 lambs, and 6.51 ± 0.65 males in Bauges mountains in France) during the mating and breeding seasons (November-May), but sexual segregation occurs in summer and rams are then solitary or roam in small groups (7.05 ± 1.25 individuals in Bauges mountains), apart from the ewes (Darmon et al., 2007; Santiago-Moreno et al., 2004). It is a sedentary species that do not perform long-distance movements, and displacements respond mainly to food availability (Darmon et al., 2007). Despite the rut occurs mainly in November in most of the Iberian populations, the mating season can extend from September-November to December-January (Rodríguez-Luengo et al., 2007; Santiago-Moreno et al., 2004).

Interbreeding with domestic sheep and the consequent genetic introgression is the main threat for European mouflon (Lauvergne et al., 1977). There is however a lack of knowledge regarding genetics of most of the European mouflon continental populations and it is suspected that several of these populations could suffer from poor genetic purity (Santiago-Moreno et al., 2004).

2.2 The wildlife-livestock interface in alpine ecosystems

2.2.1 Traditional livestock rearing in mountain areas

Alpine meadows have been historically used as seasonal pastures for domestic ruminants all over the world. This temporal source of natural forage is an important resource for livestock rearing in rural areas, and has strongly influenced mountain livelihoods. During the grazing period, shepherds move livestock herds and flocks from local and distant areas (transhumance) to communal pastures in the alpine meadows. Geometric stone formations and huts spread in meadows from the Pyrenees are signs of previous times when shepherds spent the grazing season in the mountain along with their livestock. However, this activity has been shaped by fast-changing social and economic factors and for example, in Catalonia (NE Spain), the transhumance has almost disappeared, the shepherd is no longer living with the herds and flocks during the grazing season and the number of ovine and caprine flocks have dramatically dropped in the last years (Fundació Món Rural, 2010). Despite these changes, livestock is still an important economic income in mountainous rural areas. It is estimated that about 150,200 sheep (*Ovis aries*), 11,500 goats (*Capra hircus*), 134,800 cattle (*Bos taurus*) and 8,300 horses (*Equus caballus*) seasonally graze free-range in the Catalan Pyrenees (Regions of Alt Pirineu i Aran, Berguedà and Ripollès 2009) (Institut d'Estadistica de Catalunya, 2009). A high proportion of these domestic livestock yearly dwells with wild ungulates above the timberline from April-May to October-November (Figure 2.11).
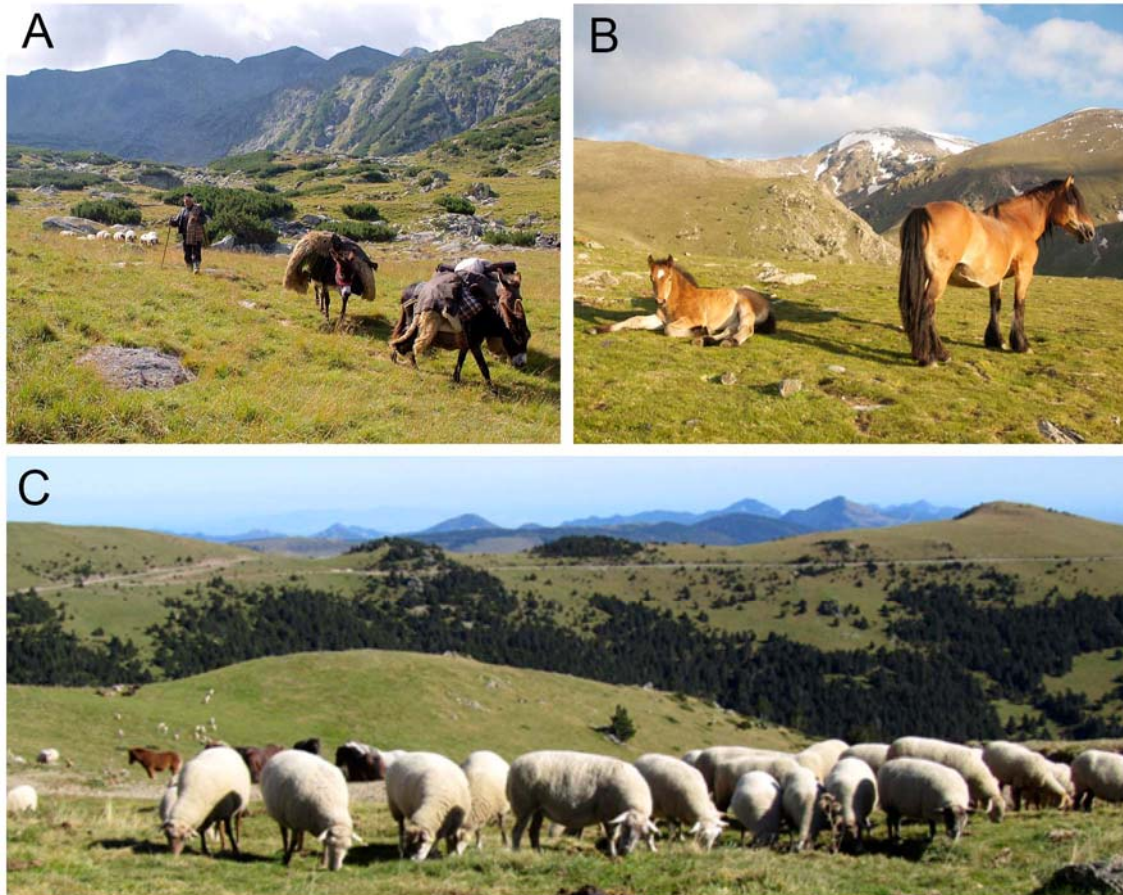
Figure 2.11 Shepherding in natural pastures is and has been an important activity in mountain areas. A) Traditional livestock rearing in mountain pastures from Retezat National Park, Romania (2013). Livestock, such as horses B) and domestic sheep C), free-range without shepherd in alpine meadows from the Pyrenees where predators are absent or scarce (National Game Reserve Freser-Setcases).

2.2.2 Interactions among wild and domestic ruminants

Wild ungulates and domestic livestock tend to avoid each other and direct contacts are generally rare (Chirichella et al., 2013; Cowie et al., 2016; Kukielka et al., 2013; Pepin and N'Da, 1992). However, the similar physiology and nutritional requirements of wild and domestic ungulates can cause spatial overlapping and abundant indirect interactions around natural resources (Kukielka et al., 2013). These hotspots for cross interactions may differ depending on the ecosystem type and the abundance/scarcity of the natural resources (Barasona et al., 2014a, 2014b).

In high mountain areas, interactions of wild ungulates and livestock occur seasonally in alpine meadows and rich pastures or down in the valley around anthropogenic food supplies in wintertime (Casaubon et al., 2012; Ryser-Degiorgis et al., 2002). Whereas interactions involving cervids are more probably to occur with cattle than with small domestic ruminants (mainly in wintertime), chamois and ibex interact mostly with small domestic ruminants (mainly in summertime) (Casaubon et al., 2012; Richomme et al., 2006; Ryser-Degiorgis et al., 2002). No studies including mouflon and livestock species interaction have been reported but its wide diet breadth and its spatial use also suggest that regular interactions occur (Darmon et al., 2014; Marchand et al., 2013). Direct contacts between close related species can also take place because of sexual attraction and mating purposes, such as ibex and domestic goat (Giacometti et al., 2004) or sheep and mouflon (Lauvergne et al., 1977) (Figure 2.12).

Figure 2.12 Interactions between wild and domestic ruminants and among wild Caprinae are regular in alpine meadows. Here a distant encounter (<20m) between Pyrenean chamois and cattle (A) and a closer interaction between Pyrenean chamois and European mouflon (B), both at the National Game Reserve of Freser-Setcases (Eastern Pyrnees, Spain). C) Close interaction between chamois and domestic sheep. D) Interbreeding between European mouflon and domestic sheep is often documented in the National Game Reserve of Freser-Setcases.

In addition, management practices of domestic herds can significantly influence on cross-species interactions. Salt deposits spread by the farmer in mountain pastures and not guarded nor enclosed domestic herds have been identified as predisposing factors for wildlife-domestic contacts (Chirichella et al., 2013; Richomme et al., 2006; Ryser-Degiorgis et al., 2002). It is interesting to notice that changes in livestock management registered in the last decades involve an increasing risk of wildlife-livestock interaction and consequently for pathogen cross-transmission.

Among wild ungulates, interactions are caused by niche overlap, which mainly relies on the feeding strategy and habitat selection of the different species (Darmon et al., 2012; La Morgia and Bassano, 2009; Loison et al., 2003). Besides, tolerance among wild Caprinae species is relatively high and interactions occur more frequently, at shorter distances and in longer periods than between domestic and wild ungulates (Ryser-Degiorgis et al., 2002) (Figure 2.13).
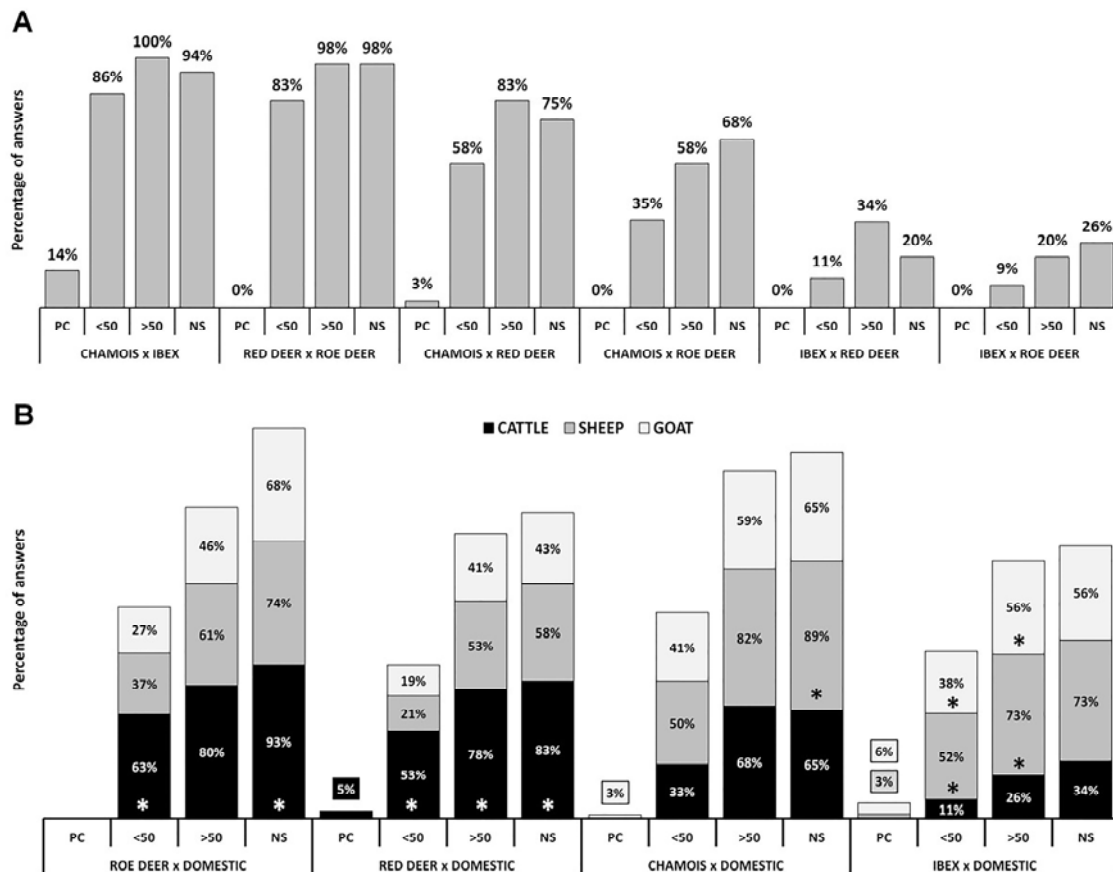
Figure 2.13 Interactions and proximity of the encounters among wild ruminants (A) and between wild and domestic ruminants (B) from Graubünden, Switzerland. PC: physical contact; < 50: encounter of < 50 m; > 50: encounter of > 50 m; NS: non-simultaneous occupation of the same area. Asterisks indicate statistically significant difference ($p < 0.05$) from the other group(s) (Casaubon et al., 2012).

2.2.3 Cross species transmission and multi-host systems

Infectious diseases are shared between wildlife and livestock and can be transmitted among them if cross interactions occur (Frölich et al., 2002; Pastoret et al., 1988; Siembieda et al., 2011). However, several conditions need to concur in an interaction between two competent hosts to be an effective contact for pathogen transmission (Ferroglio et al., 2007). These conditions vary depending on pathogen characteristics, like its environmental resistance or invasiveness, the infection dynamics in the source host which may influence the pathogen loads excreted, the target host immunity or even the characteristics of the interaction, such as the distance and the duration of the encounter (Scott

and Smith, 1994). Pathogens that do not long resist environmental conditions have higher spatial and temporal constraints for cross-transmission to occur. Consequently, indirectly-transmitted or vector-borne pathogens have more chances to spread among different host species at the wildlife-livestock interface than directly-transmitted pathogens (Astorga Márquez et al., 2014; Casaubon et al., 2012; Pruvot et al., 2014; Rossi et al., 2015).

Seasonal co-occurrence and mixing of livestock herds in alpine pastures can cause the circulation of pathogens among domestic ruminants (Braun et al., 2013; Krametter-Froetscher et al., 2007), and eventually the spill-over to wild ungulates (Belloy et al., 2003a; Casaubon et al., 2012; Martin et al., 2015). Moreover, pathogens that can be maintained in wild host populations can also be transmitted back to livestock (Mick et al., 2014). Therefore, alpine pastures provide the conditions for pathogen transmission at the wildlife-livestock interface (Garin-Bastuji et al., 2014). Incidence of host transitions depends upon the transmission type (direct, indirect or vector-borne), which can be sparse in time with recurrently historical spillover events among host populations (Kamath et al., 2016) or occur frequently and at relatively high transmission rates (Fernández-Aguilar et al., 2014).

As long as cross-transmissions are relevant, disease ecology relies in multi-host systems with different functional host populations (Fenton and Pedersen, 2005). Determining the functional roles in complex systems where several competent hosts occur can be challenging (Caron et al., 2015; Haydon et al., 2002). The use of patterns of disease incidence and pathogen prevalence may infer wrong epidemiological roles if they are not complemented with other techniques like molecular epidemiology (Viana et al., 2014). Even with pathogen genetics and the evidence of cross-species transmission, a comprehensive insight of the system may not be provided if spatio-temporal data are limited (Kamath et al., 2016; Serrano et al., 2011). Long-term studies that integrate different techniques and information, such as spatial and temporal infection dynamics and molecular epidemiology in all potential hosts, are therefore necessary to identify the maintenance host community or population (Viana et al., 2014).

2.2.4 Infection dynamics in wild mountain ungulates

Host social structure and behaviour drive aggregation and segregation of individuals and directly influence the contact rates and ultimately the transmission of infectious diseases. Despite most infectious diseases exhibit a density-dependent transmission within host populations, group-living hosts (such wild mountain ungulates) may modulate transmission of diseases in a frequency-dependent manner if contacts between subpopulations are low (Manlove et al., 2014). Dynamics of a given disease can be different among these subpopulations and some host groups may remain healthy during epidemic events. On the other hand, segregation of males in smaller social groups cause in general a sexually-biased prevalence of infectious diseases in Caprinae species (Degiorgis et al., 2000b; Rossi et al., 2007; Tschopp et al., 2005).

2.3 Infectious keratoconjunctivitis and

Mycoplasma conjunctivae

Infectious keratoconjunctivitis (IKC) is a contagious ocular condition that affects domestic and wild sheep and goats (Giacometti et al., 2002a). It is a worldwide disease known as "pink eye" in small domestic ruminants characterized by the inflammation of the conjunctiva and the cornea (Hosie, 2007). It has been extensively reported to affect different domestic and wild Caprinae species in Europe (Baas et al., 1977; Baker et al., 2001; Naglić et al., 2000), North America (Barile et al., 1972; Jansen et al., 2006; Leite-Browning, 2007), Middle East (Lysnyansky et al., 2007), South Africa (Van Halderen et al., 1994), South Asia (Shahzad et al., 2013), South-East Asia (Abdullah et al., 2014), New Zealand (Motha et al., 2003) and Australia (Barile et al., 1972; Surman, 1973). The commonest of IKC in domestic sheep does probably explain its widespread distribution. Infectious keratoconjunctivitis affecting non-Caprinae species has different origin and can be considered as a different disease with similar clinical signs (Giacometti et al., 2002a).

Caprinae species known to be susceptible to IKC include domestic sheep (Baker et al., 2001), domestic goat (Baas et al., 1977), Northern chamois (Degiorgis et al., 2000b), Southern chamois (Marco et al., 2009a), Alpine ibex (Tschopp et al., 2005), Iberian ibex (Arnal et al., 2009), European mouflon (Marco et al., 2009a), Bighorn sheep (*Ovis canadensis*) (Jansen et al., 2006) and Hymalayan thar (*Hemitragus jemlaicus*) (Daniel and Christie, 1963). Despite literature reports a limited list of species, other Caprinae species from could be also affected.

2.3.1 Aetiology

The aetiology of IKC has been extensively discussed for several decades until molecular-based techniques started to be used for diagnostics (Gauthier, 1994). There are several reasons that explain this past lack of consensus. First, eyes are in direct contact with the environment, and can thus harbour a great variety of bacteria and virus either in health or disease conditions (Dagnall, 1994a; Egwu et al., 1989). Secondly, the fastidious nature of some bacteria can yield false negative results by culture-based methods and/or would not be present at a particular stage of disease (Mayer et al., 1996; Surman, 1973). Finally, IKC is

a non-specific syndrome that can indeed be caused by diverse infectious agents in different host species. However, in Caprinae hosts *Mycoplasma conjunctivae* has been demonstrated as the main single cause of IKC according to Koch's postulates and by being associated with most of the IKC outbreaks reported (Giacometti et al., 2002a).

Mycoplasma conjunctivae was first isolated in 1968 in Australian sheep, but it was not identified until 1972 by Barile et al., who described it for the first time with specimens isolated from sheep and goats suffering from IKC (Barile et al., 1972). Since then, it has been isolated in other hosts with IKC, such as Alpine chamois (Nicolet and Freundt, 1975) or Alpine ibex (Mayer et al., 1996). Infectious keratoconjunctivitis has been also experimentally reproduced by instillation of *M. conjunctivae* in domestic sheep (Dagnall, 1993; Janovsky et al., 2001; Jones et al., 1976; Klinglerk et al., 1969; Ter Laak et al., 1988a), domestic goat (Trotter et al., 1977), Alpine ibex (Degiorgis et al., 2000a; Giacometti et al., 1998), Alpine chamois (Klinglerk et al., 1969; Nicolet and Freundt, 1975) and European mouflon (Terrier, 1998). The consistent molecular detection of *M. conjunctiva*e in most of the IKC outbreaks occurring in wild Caprinae from Europe definitely ascribed this bacteria as the main single cause of IKC (Arnal et al., 2013a; Baas et al., 1977; Degiorgis et al., 2000b; Giacometti et al., 2002b; Marco et al., 2009a; Naglić et al., 2000; Tschopp et al., 2005). The complete genome of the *M. conjunctivae* type strain HRC/581T have been sequenced (Calderon-Copete et al., 2009), and the *lppS* and *lppT* genes involved in adhesion have been characterized (Belloy et al., 2003b; Zimmermann et al., 2010).

Other infectious agents have been isolated from eyes with IKC, such as *Moraxella ovis* (formerly named *Neisseria ovis*), *Corynebacterium pyogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Lysteria monocytogenes*, *Chlamydophila psittaci, Ch. abortus*, *Mycoplasma* other than *M. conjunctivae* or *Rickettsia*-like spp. (Bijlenga et al., 1983; Blanco et al., 1982; Dagnall, 1994a; Egwu et al., 1989), but the etiologic relation of these agents with the clinical condition has not been ascertained, being mostly considered as secondary invaders or normal flora, also present in healthy eyes (Dagnall, 1994; Giacometti et al., 2002a; Polkinghorne et al., 2009). Experimental infections

with *M. ovis* and *C. psittaci* (as taxonomically considered at that time) have however demonstrated that certain strains of these bacteria can cause slight ocular disease in sheep (but not keratitis), inducing particular pathologic features such as the prominence of subconjunctival lymphoid follicles (Bankemper et al., 1990; Dagnall, 1994b; Wilsmore et al., 1990). Based on these differences, some authors considered the differentiation of non-follicular IKC, caused by *M. conjunctivae* (Jones et al., 1976), and follicular IKC caused either by *C. psittaci*, *M. ovis* or their co-infection with *M. conjunctivae* (Dagnall, 1994b; Storz et al., 1967; Wilsmore et al., 1990). However, follicular IKC cases and outbreaks have been barely reported in Caprinae (Andrews et al., 1987; Bankemper et al., 1990).

Despite other mycoplasmas can invade the eyes and cause an IKC-like syndrome, in these cases ocular disease is usually accompanied by mastitis, arthritis and/or pneumonia (Corrales et al., 2007; Gaffuri et al., 2016; Verbisck et al., 2010). Some exceptions though have been reported with *M. agalactiae*, which has been associated with an IKC outbreak in domestic sheep (Rodríguez et al., 1996) and with *Mycoplasma mycoides* subsp. *capri* large-colony serovar associated with a severe IKC case in Alpine ibex (Giangaspero et al., 2010). The isolation or detection from the eyes of other mycoplasma such as *M. arginini*, *M. ovipneumoniae, M. hyorhinis* or *Acholeplasma oculi* has not considered been related to ocular disease (Arbuckle and Bonson, 1980; Barile et al., 1972; Dagnall, 1994a; Gupta et al., 2015; Jones et al., 1976; Nicolet and Freundt, 1975).

The role of viruses in IKC of Caprinae has been poorly assessed, but virus isolation failed when it was attempted (Bijlenga et al., 1983; Van Halderen et al., 1994), and cytopathic effect has not been consistently demonstrated in cell culture exposed to clinical samples (Bijlenga et al., 1983; Costa, 1986; Mayer et al., 1996). Similarly as for non-*M. conjunctivae* mycoplasmas, several viruses can cause ocular disease in Caprinae as part of a broader syndrome in which conjunctivitis or keratoconjunctivitis is not the main feature, such as Bluetongue virus or Peste des Petits Ruminant virus (PPRV) (Elbers et al., 2008; Hamblin et al., 1998; OIE, 2008). Next-generation sequencing has revealed a number of viruses occurring in the eyes of wild ruminants, but without conclusive

information about their possible role on health disruptions (Schürch et al., 2014; Smits et al., 2013).

Other IKC-like diseases can be primary caused by other infectious agents in non-Caprinae hosts, like *Moraxella* (*Branhamella*) *bovis* and probably *M. bovoculi* in cattle, also called pink eye or infectious bovine keratoconjunctivtis (Angelos, 2015; Angelos et al., 2007), Cervid Herpesvirus-2 in reindeer (*Rangifer tarandus*) (Tryland et al., 2009), and probably *Chlamydiaceae* in deer species (Meagher et al., 1992; Taylor et al., 1996). Despite all these pathogens may cause ocular disease, differences on the aetiology, pathogenesis and epidemiology indicate that are distinct diseases and they are beyond the scope of this thesis.

2.3.2 Clinical signs and pathology

The infectious keratoconjunctivitis is can affect one or both eyes (Giacometti et al., 2002a). Four IKC stages have been described based on histological and macroscopic features in naturally diseased Alpine ibex (Mayer et al., 1997) and macroscopically in domestic sheep (Egwu, 1991; Hosie, 2007) (Figure 2.14).
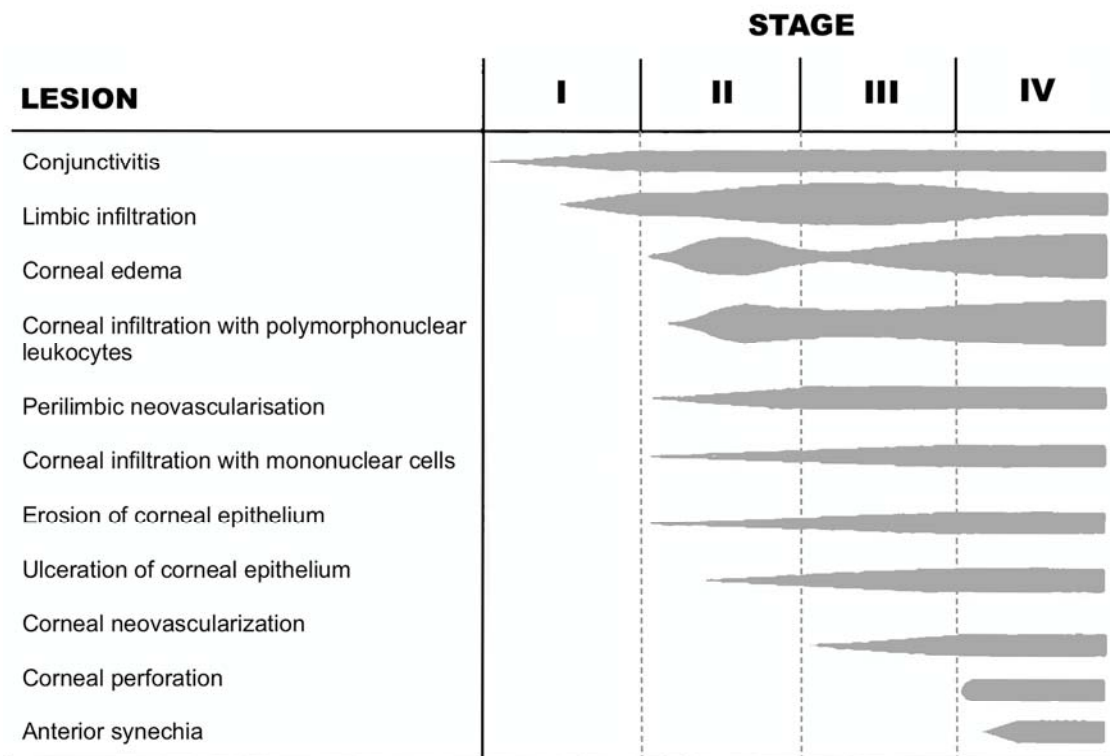
Figure 2.14 Scheme of the macroscopic clinical signs and histological features of IKC in Alpine ibex. Ocular lesions are shown upon appearance in a IKC stage gradual classification from I to IV as described (Mayer et al., 1997). The intensity of the lesions is represented by the thickness of the bars.

The initial clinical signs observed in IKC (correspondence with **IKC stage I**) is a mild conjunctivitis with hyperaemia of the conjunctiva and congestion of conjunctival vessels, generally accompanied with epiphora or slight mucopurulent effusion (Hosie, 2007). At this stage, the predominant cell infiltration in the conjunctiva and limbic area is mononuclear (lymphocytes, plasma cells, macrophages and monocytes) (Baas et al., 1977; Mayer et al., 1997).

The disease may eventually progress to more severe conjunctivitis and keratitis, affecting mainly the outer stromal layer of the eye structures (**IKC stage II**; Figure 2.15A and 2.15B). Migration of the vessels starts from the corneo-scleral margin invading the perilimbic cornea concentrically (pannus) in response to the progression of corneal erosion (Wilcock, 2008). Oedema around the perilimbic neovascularization is observed along with a more intense limbic and conjunctival cell infiltration, which in some cases can be composed by mixed mononuclear cells and neutrophils (Mayer et al., 1997). The epithelium of the central cornea may be eroded or ulcerated in some eyes, in which necrosis and oedema with severe neutrophil infiltration can be present (Mayer et al., 1997). The presence and infiltration degree of neutrophil cells in the cornea may depend on secondary bacterial infections, and may be present either by neovascularisation or by the tears through corneal damage (Wilcock, 2008). Vision can be severely impaired in those cases of corneal opacity (oedema, necrosis and cell infiltration), but spontaneous regression may still occur at this stage (Hosie, 2007).

A more advanced stage of the disease (**IKC stage III**; Figure 2.15D) shows more intense neovascularization that reaches up the centre of the cornea, and the inflammatory features observed in previous stages may affect the inner layers of the stroma. The severe cell infiltration can form aggregation in foci that produce an irregular corneal surface (Mayer et al., 1997). The corneal

epithelium is often eroded or ulcerated and oedema ranges from mild to severe. Corneas with minor epithelial erosion were reported to have more intense neovascularization and predominantly mononuclear cell infiltration. Mild epithelial pigmentation may be present in the vertex area of the cornea (Mayer et al., 1997). In general, clinical signs are more severe than in the previous stages and lachrymal discharge is more intense and purulent. However, some severe IKC stages II may be macroscopically similar to IKC stages III (Mayer et al., 1997). Domestic Caprinae may need treatment at this stage because of visual impairment (Hosie, 2007).

In the ultimate and more severe stage of IKC (**IKC stage IV**; Figure 2.15F), the cornea is opaque and the ulcer may have evolved to perforation with an evident staphyloma in the centre. Eyes at this stage show Descemet's membrane rupture and anterior synechia of incarcerated iris, which causes melanin deposition and melanophages in the vertex area (Mayer et al., 1997). In some cases, hypopyon is developed. Concurrent necrosis and regeneration processes may be intense. At this stage, lesions resolve slowly, even with treatment, and scarring may permanently persist following resolution. Eye loss may also occur.
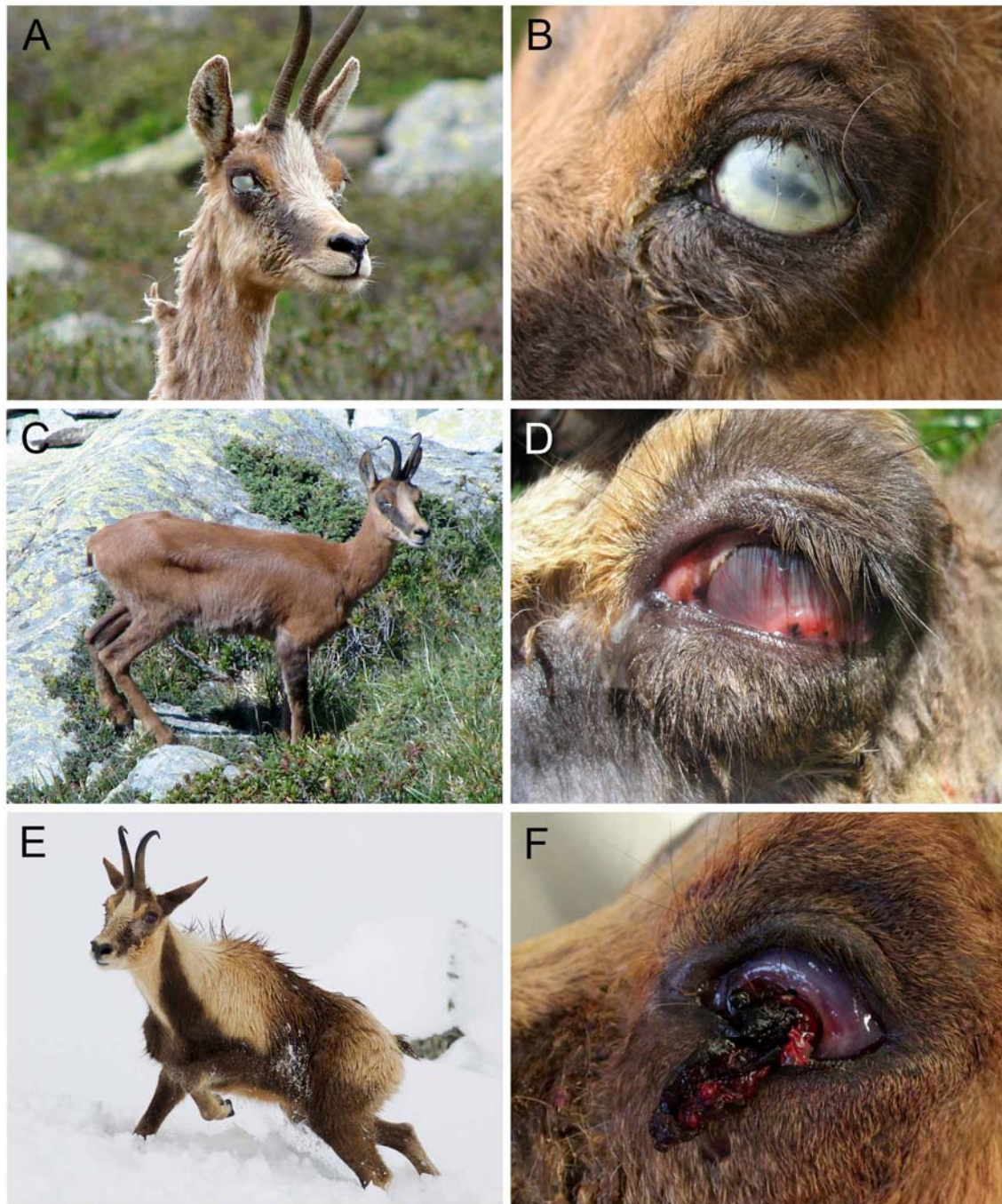
Figure 2.15 Different degrees of IKC stages in Pyrenean chamois. A) IKC stage II in an adult female with severe corneal opacity and extensive mucopurulent effusion. B) IKC stage II with severe degree of inflammatory infiltration in the cornea. C) Adult female with IKC exhibiting blepharospasm, light mucopurulent effusion and poor body condition. D) IKC stage III with a central ulcer in the cornea, neovascularization and staphyloma. E) Chronic IKC stage in adult male chamois with infra-ocular alopecia and circling movements. F) Ultimate IKC stage IV with perforation of the cornea.

Differentiation among these clinical stages only by macroscopic features may be confusing in some stage II and III cases (e.g. stage II with severe corneal oedema and inflammatory cell infiltration). Although these clinical categories consider the progression of the pathologic process, do not include chronic healing IKC stages that may also exhibit evident clinical signs (scarring in the cornea, pigmentation of the cornea or rests of neovacularization) (English, 2013). Along with ocular clinical signs, behaviour alterations consisting in circling movements have been occasionally described in wild Caprinae with severe IKC (Degiorgis et al., 2000b). No brain lesions associated to this abnormal behaviour have been reported and it is considered to be caused by disorientation and stress in blind animals (Figure 2.16) (Giacometti et al., 2002a).

Figure 2.16 Severely IKC affected Alpine chamois in the snow, which shows the tracks of the repetitive circular movements occasionally described in severely affected individuals (Hofmann and Rapperswil, 1999).

Mycoplasma conjunctivae has been also isolated from lung lesions in one chamois and it was proposed that it may also infect the mucous membranes of the upper respiratory tract with an eventual involvement of the lung tissue

(Nicolet and Freundt, 1975). Brain lesions and neurologic clinical signs were concurrently found with an IKC outbreak in chamois and ibex from the Italian Alps (Bassano et al., 1994; Lanfranchi et al., 1985). However, pathologic examinations in most of the IKC cases from epizootics have yielded no evidence of affection in other organs or tissues a part from the eyes (Arnal et al., 2013a; Degiorgis et al., 2000b; Mayer et al., 1997). Atypical strains of mycoplasmas that have integrated prophages from other specimens may however show different virulence and tissue tropism than typical strains (Tardy et al., 2012).

2.3.3 Pathogenesis and immunity

The incubation period for *M. conjunctivae* ranges from 12 hours to four days, with a mean of two days, although exceptionally it can reach up to 21 days (Degiorgis et al., 2000a; Jones et al., 1976; Ter Laak et al., 1988a; Van Halderen et al., 1994). Nevertheless, clinical signs may never be expressed (Degiorgis et al., 2000a). The initial load of *M. conjunctivae* is determinant for the clinical reproduction of the disease in experimental infections (Dagnall, 1993). Clinical progression is widely variable and may extend until 12-15 weeks in domestic Caprinae (Baas et al., 1977; Dagnall, 1993; Janovsky et al., 2001) and six weeks post-infection (p.i.) in Alpine ibex (Giacometti et al., 1998). *Mycoplasma conjunctivae* may persist in the eyes from two to six months beyond clinical signs disappear (Giacometti et al., 1998; Janovsky et al., 2001). This persistence may depend on the strain involved (Janovsky et al., 2001). Although spontaneous regression of IKC may occur at any disease stage, clinical relapse is a common feature of IKC (Baas et al., 1977; Giacometti et al., 1998; Janovsky et al., 2001; Ter Laak et al., 1988b).

Considering that clinical recovery is the most common course of IKC, adaptive immune response is probably effective to cope with the infection (Hosie, 2007). Specific systemic immunoglobulin G (IgG) is detected from two to four weeks p.i. against antigens of 175, 73, 68, 60 and 33 kDa. A similar but weaker IgG response is observed in conjunctival washes (Degiorgis et al., 2000a; Grattarola et al., 1999). However, systemic immune involvement is not always observed

after *M. conjunctivae* infection (Baas et al., 1977), and weaker humoral responses have been reported in experimental infections than in natural IKC outbreaks (Degiorgis et al., 2000a; Trotter et al., 1977). Cell-mediated response or other local mechanisms may therefore necessarily occur to resolve the infection (Trotter et al., 1977). Based on IgG in sera, population immunity is around 50-60% in endemic sheep flocks (Janovsky et al., 2001). Chamois populations with current or recently past IKC outbreaks had shown low IgG seroprevalence (8% and 3%, respectively), suggesting that immunity do not last long in this species (Giacometti et al., 2002b). Acquired immunity also seems to be insufficient to resist re-infections under experimental conditions (Costa, 1986; Trotter et al., 1977), and field observations support that re-infections with milder clinical signs may occur in a short-time period (Crampe, 2008). Asymptomatic consecutive *M. conjunctivae* infections with two different strains in a two-year interval have been also observed in Alpine ibex (Mavrot et al., 2012a).

2.3.4 Predisposing factors and secondary infections

Both biotic and abiotic factors have been proposed to influence disease expression (Hars and Gauthier, 1984). *Moraxella ovis* and *Lysteria monocytogenes* were suggested to predispose the onset of clinical signs associated to *M. conjunctivae* in some sheep flocks (Akerstedt and Hofshagen, 2004; Dagnall, 1994a, 1994b), but not in others (Ter Laak et al., 1988a). *Staphylococcus aureus* was also isolated more frequently in severely than mildly affected eyes (Egwu et al., 1989). However, overgrowth of secondary invaders in diseased eyes may not be necessarily related with pathogenesis or may depend on the virulence of the strains involved (Dagnall, 1994b). The vaccination with attenuated PPRV vaccine was suggested to have triggered a severe and unusual IKC outbreak caused by *M. conjunctivae* in an endemic goat farm, acting either in co-infection or as a stressor (Hadani et al., 2013).

Harsh environmental conditions of snow and high winds may also drive the onset of clinical signs in sheep flocks, which gives rise to the colloquial term of "snow blindness" in UK (Hosie, 2007; National Animal Disease Information

Service UK, 2017). In wild Caprinae (i.e. Alpine chamois and ibex), altitude may likewise negatively influence IKC course (Mavrot et al., 2012b). All these factors are probably related with an increased ultraviolet radiation due to altitude (Marín et al., 2005) and snow reflectance (Dolin and Johnson, 1994). Ultraviolet radiation can directly produce eye damage and is considered a predisposing factor for infectious keratoconjunctivitis in humans (Ellerton et al., 2009). Air dryness in colder environments (also at higher altitudes) can also contribute to eye moisture deficiency and predispose to bacterial overgrowth (Dart, 1988). Neither the onset nor the severity of the clinical signs is influenced by poor physical condition or exogenous corticosteroids administration in sheep (Dagnall, 1993; Egwu et al., 1989; Jones et al., 1976).

2.3.5 Effects of Infectious Keratoconjunctivitis on animal health and livestock production

Blindness can cause a great distress to sheep and goats affected by IKC and may imply the need of therapeutic treatment in severe cases (Hosie, 2007). Epizootic IKC spreads fast and outbreaks generally affect the whole flock or herd, and cause occasional economic losses and disturbance to the farmer owing to the special nursing care needed during the blindness period (Naglić et al., 2000). Significance of IKC in wild hosts is however of more concern, since IKC outbreaks with severe cases of irreversible blindness recur in wild host populations (Gauthier, 1991). Mortality is derived from blindness and the treacherous mountain terrain where wild Caprinae occur, as well as from predation in areas where large predators inhabit (predation can reach up to 50% of the total mortality related to IKC) (Degiorgis et al., 2000b; Jansen et al., 2007). Although mortality in IKC epidemics does not usually exceed 12-15% (Gauthier, 1991; Hars and Gauthier, 1984), it may locally rise up to 27% (Degiorgis et al., 2000b). Important demographic effects are also described during IKC outbreaks and in the post-epizootic period, with a lower reproductive index in chamois populations that extend population recovery from three to five years (Arnal et al., 2013a; Loison et al., 1996). A long-term cohort effect may also occur in chamois males born during epizootics, which have smaller mass

and horn length (Loison et al., 1996). It is therefore considered one of the main infectious diseases that affect chamois (Serrano et al., 2015) and ibex (De Danieli and Sarasa, 2015).

2.3.6 Historical records of IKC outbreaks in wild Caprinae

Because IKC is highly noticeable, records of epizooties have been easily registered and tracked for a long time in Europe. The first detailed record of an IKC outbreak in wild Caprinae dates from 1916 in Alpine chamois from Bavaria (Germany) that also affected the neighbouring Tirol (Austria) and extended to the East, ending in Lower Austria in 1920 (Steineck, 1985; Stroh, 1919). A concurrent outbreak also occurred in the Argentera Massif from the Italian Alps in 1915-1918 (Gauthier, 1991). Since then, several outbreaks affecting Alpine chamois, Alpine ibex and mouflon have occurred in the Alpine arc involving several countries (Degiorgis et al., 2000b; Grattarola et al., 1999; Sarrazin et al., 1990; Tschopp et al., 2005).

In the Pyrenees, the first description of IKC dates from 1957 as isolated cases of disease in Pyrenean chamois (Sánchez-Belda and Martínez-Ferrando, 1985). However, IKC outbreaks were not registered until 1980 when a large epizooty arose in the Central Pyrenees affecting both the Spanish and French sides (Catusse, 1982). The Pyrenean chamois populations were then abundant and the outbreak rapidly extended towards West and East throughout almost the whole Pyrenees (Müller, 1984), reaching the Eastern Pyrenees in 1981-1983 (Gauthier, 1991). Several IKC isolated cases and other smaller outbreaks were recorded in the next years in the Central and Eastern Pyrenees, arriving for the first time to the Eastern pre-Pyrenees (Cadí NGR, former Cadí-Moixeró NGR) in 1991 and to the easternmost part of the Pyrenees (Freser-Setcases NGR) in 1995. Sporadic IKC cases and local outbreaks have since then been recorded in some of the previously affected areas (Marco et al., 2009a; Pañella et al., 2010). In 2006-2008 a large IKC outbreak arose again in the Central Pyrenees and extended throughout the whole Central Pyrenees as the previous 1980-1983 outbreak (Arnal et al., 2013a; Crampe, 2008) (Annex I).

2.3.7 Seasonal trends and individual risk factors

Infectious keratoconjunctivitis may occur all year round in host populations and herds, but natural outbreaks in wild Caprinae show peaks of disease during the course of epizootics. Despite these rises of incidence showed no seasonal patterns in some IKC outbreaks (Degiorgis et al., 2000b), most of the outbreaks have higher IKC cases and mortality in spring and summer, which matches the vector season and breeding period (Arnal et al., 2013a; Gauthier, 1991; Giacometti et al., 2002b; Loison et al., 1996; Tschopp et al., 2005). A possible second rise may occur in autumn, coinciding with the rut of wild Caprinae and the consequent higher proportion of IKC-affected males as compared to other seasons (Degiorgis et al., 2000b; Giacometti et al., 2002b; Tschopp et al., 2005).

All age classes can develop IKC in domestic sheep, and lambs may develop disease since they are only five to ten days old (Jones et al., 1976). However, IKC also typically causes more severe clinical signs in adult sheep than in lambs (Greig, 1989; Hosie, 2007; Jones et al., 1976). The influence of age on *Mycoplasma conjunctivae* prevalence has not been investigated up to date in sheep flocks.

Younger individuals (kids, yearlings and juveniles under four years old) and adult females are the most represented age and sex classes found dead during IKC outbreaks in wild Caprinae (Degiorgis et al., 1999; Giacometti et al., 2002b). However, when the numbers are corrected according to the age and sex class structure of the population, no age differences but a sex-biased mortality toward females is found (Arnal et al., 2013a). Furthermore, a closely monitored chamois population with marked individuals from the National Park of the Pyrenees (France) showed that mortality was correlated with age and a high number of chamois orphans were observed. Clinical signs were also in general milder in kids than in their respective mothers (Crampe, 2008). Similar observations were performed in the Spanish Pyrenees when affected by the same IKC outbreak, where solitary kids, kids with males and females with two and three kids were observed (Arnal et al., 2013a). The lower reproductive performance of severely IKC-affected chamois populations during the epizootic and in the post-epizootic period may rely in this higher susceptibility of adult

females and the sex-biased mortality (Arnal et al., 2013a; Loison et al., 1996). Despite these age-class differences, significant higher prevalence of *M. conjunctivae* has been reported in yearling than in adult ibexes (Mavrot et al., 2012a).

2.3.8 Transmission of *Mycoplasma conjunctivae* and epidemiological scenarios

Mycoplasma conjunctivae is an obligate microparasite that barely resists environmental conditions (Calderon-Copete et al., 2009). Therefore, transmission mainly occurs by direct contact through aerosols and/or contaminated excretions (Prave et al., 1987; Ter Laak et al., 1988a). Direct transmission has been demonstrated in experimental infections from challenged animals to control contact animals in two or three days post-infection in Alpine chamois (Prave et al., 1987), six to sixteen days p.i. in sheep (Ter Laak et al., 1988a) and 20 to 22 days p.i. in Alpine ibex (Degiorgis et al., 2000a; Giacometti et al., 1998). However, eye-frequenting insects are suspected to act as vectors, enhance transmission and enable cross-species transmission in natural ecosystems (Belloy et al., 2003a).

In domestic livestock, the emergence of IKC outbreaks has been associated with the introduction of *M. conjunctivae* in a naïve flock or herd along with healthy carriers (Egwu et al., 1995; Naglić et al., 2000), or with IKC-affected individuals introduced in herds with sporadic clinical cases (Baas et al., 1977). IKC is highly contagious within herds and well connected host groups or subpopulations (Baas et al., 1977; Crampe, 2008; Gauthier, 1991). Epizootics are consequently characterized by a high transmission rate that causes high morbidity, affecting 85-95% of the population (Degiorgis et al., 2000b; Gauthier, 1991; Hars and Gauthier, 1984; Loison et al., 1996). Outbreaks in wild Caprinae can affect locally (Tschopp et al., 2005) or spread throughout mountain ranges for several years, affecting host populations from consecutive massifs, covering huge areas and moving more than 100 km in straight line (Arnal et al., 2013a; Gauthier, 1991; Gelormini et al., 2016). The epizootic spread of IKC has been reported to move forward with an average speed of 15 km per year (Degiorgis

et al., 2000b). Connectivity of wild host populations may influence the spread of IKC outbreaks (Loison et al., 1999), but high altitudes and slopes do not necessarily stop epizootics and only deep valleys (<1200m) may act as barriers (Crampe, 2008; Tschopp et al., 2005). A second IKC outbreak in a short-time period in the same herd or population may entail milder clinical signs (Baas et al., 1977; Gauthier, 1991).

Despite IKC outbreaks are noticeable events highly reported, a low and sporadic incidence of mild IKC is more commonly observed in domestic sheep flocks (Hosie, 2007; Janovsky et al., 2001). *Mycoplasma conjunctivae* has been reported in 82-85% of the sheep flocks sampled in the Pyrenees (Arnal et al., 2013a) and 85.7% of the flocks sampled in the Swiss Alps (Janovsky et al., 2001). Sheep flock surveillance for *M. conjunctivae* through real time PCR revealed an overall prevalence of 21.3% - 29.2%, but did not report flock prevalence or the relationship between *M. conjunctivae* detection and clinical signs, nor the percentage of asymptomatic infections (Arnal et al., 2013a). *Mycoplasma conjunctivae* has been isolated from 7% and 8% of asymptomatic sheep in flocks with and without IKC clinical cases, respectively (Akerstedt and Hofshagen, 2004).

Similar non-epidemic IKC has been also reported in chamois populations that have recently passed a severe IKC outbreak (based on visual detection of the disease) (Arnal et al., 2013a) and in some wild Caprinae Swiss populations (namely Alpine chamois and ibex). Contrary to reports in sheep, a *M. conjunctivae* prevalence as low as 3.8% was associated with sporadic IKC cases in Alpine chamois (Holzwarth et al., 2011). Relatively high percentage (19.1%) of Alpine ibex has been also described with *M. conjunctivae* infection without clinical signs or only mild IKC, meanwhile IKC epizootic occurred in sympatric Alpine chamois populations (Ryser-Degiorgis et al., 2009). Other studies reported non-epidemic scenarios in Alpine ibex and Alpine chamois, but without a clear information of the *M. conjunctivae* dynamics or the population prevalence (Gauthier, 1991; Mavrot et al., 2012a).

2.3.9 *Mycoplasma conjunctivae* cross-species transmission and persistence in host populations

The ecology of *M. conjunctivae* may become complex in alpine areas where several competent hosts species cohabit and interact with each other (Ryser-Degiorgis et al., 2002). Simultaneous IKC affection in different Caprinae species from the same areas early suggested that cross-species transmission of the etiologic agent may occur in mountain environments (Nicolet and Freundt, 1975). The development of molecular epidemiology techniques based on the variable domain of the *lppS* gene demonstrated afterwards that *M. conjunctivae* strains causing IKC in chamois were similar to the strains found in local sheep (Belloy et al., 2003a). Subsequent studies also showed that *M. conjunctivae* strain clusters were shared by different wild host species (namely Alpine chamois and Alpine ibex) (Mavrot et al., 2012a; Ryser-Degiorgis et al., 2009), as well as by domestic sheep and chamois (Zimmermann et al., 2008), indicating that cross-species transmission of *M. conjunctivae* may be common in the European Alps. All these findings suggest that *M. conjunctivae* rely in a multi-host system in alpine areas and that different functional roles may occur among competent host populations (Dobson, 2004). Whereas *M. conjunctivae* can be maintained endemically in sheep flocks with mild or asymptomatic infections (Akerstedt and Hofshagen, 2004; Janovsky et al., 2001), severe IKC outbreaks followed by the fading out of *M. conjunctivae* have been reported in chamois populations from the Alps (Giacometti et al., 2002b; Loison et al., 1996; Tschopp et al., 2005). These observations converged to the hypothesis that sheep could be the source of IKC outbreaks for the more susceptible wild Caprinae, acting as a reservoir host in alpine ecosystems (Giacometti et al., 2002a; Hosie, 2007). Within wild Caprinae, Alpine ibex have been proposed to play a more relevant role for *M. conjunctivae* maintenance in wild host communities as compared with Alpine chamois, based on a higher rate of asymptomatic infections (Ryser-Degiorgis et al., 2009). Conversely, other reports showed similar *M. conjunctivae* prevalence of asymptomatic infections in Alpine chamois (5.6%) and Alpine ibex (5.8%) associated to epidemic and non-epidemic IKC occurrence, and suggested that *M. conjunctivae* persistence in wild Caprinae should not be excluded (Mavrot et al., 2012a). However,

sporadic IKC occurrence in wild host populations has been described after IKC outbreaks without the implication of *M. conjunctivae* persistence at medium or long term (Arnal et al., 2013a), and *M. conjunctivae* prevalence trend in wild host populations has never been evaluated to test if these non-epidemic scenarios are temporal. The long-term dynamics of *M. conjunctivae* infection along with the molecular epidemiology of the strains involved should be assessed on all competent hosts, both domestic and wild Caprinae, to establish conclusive functionality of the host populations in the system (Fenton and Pedersen, 2005). The limited epidemiological data available have not allowed to confirm whether *M. conjunctivae* can persist in wild host populations or not(Holt et al., 2003; Viana et al., 2014).

3. HYPOTHESIS AND OBJECTIVES

The infectious keratoconjunctivitis (IKC) is an old-known disease of wild mountain ungulates, but several aspects of its epidemiology are poorly understood and remain unknown. The sporadic arising of IKC epizootics and subsequent fading out in wild Caprinae had early suggested that domestic livestock was the reservoir source of the disease (Nicolet and Freundt, 1975). Furthermore, interspecific transmission have been demonstrated in Alpine ecosystems associated with an IKC outbreak (Belloy et al., 2003a). Accordingly, persistence of *Mycoplasma conjunctivae* in wild host populations have been disputed and generally considered hardly possible (Deutz et al., 2004; Giacometti et al., 2002a, 2002b). Recent investigations however showed that *M. conjunctivae* can also cause asymptomatic infections in wild Caprinae including non-epizootic incidence of IKC cases in free-ranging populations (Mavrot et al., 2012a), and was suggested that *M. conjunctivae* may be maintained in some wild host populations. Therefore, contrasted hypotheses have been formulated about the functional roles of the different host species in multi-host alpine systems.

Studies on IKC have been classically performed only in clinical cases of dead or severely affected animals, which provide useful information of the aetiology and the pathogenic effect of the disease, but limited information on the dynamics of *M. conjunctivae* in the population. Targeted surveillance on *M. conjunctivae* in free-ranging host populations by using highly-sensitive molecular techniques is scarce and is needed to improve the knowledge of this disease.

The objectives of the present thesis are:

1. To evaluate the status regarding *M. conjunctivae* of small domestic ruminant flocks that graze seasonally in areas where wild Caprinae inhabit, and assess its health impact in domestic hosts (**Study I and II**).

2. To determine *M. conjunctivae* infection dynamics in wild host populations from alpine systems in Northern Spain and specifically assess its persistence in these populations (**Study III**).

3. To assess *M. conjunctivae* cycles and functional roles of host populations in the alpine systems from Northern Spain (**Study III**).

4. To assess host-mycoplasma interactions in terms of clinical signs, molecular detection and immune response to infer epidemiological patterns and detect potential driving factors involved in *M. conjunctivae* infection (**Study I, II, III, IV**).

5. To explore non-ocular locations of *M. conjunctivae* with potential relevance in transmission and persistence in its hosts (**Study V**).

4. STUDIES

4.1 Study I:

Mycoplasma conjunctivae in domestic small ruminants from high mountain habitats in Northern Spain

BMC Veterinary Research, (2013) 9:253

Abstract

Infectious keratoconjunctivitis (IKC) is a clinical condition affecting eyes of domestic and wild Caprinae worldwide, and *Mycoplasma conjunctivae* is considered the primary causative agent of IKC in sheep, goats and wild Caprinae. Domestic ruminants from high mountain habitats share grazing areas with wild mountain ungulates, such as chamois (*Rupicapra* spp.), Alpine ibex (*Capra ibex*) and European mouflon (*Ovis aries musimon*), and domestic sheep seem to act as *M. conjunctivae* reservoir. In this study, the presence of *M. conjunctivae* in domestic sheep and goats from the two main mountain ranges of Northern Spain, the Pyrenees and the Cantabrian Mountains, has been investigated.

Eye swabs were obtained from 439 domestic small ruminants selected from flocks that seasonally graze in alpine meadows during three consecutive years (2011-2012-2013). Seventy-nine out of the 378 domestic sheep (20.9%) tested positive to a *M. conjunctivae* specific real time-PCR (qPCR) in at least one eye, whereas all the 61 sampled domestic goats were negative. Statistically significant higher prevalence and higher proportion of infected flocks (P<0.001) was observed in the Pyrenees (25.7%; 12 flocks out of 13), where *M. conjunctivae* is widespread and probably endemic in domestic sheep, than in the Cantabrian Mountains (7.8%; one flock out of six). Twenty-five sheep (three from the Pyrenees and 22 from the Cantabrian Mountains) which showed clinical signs consistent with infectious keratoconjunctivitis (IKC) were negative by qPCR. In contrast, 62 out of the 71 (87.3%) *M. conjunctivae*-positive sheep from the Pyrenees and the eight positive sheep from the Cantabrian Mountains were asymptomatic.

This study provides qPCR-based evidences of *M. conjunctivae* maintenance in domestic sheep, as well as a relationship between prevalence in domestic sheep and previously reported *M. conjunctivae* and IKC in wild ruminants. Domestic goats do not seem to play an important role in the epidemiology of *M. conjunctivae* in alpine habitats from Northern Spain.

Introduction

Infectious keratoconjunctivitis (IKC) is a clinical condition affecting eyes of domestic and wild Caprinae worldwide. Several infectious agents such as *Mycoplasma conjunctivae*, *Chlamydophila psittaci* or *Moraxella ovis* (formerly *Branhamella ovis*) have been isolated from eyes of small ruminants affected by IKC (Giacometti et al., 2002a). However, *M. conjunctivae* is considered the primary causative agent of IKC in sheep, goats and wild Caprinae (Giacometti et al., 2002a; Hosie, 2007; Ter Laak et al., 1988b; Trotter et al., 1977). Susceptibility to *M. conjunctivae* infection differs among host species. While in sheep and goats IKC usually appears in form of transitory blindness causing little concern and economic consequences, pathogenicity to wild species is generally high though variable, causing outbreaks with morbidity and mortality up to 30% (Giacometti et al., 2002a).

The epidemiology of *M. conjunctivae* is particularly worth investigating in mountain habitats, where domestic ruminants share grazing areas with susceptible wild mountain ungulates during late spring (May-June) to early fall (September-October), such as chamois (*Rupicapra* spp.), Alpine ibex (*Capra ibex*) and European mouflon (*Ovis aries musimon*) (Giacometti et al., 2002a; Marco et al., 2009a). Interspecific transmission may occur (Belloy et al., 2003a), and domestic sheep seem to play a key role as a reservoir host for *M. conjunctivae* in such a complex scenario of host interaction (Giacometti et al., 2002b; Janovsky et al., 2001). Several outbreaks of IKC have been described in domestic sheep and goats worldwide (Baas et al., 1977; Barile et al., 1972; Naglić et al., 2000; Ter Laak et al., 1988b), but few active surveillance studies have been conducted in small domestic ruminants, particularly in high mountain habitats.

In this study, the presence of *M. conjunctivae* in domestic sheep and goats from the two main mountain ranges of Northern Spain, the Pyrenees and the Cantabrian Mountains, has been investigated. *M. conjunctivae* and IKC outbreaks have been reported in Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) in the Pyrenees (Arnal et al., 2013a; Marco et al., 2009a; Pañella et

al., 2010), but not in Cantabrian chamois (*Rupicapra pyrenaica parva*) from the Cantabrian mountains. Therefore, both study areas represent two different epidemiological scenarios.

Materials and methods

Conjunctival swabs were obtained from below the nictitating membrane from both eyes in 439 physically restrained small ruminants (19 flocks; 378 sheep, 61 goats) from the Catalan Pyrenees (Eastern and Central Pyrenees; 13 flocks; 276 sheep and 24 goats) and the southern side of the Cantabrian Mountains (6 flocks; 102 sheep and 37 goats). Flocks that graze in alpine meadows were selected according its potential contact with wild hosts susceptible to *M. conjunctivae* infection, namely Pyrenean chamois and European mouflon, in the National Game Reserves (NGR) of Freser-Setcases, Cadí, Cerdanya, and Alt Pallars in the Pyrenees, and Cantabrian chamois in the National Park of Picos de Europa and NGR of Mampodre and Riaño in the Cantabrian Mountains (Figure 4.1.1 and 4.1.2). Handling procedures were designed to reduce stress and health risks for subjects, according to European (86/609) and Spanish laws (R.D. 223/1988, R.D.1021/2005), and current guidelines for ethical use of animals in research (Kilkenny et al., 2010). A mean number of 20 sheep and goats were randomly sampled within each flock from November to May in three consecutive housing periods (2010-2011, 2011-2012 and 2012-2013) (Table 4.1.1). Two flocks in each region were sampled in at least two periods. All the goats were sampled in mixed goat and sheep flocks, except for 20 goats from a goat flock in the Cantabrian Mountains. Clinical signs compatible with IKC were recorded at sampling and swabs were stored at -20ºC until analyzed.

Figure 4.1.1 A) Domestic sheep flock that grazes in National Game Reserve in Alt Pallars during sampling in the winter shelter B) Sampling of domestic sheep flock in the Cantabrian Mountains. C) and D) are ones of the more severe IKC cases observed in domestic sheep from the Pyrenees during the study period, with epiphora, conjunctival hyperaemia, peripheral corneal oedema, and neovascularization.

During the study period, no outbreak of IKC was detected neither in domestic ruminants or wild mountain ungulates in the study area, though IKC cases in Pyrenean chamois are observed every year (**Study III**).

At the laboratory, swabs were thawed and mixed with 0.5 ml of lysis buffer (100 mM Tris–HCl, pH 8.5, 0.05% Tween 20, 0.24 mg/ml proteinase K) in microcentrifuge tubes and cells were lysed for 60 minutes at 60 °C and 15 minutes at 97 °C. The presence of *M. conjunctivae* was determined by a TaqMan real-time PCR (qPCR) with an exogenous external positive control in each reaction, as previously described (Vilei et al., 2007) (Annex II). For analysis of *Mycoplasma agalactiae* as a potential cause of IKC, the real time

PCR method based on the cytadhesin P40 (Fleury et al., 2002) was used as described (Oravcová et al., 2009) (Annex II).

Chi-square tests were performed in order to detect statistically significant differences in *M. conjunctivae* prevalence both at flock and individual level according to area and clinical status, using the PROC FREQ of the SAS[®] 9.1.3 System for Windows (SAS Institute, Cary, NC, USA).

Results

Mycoplasma conjunctivae was detected in at least one eye of 79 out of the 378 domestic sheep (20.9%) analyzed. Both individual and flock prevalence were significantly higher (p<0.001) in the Pyrenees (25.7%; 71 of 276 sheep; 12 positive flocks out of 13) than in the Cantabrian Mountains (7.8%; 8 of 102 sheep; one positive flock out of six) (Table 4.1.1 and Figure 4.1.2). Mean prevalence within infected sheep flocks was 29.9% (range 6.7% to 65%) in the Pyrenees and 32% in the only positive flock from the Cantabrian Mountains. The flocks sampled in two periods did not change their status regarding the presence of *M. conjunctivae* (one positive and one negative flock in each region). Moreover, the five sheep sampled twice in the *M. conjunctivae*-positive flock from the Pyrenees kept their status (two positive and three negative sheep) in both sampling periods which were separated by 12 months.

Table 4.1.1 Results of *Mycoplasma conjunctivae* prevalence assessed by qPCR as described in (Vilei et al., 2007).

Sampling period	2010-2011		2011-2012		2012-2013		Total		
	Prevalence	Flocks	Prevalence	Flocks	Prevalence	Flocks	Prevalence	CI 95 %	Flocks*
Goats									
Pyrenees	NS	NS	0.0 (0/10)	0/5	0.0 (0/14)	0/3	0.0 (0/24)	-	0/7
Cantabrian Mountains	0.0 (0/5)	0/1	0.0 (0/4)	0/1	0.0 (0/28)	0/2	0.0 (0/37)	-	0/2
Total	0.0 (0/5)	0/1	0.0 (0/14)	0/6	0.0 (0/42)	0/5	0.0 (0/61)	-	0/9
Sheep									
Pyrenees	NS	NS	26.1 (29/111)	6/7	25.4 (42/165)	7/8	25.7 (71/276)	20.5 - 30.9	12/13
Cantabrian Mountains	25.0 (3/12)	1/2†	0.0 (0/18)	0/2	6.9 (5/72)	1/4†	7.8 (8/102)	2.6 - 13.0	1/5
Total	25.0 (3/12)	1/2	22.5 (29/129)	6/9	19.8 (47/237)	8/12	20.9 (79/378)	16.8 - 25.0	13/18
Total	17.6 (3/17)	1/2	20.3 (29/143)	6/10	16.8 (47/279)	8/13	18.0 (79/439)	14.4 -21.6	13/19

NS: Not sampled.

* Total number of flocks may not coincide with the sum of all the periods because some of the flocks were re-sampled in more than one period.

† In the Cantabrian Mountains there was a single *M. conjunctivae*-positive flock, sampled in both periods (2010-2011 and 2012-2013).
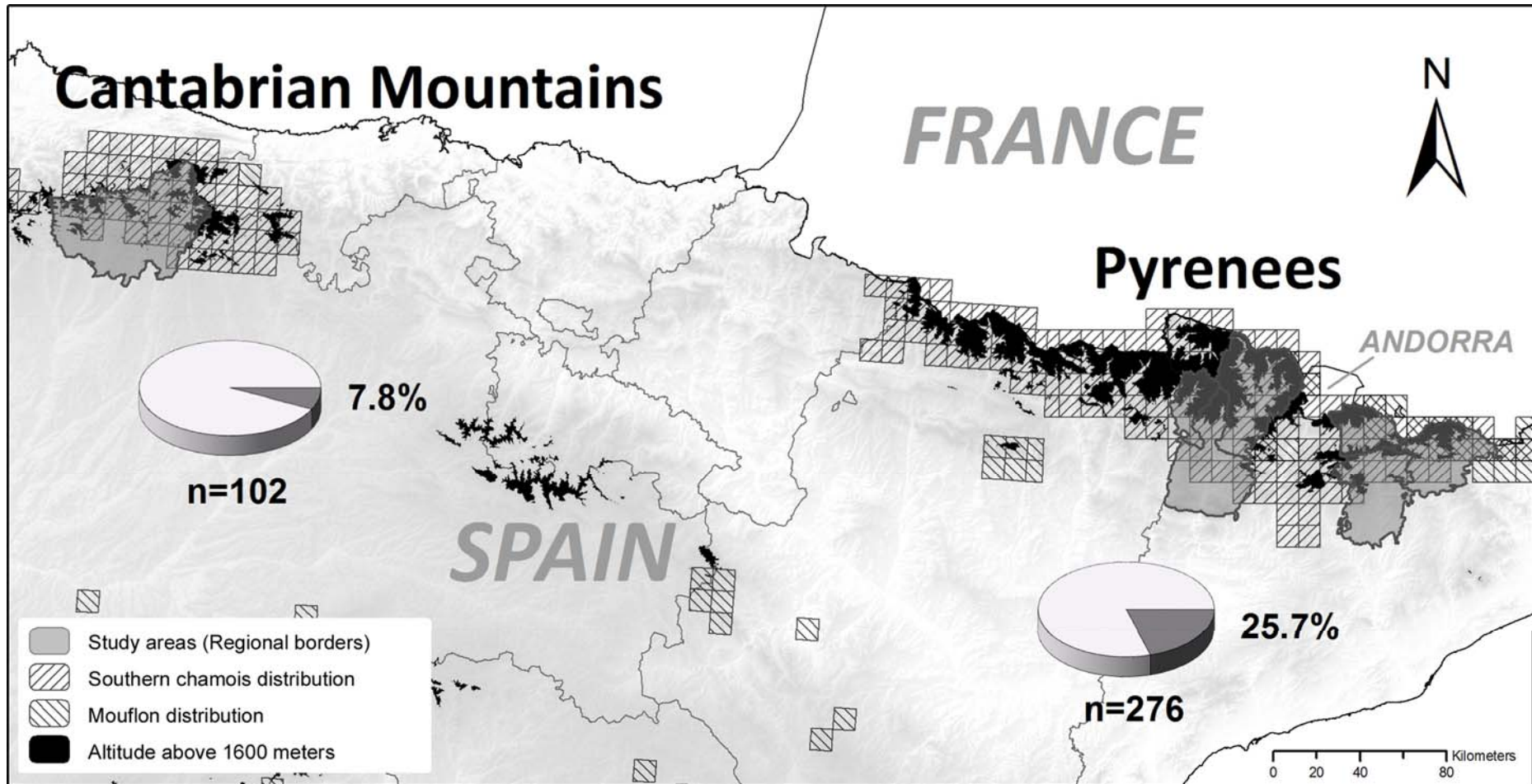
Figure 4.1.2 Pie charts showing the prevalence of *M. conjunctivae* in domestic sheep in the Cantabrian Mountains and the Pyrenees. Altitude above 1600 meters is shown in black. Borders for the regions where the sampled flocks graze in alpine meadows are shown in darker grey. Southern chamois and mouflon distribution squares correspond to a 10x10 Km grid.

Clinical signs consistent with IKC, such as ocular discharge, epyphora, mild conjunctivitis and/or corneal opacity were registered in 4.3% (12/276) of the examined domestic sheep from the Pyrenees and in 21.6% (22/102) in the Cantabrian Mountains (Figure 4.1.1). Most of the cases of IKC in sheep from the Pyrenees were associated with the presence of *M. conjunctivae* (75%), whereas none of the 22 sheep and three goats that showed clinical signs in Cantabrian Mountains had *M. conjunctivae* in their eyes. As *Mycoplasma agalactiae* has been reported to be a mycoplasmal cause of IKC in domestic ruminants (Rodríguez et al., 1996), samples of these sheep were also analyzed by real time PCR for the presence of this pathogen. This analysis did not detect *M. agalactiae* in the eye swabs of these animals. In contrast, 62 out of the 71 (87.3%) *M. conjunctivae*-positive sheep from the Pyrenees and the eight positive sheep from the Cantabrian Mountains were asymptomatic. In most flocks, IKC was unnoticed by the owners, as clinical cases were found to be occasional and mild. The clinical signs of the two sheep from the Pyrenees which were positive to *M. conjunctivae* in two samplings periods evolved from lachrymation and bilateral conjunctivitis in the first sampling to apparently healthy eyes in the second sampling in one sheep, and conversely for the other sheep.

M. conjunctivae was not detected in any of the goats sampled in both study areas (Table 4.1.1), although three goats from one flock in the Cantabrian Mountains showed mild clinical ocular signs.

Discussion

The high prevalence of *M. conjunctivae* in domestic sheep from the Pyrenees and its maintenance over sampling periods (Table 4.1.1) indicate that *M. conjunctivae* infection is widespread among domestic sheep in the Pyrenees, endemic and self-maintained within domestic sheep flocks. This agrees with previous data on *M. conjunctivae* prevalence (25.8%) in domestic small ruminants from Central Pyrenees (Arnal et al., 2013a) and the wide distribution of *M. conjunctivae* infection in domestic sheep throughout Europe (Hosie, 2007; Janovsky et al., 2001). In contrast, the lower prevalence found in the Cantabrian Mountains and the fact that *M. conjunctivae* was detected in only one flock in this region suggest that *M. conjunctivae* is currently less

common in this area. Moreover, the only *M. conjunctivae*-positive flock in the Cantabrian Mountains seasonally migrates to Caceres (South-Western Spain) in winter. Hence, the origin of *M. conjunctivae* in this flock is unclear and could lead to an overestimation of the endemic situation of *M. conjunctivae* in this region. Furthermore, the occurrence of *M. conjunctivae* relative to IKC cases in the two study areas strongly differed. Whereas there is a good agreement between IKC cases and the presence of *M. conjunctivae* in the Pyrenees, this is not the case in the Cantabrian Mountains.

The prevalence of *M. conjunctivae* among asymptomatic sheep was higher than previous data obtained by traditional culture methods (Akerstedt and Hofshagen, 2004). However, strain pathogenicity or individual host factors such as immunity may influence the outcome of clinical disease. Spontaneous clinical recoveries and relapses are a common feature of IKC in sheep (Hosie, 2007), which agrees with the clinical evolution of the two positive sheep from the Pyrenees sampled twice, further suggesting the role of sheep as a maintenance host of *M. conjunctivae* in this area, as previously suggested in Switzerland assessed by serology (Janovsky et al., 2001) and Central Pyrenees (Arnal et al., 2013a).

Several IKC outbreaks caused by *M. conjunctivae* have been reported in domestic goats (Baas et al., 1977; Barile et al., 1972; Ter Laak et al., 1988b), and IKC has been experimentally reproduced in goats by inoculating *M. conjunctivae* previously isolated from a goat with IKC (Trotter et al., 1977). However, the absence of *M. conjunctivae* occurrence in goats in this study, even in mixed flocks with high prevalence of *M. conjunctivae* in sheep, suggests a lower susceptibility to *M. conjunctivae* infection or a host specificity of the strains circulating in the area among domestic sheep. Therefore, domestic goats do not seem to contribute to *M. conjunctive* epidemiology in non-epidemic IKC in mountain habitats from Northern Spain. The finding of sheep and goats negative for *M. conjunctivae* and *M. agalactiae* but showing clinical signs consistent with IKC suggests a possible implication of other pathogens, such as *Chlamydophila psittaci*, *Moraxella ovis*, or *Listeria monocytogenes* previously detected in domestic small ruminants with IKC (Akerstedt and Hofshagen, 2004; Dagnall, 1994; Giacometti et al., 2002a). The higher occurrence of *M. conjunctivae*-negative sheep and goats with clinical signs of keratoconjunctivitis in the Cantabrian Mountains suggests that other pathogens may

be more relevant than *M. conjunctivae* for keratoconjunctivitis in this region. Although *M. conjunctivae* is considered the main etiological agent of IKC outbreaks in both domestic small ruminants and wild mountain ruminants (Giacometti et al., 2002a; Naglić et al., 2000; Trotter et al., 1977), pathogens or conditions associated with non-epidemic IKC warrant further research, as previously suggested in wild mountain ungulates (Mavrot et al., 2012a).

The widespread and consistent presence of *M. conjunctivae* in domestic sheep from the Pyrenees and the less relevant role of this pathogen in the Cantabrian Mountains seem to correspond to previous reports of IKC in wild sympatric susceptible hosts, such as European mouflon and Pyrenean chamois in the Pyrenees (Arnal et al., 2013a; Giacometti et al., 2002a; Marco et al., 2009a; Pañella et al., 2010), but not in Cantabrian chamois from the Cantabrian Mountains. Phylogenetical analyses of *M. conjunctivae* strains circulating in domestic sheep and wild mountain ruminants would help clarifying the specific role of different host species in the epidemiology of IKC from the studied areas, particularly in the Pyrenees.

This study provides qPCR-based evidence of *M. conjunctivae* maintenance in domestic sheep, as well as a relationship between prevalence in domestic sheep and previously reported *M. conjunctivae* and IKC in wild ruminants. This finding adds new relevant information into the epidemiology of *M. conjunctivae* in the domestic-wildlife interface. Domestic goats do not seem to play an important role in the epidemiology of *M. conjunctivae* in alpine habitats from Northern Spain.

4.2 Study II:

Infectious keratoconjunctivitis and occurrence of *Mycoplasma conjunctivae* and *Chlamydiaceae* in small domestic ruminants from the Central Karakoram, Pakistan

Under review

Abstract

Infectious keratoconjunctivitis (IKC) is a contagious eye disease primarily caused by *Mycoplasma conjunctivae* in domestic and wild Caprinae. *Chlamydophila* spp. have also been detected in ruminants with IKC. The objectives of this study are to investigate the ocular infection of *M. conjunctivae* and *Chlamydiaceae* and assess its interaction in relation to IKC in sheep and goats from remote communities around the Central Karakoram National Park in Pakistan, performing a combination of cross-sectional and case-control study design.

Mostly asymptomatic and endemic infections of *M. conjunctivae* and *Chlamydiaceae* were found in sheep (19.3% and 4.5%, respectively) and goats (9.5% and 1.9%, respectively) from all communities, assessed by qPCR. Prevalence significantly differed between species only for *M. conjunctivae* (p= 0.0184), which was also more prevalent in younger sheep (p<0.01). *Chlamydophila pecorum* was identified by sequencing and was related with IKC only when coinfection with *M. conjunctivae* occurred, which suggest a synergic interaction. Cluster analysis of *M. conjunctivae* strains revealed higher diversity of strains than expected, evidenced interspecific transmission and suggested a higher local livestock trade than previously assumed.

These results highlight the widespread occurrence of *M. conjunctivae* in sheep worldwide and its implications for wildlife should be assessed from a conservation perspective.

Introduction

Infectious keratoconjunctivitis (IKC) is a common contagious ocular disease among ruminants (Giacometti et al., 2002a). In small domestic ruminants, IKC is usually bilateral and produces ocular discharge, epyphora, mild conjunctivitis and/or corneal opacity, causing transitory blindness in most of the cases. However, IKC outbreaks may course with more severe clinical signs, including affection of the cornea leading to ulceration and perforation of the eye if no treatment is applied (Hosie, 2007).

Mycoplasma conjunctivae has been associated to most of the IKC outbreaks reported in small domestic ruminants and wild *Caprinae* worldwide, and is considered the primary pathogen of this condition (Giacometti et al., 2002a; Ter Laak et al., 1988a; Trotter et al., 1977). However, *M. conjunctivae* is also commonly detected in the eyes of asymptomatic sheep and is eventually endemic in sheep flocks throughout Europe (Akerstedt and Hofshagen, 2004; Fernández-Aguilar et al., 2013; Janovsky et al., 2001). *M. conjunctivae* infection is therefore not consistent with IKC in sheep, indicating that other factors may determine the development of clinical signs in non-epizootic conditions (Fernández-Aguilar et al., 2013). Other infectious agents such as *Moraxella* (*Branhamella*) *ovis*, *Chlamydophila* spp. and *Lysteria monocytogenes* have been isolated from the eyes of clinically affected sheep and may act opportunistically as secondary invaders and contribute to the onset of IKC (Akerstedt and Hofshagen, 2004; Dagnall, 1994b). However, *Chlamydophila* ocular infections have been occasionally associated with ocular disease in small domestic ruminants (Andrews et al., 1987; Gupta et al., 2015; Walker et al., 2015; Wilsmore et al., 1990) and wild ruminants (Meagher et al., 1992; Taylor et al., 1996), and therefore their potential role as primary pathogens for IKC has been discussed (Gupta et al., 2015; Walker et al., 2015). Although chlamydial infections are frequent in the eyes of diseased and asymptomatic sheep and goats (Osman et al., 2013; Polkinghorne et al., 2009), ocular co-infections with *M. conjunctivae* has not always been addressed in IKC control-case studies to properly assess a causal relationship. The finding of both infectious agents in clinical eyes described in some studies did neither provide a conclusive etiological information (Arnal et al., 2013a; Holzwarth et al., 2011; Lysnyansky et al., 2007).

In mountain ecosystems, sheep and goats share alpine pastures with other IKC-susceptible wild species such as chamois (*Rupicapra* spp.), Alpine ibex (*Capra ibex*)

or Himalayan tahr (*Hemitragus jemlahicus*) (Giacometti et al., 2002a; Ryser-Degiorgis et al., 2002). IKC outbreaks in wild free-ranging ruminants can cause significant mortality and demographic impact on affected herds (Arnal et al., 2013a; Loison et al., 1996). Although host specificity of certain *M. conjunctivae* strains or genotypes has been suggested (Zimmermann et al., 2008), interspecific transmission from domestic to wild ruminants can occur (Belloy et al., 2003a), and domestic sheep may play a key role as a *M. conjunctivae* reservoir host for wild ruminants (Fernández-Aguilar et al., 2013; Giacometti et al., 2002a; Janovsky et al., 2001). Therefore, asymptomatic or mildly symptomatic small domestic ruminants can be at the origin of IKC outbreaks in wild ruminants, particularly if the latter have not previously been in contact with *M. conjunctivae*.

Furthermore, IKC outbreaks in livestock can cause occasional economic losses for farmers, as well as a detrimental impact on animal welfare (Naglić et al., 2000). In developing countries, livestock production is an important economic income in rural areas. In Pakistan there are 29.1 million sheep and 66.6 million goats. In 2013-2014 livestock production represents 55.9% of the agriculture and 11.8% of the Gross Domestic Product (Anonymous, 2014).

The objectives of this study are to investigate the presence of *M. conjunctivae* and *Chlamydiaceae* in the eyes of sheep and goats from two isolated valleys in the buffer zone of the Central Karakoram National Park (CKNP) in Pakistan and to evaluate its health significance in relation to IKC. Factors affecting prevalence are explored and cluster analysis of the *M. conjunctivae* strains are also performed to establish epidemiological associations and evaluate the strains diversity in this remote area.

Materials and Methods

Study design and sample collection

This study was based on a combination of cross-sectional sampling strategy to estimate the apparent prevalences of *M. conjunctivae* and *Chlamydiaceae* and a case-control design to evaluate their influence on the clinical condition of IKC. From March 2013 to April 2014, eye swabs were collected from 334 small domestic ruminants (176 sheep and 158 goats) belonging to six communities at the boundaries of the CKNP (Figure 4.2.1). The minimum sample size required for pathogen

prevalence estimation was 196, calculated with the WinEpiscope 2.0 software (Thrusfield et al., 2001) with an expected prevalence of 15% (CI of 95%, 5% accepted error) (Fernández-Aguilar et al., 2013) in unknown total population, and was achieved depending on the availability for sampling. In particular, a team of veterinarians (including three of the Authors) and livestock assistants either operated at a congregation point where local sheep and goat owners had been invited via Muezzin's announcement, or freely moved across a village looking for owners' consent to sampling. All individuals in available flocks were sampled.
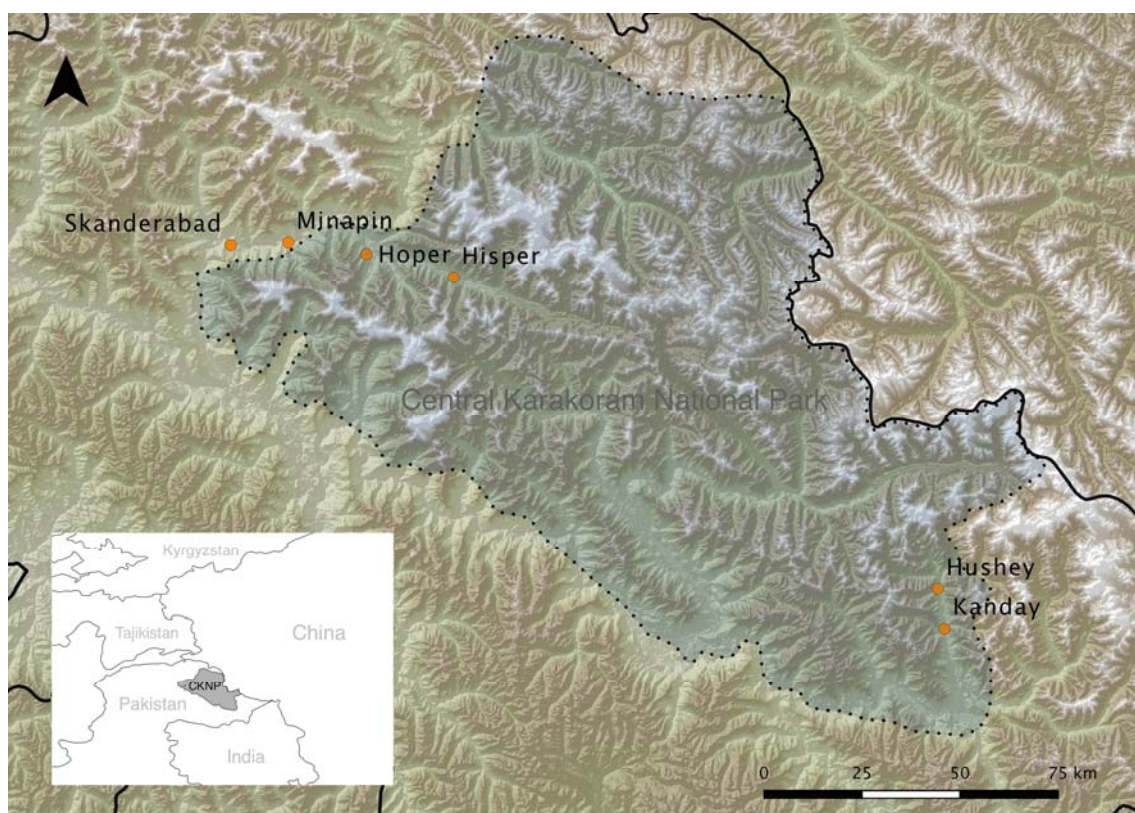
Figure 4.2.1 Map showing the location of the Central Karakoram National Park (CKNP), in Pakistan, and the six communities were the study was performed, located in two main valleys, Hunza Nagar Valley in the North-West and the Hushey Valley in the South-East.

Central Karakoram National Park is the largest protected area of Pakistan and one of the largest worldwide, covering over 10,000 km^2 in the Gilgit-Baltistan district (Figure 4.2.1). The sampled communities are located in two of the main valleys in the area, namely the Hunza Nagar Valley in the North-West (36º15'4.25'' N, 74º32'9.89''E), and the Hushey Valley in the South-East (35º27'51.64''N, 76º21'4.23''E), at

elevations ranging between 1,900 m and 3,500 m (Figure 4.2.1). Local climate is dry (rainfall <200 mm/year), with a relatively greater precipitation in winter and spring, arid continental climate in summer and sudden onsets of cold weather in early autumn. Winter snowfalls are not abundant but temperatures frequently reach -15°C.

Based on data provided by official veterinarians at the local Gilgit-Baltistan Livestock Department, there was a census of 7,173 sheep and 6,167 goats in the sampled communities at the beginning of the last decade. Typically, sheep and goats are kept in small (10-30 heads) mixed household flocks, which are seasonally merged into larger summer flocks of few hundred individuals. In the investigated area, small domestic ruminants share habitat with approximately 6,000 cattle (cows, yaks and hybrids) and a limited number of horses and donkeys. Two wild *Caprinae* species are also found, the relatively common Asian ibex (*Capra sibirica*), and the rare and localized Flare-horned Markhor (*Capra falconeri*). Seasonal contacts between wild and domestic *Caprinae* occurs mainly in summer, as witnessed to authors by members of the sampled communities. Sheep and goats in this study were sampled while kept in the surroundings of the investigated communities, before or just after the traditional summer transhumance to the high pastures.

Swabs were collected under the third eyelid from both eyes, transported refrigerated and stored frozen at -20ºC until analyzed. Ocular clinical signs were recorded at sampling considering the clinical condition of IKC whenever signs of ocular damage, inflammation or discharged occurred. Species, age, sex and body condition score (BCS) were also recorded (Russel et al., 1969). Age was determined in 141 of the 158 goats and in 166 of the 176 sheep by definitive incisor teeth eruption (Food and Agriculture Organization of the United Nations, 1994). All applicable institutional and/or national guidelines for the care and use of animals were followed.

Mycoplasma conjunctivae detection and LPPS sequencing

At the laboratory, eye swabs were placed into sterile tubes with 0.5 ml of lysis buffer (100 mM Tris–HCl, pH 8.5, 0.05 Tween 20, 0.24 mg/mL proteinase K). After mixing with a vortex, cells were lysed for 60 minutes at 60ºC and then heated to 97ºC for 15 minutes in order to inactivate Proteinase K.

The lysates obtained were tested for the presence of *M. conjunctivae* DNA with a TaqMan qPCR, using the primers LPPS-TM-L, LPPS-TM-R, and the probe LPPS-

TM-FT as described (Vilei et al., 2007) (Annex II). For cluster analysis a subtyping of the *M. conjunctivae* strains based on the *lppS* gene was attempted with a selection of 26 samples which showed the lowest Ct values at the TaqMan qPCR. DNA was amplified by nested PCR according to the method described (Belloy et al., 2003a) with minor modifications of the primers (Annex II). All PCR products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Rotkreuz, Switzerland) for subsequent DNA sequence analysis. DNA sequence determination was performed using the BigDye termination cycle sequencing kit (Applied Biosystems, Forster City, CA, USA) with the sequencing primers Ser_start2, Ser_start0 and Ser_end0 (Annex II). The sequences obtained were trimmed to contain the variable part of gene *lppS* and flanking regions corresponding to the nucleotides 3935-5035 of *lppS* of the type strain HRC/581 of *M. conjunctivae* (accession number AJ318939) (Annex II). Cluster relationships between strains were assessed by generation of phylogenetic trees, based on the sequence of using the MEGA 6 software with the following parameters: gap opening penalty 15; gap extension penalty 6.6, DNA weight matrix IUB, transition weight 0.5 including the corresponding DNA sequence data from *lppS* of HRC/581 for comparison.

Chlamydiaceae detection and identification

For the detection of *Chlamydiaceae* species in the eye swabs lysates, a SYBR green-based qPCR assay was performed using the primers Chuni-1F and Chuni-2R (Nordentoft et al., 2011). Each reaction consisted of 2,5 μl of DNA sample, 12,5 μl of SYBR®Green PCR Master Mix 2x (Applied Biosystems, Warrington, United Kingdom), 400 nM of each forward and reverse primer and nuclease-free water to a total volume of 25 μl. PCR was performed following reported cycling conditions (Nordentoft et al., 2011) (Annex II). Samples were assayed per duplicate and were assayed with an exogenous Internal Positive Control (IPC; Applied Biosystems, Foster City, CA, USA) to detect eventual PCR inhibitors.

The positive samples were analyzed further with a PCR that targets the Chlamydiales specific 298bp signature of the 16S rRNA gene using the primers 16SIGR and 16SIGF (Everett et al., 1999) (Annex II). Each reaction consisted of 2,5 μl of test sample, 25 μl of AmpliTaq Gold® 360 Master Mix (Applied Biosystems, Warrington, United Kingdom), 400 nM of each primer and nuclease-free water up to 50 μl.

Amplifications were performed starting with an initial denaturation at 95ºC for 10 min, 40 cycles that consisted in denaturation at 95ºC for 30 s, annealing at 51ºC for 30 s and extension at 72ºC for 30 s, followed with a final extension step at 72ºC for 7 min. All PCR reactions were run on an ABI 7500 instrument (Applied Biosystems Foster City, CA, USA). Purified amplicons (Minelute Gel Extraction Kit, Qiagen, Hilden, Germany) were sequenced for its identification with Big Dye Terminator v.3.1 Kit and the ABI 3130xl Genetic Analyzer (Applied Biosystems, Warrington, United Kingdom). The sequences obtained were introduced in the BLAST server from the National Centre for Biotechnology website (http://www.ncbi.nlm.nih.gov/blast/) to compare with the sequences available in GenBank.

Statistical analyses

A tree modeling approach was performed in order to identify factors that drive the *M. conjunctivae* and *Chlamydiaceae* infection. Conditional inference trees estimate relationship by binary recursive partitioning in which associations between variables are defined by p-values. It is a robust statistical tool capable to deal with variables of different nature and are suitable for complex epidemiological data (Friedman and Meulman, 2003; Hothorn et al., 2006). *M. conjunctivae* and *Chlamydiaceae* infection were considered as response variables (Bernoulli distribution) in two independent classification trees, whereas host (sex, age and BCS) and population variables (community and valley) were included as explanatory variables in each tree. The analyses were performed separately for sheep and goats. A conditional inference tree approach was also used to identify risk factors for IKC, including the occurrence of *M. conjunctivae* and *Chlamydiaceae* infection (i.e. discrete nominal variables with two categories positive/negative) and individual host factors (sex, age and BCS) as explanatory variables. These analyses were also performed separately for each ruminant species. Differences of Ct values of the *M. conjunctivae* qPCR between asymptomatic and clinical sheep and goats were assessed by Wilcoxon signed-rank test. Prevalences of *M. conjunctivae* and *Chlamydiaceae* were compared between species and communities using tests of proportions and setting statistical significance at 0.05. Statistical analyses were performed with R software (R Development Core Team 3.1.3, 2015), using the "party" package for the trees and the "EpiR" package to calculate the prevalence estimates (Stevenson et al., 2012).

Results

Mycoplasma conjunctivae had 14.7% prevalence (CI_{95} 11.3-18.9, 49/334) in the sampled domestic ruminants. Prevalence was significantly (p= 0.01842) higher in sheep (19.3%, CI_{95} 14.2-25.8, 34/176) than in goats (9.5%, CI_{95} 5.8-15.1, 15/158), both overall and for each sampling site (Table 4.2.1 and 4.2.2). *Chlamydiaceae* prevalence was lower than *M. conjunctivae*, both overall (3.3%, CI_{95} 1.8-5.8, 11/334) and for each species separately (sheep 4.5%, CI_{95} 2.3-8.7, 8/176 and goats 1.9%, CI_{95} 0.6-5.4, 3/158). Conversely to *M. conjunctivae*, the prevalence of *Chlamydiaceae* did not significantly differ between species. *M. conjunctivae* was detected in all investigated communities in sheep, and in four of them in goats, whereas *Chlamydiaceae* were detected in five communities in sheep and in three communities in goats (Table 4.2.1 and 4.2.2).

Table 4.2.1 Summary of samples (S), clinical signs (KC) and prevalence (percentage and number of positives) of *Mycoplasma conjunctivae* and *Chlamydiaceae* in the eyes of small domestic ruminants from the Central Karakoram National Park area, Pakistan. * Mean *M. conjunctivae* prevalence was significantly (p= 0.01842) higher in sheep than in goats both overall and for each sampling community.

Community	Goats				Sheep			
	S	KC	*M. conjunctivae*	*Chlamydiaceae*	S	KC	*M. conjunctivae*	*Chlamydiaceae*
Hisper	23	0	0.0% (0)*	0.0% (0)	9	0	11.1% (1)*	0.0% (0)
Hoper	22	1	22.7% (5)*	4.6% (1)	10	2	30.0% (3)*	10.0% (1)
Hushey	36	0	5.6% (2)*	2.8% (1)	43	5	18.6% (8)*	4.6% (2)
Kanday	61	1	11.5% (7)*	0.0% (0)	73	5	17.8% (13)*	4.1% (3)
Minapin	2	0	0.0% (0)*	0.0% (0)	15	0	26.7% (4)*	6.7% (1)
Skanderabad	14	0	7.1% (1)*	7.1% (1)	26	0	19.2% (5)*	3.9% (1)
Total	158	2	9.5% (15)*	1.9% (3)	176	12	19.3% (34)*	4.5% (8)

Table 4.2.2 Summary of samples analyzed (n), positives to qPCR (Pos.) and prevalence (%) of *Mycoplasma conjunctivae* and *Chlamydiaceae* in eye swabs of small domestic ruminants from the Central Karakoram National Park area, showed by ruminant species and by ocular clinical signs (KC).

		M. conjunctivae			*Chlamydiaceae*	
	n	Pos.	Prevalence (CI$_{95}$)	Pos.	Prevalence (CI$_{95}$)	
Sheep						
With KC	12	4	33.3% (13.8-60.9)	2*	16.7% (4.7-44.8)	
Without KC	164	30	18.3% (13.1-24.9)	6	3.7% (1.7-7.7)	
Total	176	34	19.3% (14.2-25.8)	8	4.5% (2.3-8.7)	
Goats						
With KC	2	1	50.0% (2.6-97.4)	0	0% (0-65.8)	
Without KC	156	14	9.0% (5.4-14.5)	3	1.9% (0.6-5.5)	
Total	158	15	9.5% (5.8-15.1)	3	1.9% (0.6-5.4)	
Total	334	49	14.7% (11.3-18.9)	11	3.3% (1.8-5.8)	

*These two sheep were also positive to *M. conjunctivae*.

Among the 34 *M. conjunctivae*-positive sheep, four (11.8%) had keratoconjunctivitis with ocular discharge, whereas the remaining thirty sheep (88.2 %) had no clinical signs at the time of sampling (Table 4.2.2). One of the fifteen *M. conjunctivae*-positive goats showed severe signs of keratoconjunctivitis (bilateral cornea perforation) associated with the ocular presence of *M. conjunctivae* (Figure 4.2.2), whereas the remaining fourteen (93.3%) *M. conjunctivae*-positive goats did not show any clinical signs. Median Ct values of the *M. conjunctivae* qPCR showed no statistical differences in sheep with IKC (median 30.2) and without (median 31.7), and in goats with IKC (26.3 in the only IKC case) and without (31.3). On the other hand, eight sheep (4.5%, 8/176) and one goat (0.6%, 1/158) with ocular clinical signs tested negative to the *M. conjunctivae* qPCR (Table 4.2.2). Three sheep of the eleven domestic small ruminants (eight sheep and three goats) positive to *Chlamydiaceae* were also positive to *M. conjunctivae*. Keratoconjunctivitis clinical signs in the *Chlamydiaceae*-positive small ruminants were observed only when co-infection with *M. conjunctivae* occurred, in two out of these three sheep.

Figure 4.2.2 Image of a goat exhibiting a severe stage of infectious keratoconjunctivitis, with bilateral panophtalmitis, lacrymation, hypopyon and perforation of the cornea. *Mycoplasma conjunctivae* was confirmed in eye swabs by qPCR.

Decision tree analyses indicated that *M. conjunctivae* prevalence was significantly (p < 0.01) higher in sheep younger than one year than in older sheep (Table 4.2.3). Differences by age category were not observed in goats and no other factors considered in the tree analysis were identified to influence significantly on *M. conjunctivae* prevalence. No age-related differences were observed in both hosts for *Chlamydiaceae*. On the other hand, no risk factors for IKC were identified among the explanatory variables considered in the tree, including *M. conjunctivae* and *Chlamydiaceae* occurrence in the eyes of sheep and goats.

Table 4.2.3 Results of *Mycoplasma conjunctivae* and *Chlamydiaceae* qPCR (prevalence %; positive/tested) showed by age in 141 goats and 166 sheep in which age was determined.

	Age category	
	0-1 years	**Older than 1 year**
M. conjunctivae		
Goats	16.3% (8/49)	3.3% (3/92)
Sheep	30.0% (24/80)*	10.5% (9/86)*
Total	24.8% (32/129)	6.7% (12/178)
Chlamydiaceae		
Goats	2.0% (1/49)	2.2% (2/92)
Sheep	5.0% (4/80)	4.7% (4/86)
Total	3.9% (5/129)	3.4% (6/178)

* Statistically significant differences ($p < 0.01$).

Eleven PCR amplified fragments of the *lppS* gene of *M. conjunctivae* were sequenced from seven goats and from four sheep taken from four different communities. Sequence analyses allowed to differentiate seven *M. conjunctivae* strains that were all phylogenetically related to *M. conjunctivae* strains from sheep (2820s) and Alpine chamois (*R. rupicapra*) (2778c, 2784c) that were isolated in the Alps and related to IKC outbreaks in chamois and sheep (Belloy et al., 2003a) (Figure 4.2.3). Among the strains sequenced, only one (P117) was associated with clinical keratoconjunctivitis in a sheep. In four goats (P119, P124, P122, P136) from Hoper community a common strain was identified. Furthermore, a common strain was found in a sheep (P98) and a goat (P16) from Minapin and Kanday communities, respectively (Figure 4.2.3). The EMBL/Gen Bank accession numbers for the *lppS* gene fragments of the different strains are also shown in Figure 4.2.3.
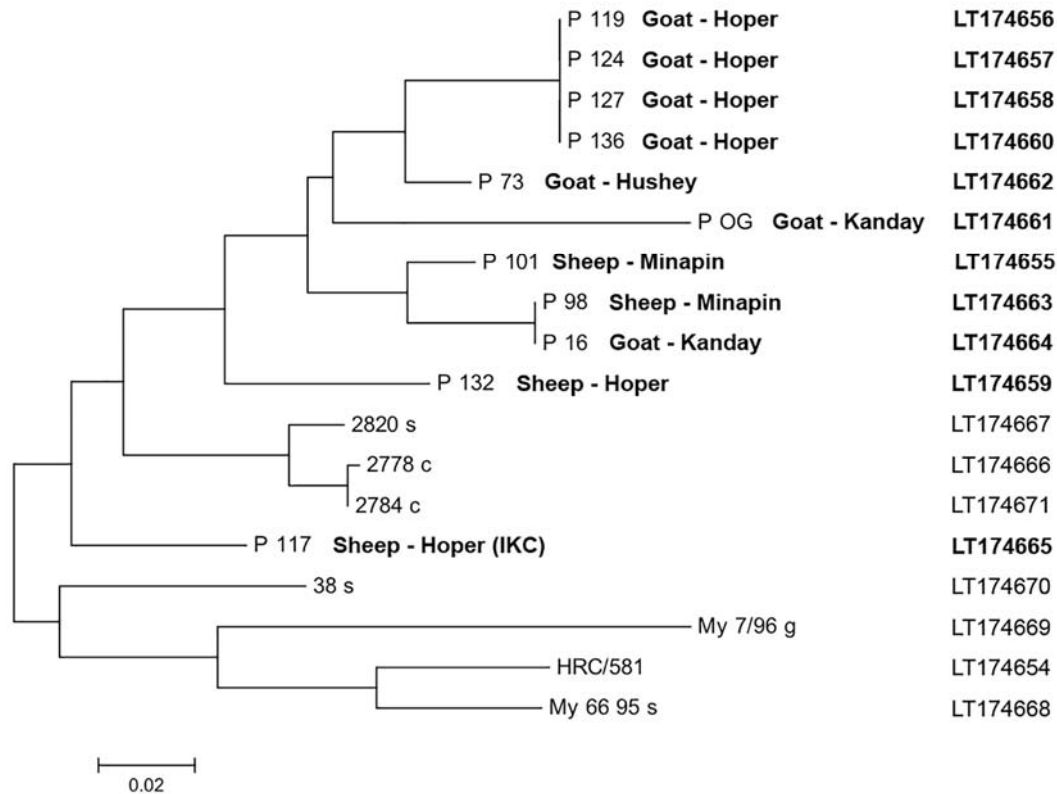
Figure 4.2.3 Phylogenetic representation of the *M. conjunctivae* strains sequenced from sheep and goats from the CKNP area (number starting with P) in comparison with the type strain of *M. conjunctivae* (HRC/581) and strains from sheep in the Eastern Swiss Alps (38 s and 2820 s), from chamois in the Austrian Alps (2778 c and 2784 c), from sheep in Croatia imported from Australia (My66 95) and from a goat imported to Croatia from the Southern French Alps (My 7/96). The species and the community are specified next to the strain reference. IKC indicates the presence of ocular clinical signs at sampling. The EMBL/Gen Bank accession numbers for the *lppS* gene fragments of the different strains identified in the study are indicated in bold, whereas the accession numbers for the reference strains are indicated in plain text.

Sequences of the *Chlamydiaceae* 16S rRNA were obtained from five *Chlamydiaceae*-positive samples (four sheep and one goat from two different communities) and all had the highest similarity (96-100) with *Chlamydophila* (*Cp.*) *pecorum* 16S ribosomal RNA complete sequence (accession number NR_121750.1).

Discussion

Mycoplasma conjunctivae and *Chlamydiaceae* were detected in asymptomatic and clinically affected eyes of sheep and goats from the Central Karakoram National Park, Gilgit-Baltistan district of Pakistan. These results agree with the previously reported sporadic IKC cases in sheep flocks with endemic *M. conjunctivae* infections and its capability to establish asymptomatic ocular infections (Fernández-Aguilar et al., 2013; Jones et al., 1976). Reports of non-epidemic IKC in goats are scarce (Surman, 1973) and most of the IKC descriptions refer to outbreaks (Baas et al., 1977; Ter Laak et al., 1988b). However, the results obtained in this study suggest that *M. conjunctivae* is endemic in mixed sheep-goat flocks of the CKNP area. Although *M. conjunctivae* has been previously reported in sheep from Pakistan (Shahzad et al., 2013) and several other locations worldwide (Barile et al., 1972; Lysnyansky et al., 2007; Motha et al., 2003; Van Halderen et al., 1994), its detection had not been reported in traditionally reared flocks from such remote and isolated communities. This finding confirms that *M. conjunctivae* is probably one of the most common and geographically widespread pathogens found in sheep.

Mycoplasma conjunctivae has been traditionally studied as the main etiological agent in IKC outbreaks characterized by high morbidity affecting all age classes and severe clinical signs (Motha et al., 2003; Naglić et al., 2000; Shahzad et al., 2013). Asymptomatic carriers may have lower *M. conjunctivae* loads (Mavrot et al., 2012a), which altogether with the fastidious nature of *M. conjunctivae* may have complicated its identification, resulting eventually in the underestimation of its frequency by traditional methods of isolation (Akerstedt and Hofshagen, 2004; Ter Laak et al., 1988b). Similar prevalence to those found in sheep and goats from the CKNP area have been reported in sheep from Northern Spain (25.7%-29.2%) when assessed by qPCR, with also a similar percentage of asymptomatic *M. conjunctivae*-positive sheep (87.3%) (Arnal et al., 2013a; Fernández-Aguilar et al., 2013). Since *M. conjunctivae*-infected sheep from Pakistan were mostly asymptomatic, no statistically significant relationship could be established between *M. conjunctivae* infection and IKC. In these endemic and mostly subclinical infections, the development of clinical signs may depend on other factors, such as host immunity, strain virulence or concurrent

infections (Akerstedt and Hofshagen, 2004; Fernández-Aguilar et al., 2013). Ocular clinical signs were however not related with the Ct values of the *M. conjunctivae* qPCR and several asymptomatic infections exhibited low Ct values (i.e. inverse to mycoplasmal loads), which suggests that a different host-mycoplasma interaction than the previously described in wild Caprinae occurred in sheep and goats (Mavrot et al., 2012a; Ryser-Degiorgis et al., 2009),

The higher prevalence of *M. conjunctivae* in sheep younger than one year as compared to the older ones indicates that acquired immunity may influence the course of the infection in sheep. Infectious keratoconjunctivitis typically causes more severe clinical signs in adult sheep than lambs (Hosie, 2007; Jones et al., 1976). However, *M. conjunctivae* persistence in the eyes do not necessarily have to be related with clinical signs as broadly described in experimental infections (Giacometti et al., 1998; Janovsky et al., 2001). Higher prevalence of *M. conjunctivae* in young animals have been also described in Alpine ibex (Mavrot et al., 2012a), which suggests that younger animals/lambs may be more important for the *M. conjunctivae* maintenance in the herd/population. Endemic and mostly asymptomatic *M. conjunctivae* infections had no apparent effect on BCS, which is probably influenced by other factors not assessed in this study. The lack of identifying risk factors for IKC may be because of the resulting limited ocular disease cases found in the present study.

The higher prevalence of *M. conjunctivae* observed in sheep as compared to goats corresponds to previous reports from other geographic areas (Fernández-Aguilar et al., 2013). This higher prevalence of *M. conjunctivae* in sheep may be related to host specificity of the prevalent local strains, or to a higher density, aggregation and intraspecific contact among sheep than among goats within mixed flocks. Overall, the results of this study and previous descriptions suggest that sheep are better hosts than goats for the endemic and mostly asymptomatic maintenance of *M. conjunctivae*. However, high pathogenic strains of *M. conjunctivae* were isolated during IKC outbreaks in both ruminant species (Baas et al., 1977; Naglić et al., 2000), and goats can also develop severe IKC in endemic infections as observed in this study (Figure 4.2.2).

Prevalence of *Chlamydiaceae* was lower than reported in previous studies in small domestic ruminants (Osman et al., 2013; Polkinghorne et al., 2009). The

differences could be in part because different sampling and diagnostic methodology were used. Several *Chlamydophila* species, such as *Cp. abortus*, *Cp. pecorum*, *Cp. psittaci* and *Cp. suis* have been identified in the eyes of livestock without correlation with ocular clinical signs (Gupta et al., 2015; Osman et al., 2013; Polkinghorne et al., 2009). On the other hand, *Cp. pecorum* has been occasionally associated with keratoconjunctivitis and polyarthritis in sheep and goats (Jelocnik et al., 2014) and IKC was successfully induced experimentally with *C. psittaci* infection (as taxonomically considered at that time) (Wilsmore et al., 1990). Chlamydial keratoconjunctivitis outbreaks have also been reported both in domestic and wild ruminants (Andrews et al., 1987; Meagher et al., 1992). Since *Chlamydiaceae* and particularly *Cp. pecorum* infection in sheep and goats from CKNP area were exclusively associated with clinical signs of IKC in case of coinfection with *M. conjunctivae*, it is probable that they acted synergically with *M. conjunctivae* as a secondary infectious agent for the development of clinical disease. However, the few number of coinfection cases found in this study does not allow inferring conclusions on virulence synergism with a statistical approach. The relative etiological importance of *Chlamydiaceae* may also rely on other factors, such as the strain pathogenicity (Jelocnik et al., 2014). Similar concurrent infections of *M. conjunctivae* and *Chlamydiaceae* have been reported in domestic sheep (Gupta et al., 2015; Lysnyansky et al., 2007) and free-ranging chamois (Arnal et al., 2013a; Holzwarth et al., 2011) in both diseased and asymptomatic individuals. According to these results, the interaction of *Chlamydophila spp.* with *M. conjunctivae* should be considered in etiological investigations of ocular disease in small domestic ruminants, although experimental evidence would probably be necessary to assess whether such coinfections determine the onset of IKC.

The diversity of the *M. conjunctivae* strains identified in the CKNP area suggests that *M. conjunctivae* has been present for a long time in Northern Pakistan. However, the relatively close relationship of the *M. conjunctivae* strains found in this study with strains described in sheep and wild *Caprinae* from the Alps (Central Europe) suggests that *M. conjunctivae* might have been introduced in this area along with its hosts, as it was described in Croatia in the

early 1990s (Belloy et al., 2003a). Furthermore, the molecular identification of the same strain infecting sheep and goats confirms interspecific transmission and the need to approach the epidemiological study of IKC considering all the susceptible hosts, taking into account the differences in host-pathogen interaction (Fernández-Aguilar et al., 2013). Cluster analyses also revealed that the same *M. conjunctivae* strain was present in the communities of Kanday and Minapin, separated by 190 Km straight-line distance and mountains ranging 5,500-8,000 meters of altitude (Figure 4.2.1). Livestock trade in Northern Pakistan is mainly on a local scale, and it can be assumed that trade movements between Kanday and Minapin are limited due to the great physical and cultural distance and the deriving lack of relationships. However, the molecular analyses of *M. conjunctivae* in sheep and goats mirror more complex trade habits and suggest a long endemic presence of *M. conjunctivae* in the small domestic ruminants of the CKNP area.

The introduction of *M. conjunctivae* asymptomatic carriers into naïve populations supposes a risk of severe IKC outbreaks in sheep flocks (Motha et al., 2001; Naglić et al., 2000). Similarly, interspecific transmission of *M. conjunctivae* from domestic to wild mountain ruminants may result into massive IKC outbreaks in wildlife as demonstrated in Alpine chamois (Belloy et al., 2003a). Therefore, domestic sheep and goat populations of the CKNP area, asymptomatically infected with *M. conjunctivae*, may represent a potential source of infection and IKC outbreaks in sympatric wild hosts, namely the Asian ibex and the Flare-horned Markhor. To the authors' knowledge, there is currently no robust information about IKC in wild ruminants from the CKNP. Syndromic surveillance of IKC in local flocks, however would improve rapid detection of any major risk of spill over of aggressive *M. conjunctivae* strains at the livestock-wildlife interface.

In conclusion, mostly asymptomatic and endemic infections of *M. conjunctivae* and *Chlamydiaceae* were found in sheep and goats populations of the CKNP area in the Gilgit-Baltistan district of Pakistan. *Chlamydiaceae* was associated with ocular clinical signs only when coinfection with *M. conjunctivae* occurred, but further studies are needed to better assess the effect of such coinfections in

eye disease. Cluster analysis of *M. conjunctivae* strains revealed higher diversity of strains than expected, evidenced interspecific transmission and suggested a higher local livestock trade than previously assumed. *M. conjunctivae* is probably one of the most common pathogens found in sheep worldwide and its implications should be assessed from a conservation perspective if sheep are allowed to share pastures with endangered or vulnerable ruminant species.

4.3 Study III:

Long-term dynamics of *Mycoplasma conjunctivae* at the livestock-wildlife interface in the Pyrenees

Under review

Abstract

Functional roles of domestic and wild host populations in infectious keratoconjunctivitis (IKC) epidemiology have been extensively discussed claiming a domestic reservoir for the more susceptible wild hosts, however, based on limited data. With the aim to better assess IKC epidemiology in complex host-pathogen alpine systems, the long-term infectious dynamics and molecular epidemiology of *Mycoplasma conjunctivae* was investigated in all host populations from six study areas in the Pyrenees and one in the Cantabrian Mountains (Northern Spain).

Detection of *M. conjunctivae* was performed by qPCR on 3600 eye swabs collected during seven years from hunted wild ungulates and sympatric domestic sheep (n=1800 animals), and cluster analyses of the strains were performed including previous reported local strains. *Mycoplasma conjunctivae* was consistently detected in three Pyrenean chamois (*Rupicapra p. pyrenaica*) populations, as well as in sheep flocks (17.0%) and occasionally in mouflon (*Ovis aries musimon*) from the Pyrenees (22.2% in one year); statistically associated with ocular clinical signs only in chamois. Chamois populations showed different infection dynamics with a low but steady prevalence (4.9%) and significant yearly fluctuations (0.0% – 40.0%). Persistence of specific *M. conjunctivae* strain clusters in wild host populations is demonstrated for six and nine years. Cross-species transmission between chamois and sheep and chamois and mouflon were also sporadically evidenced.

Overall, independent *M. conjunctivae* sylvatic and domestic cycles occurred at the wildlife-livestock interface in the alpine ecosystems from the Pyrenees with sheep and chamois as the key host species for each cycle, and mouflon as a spill-over host. Host population characteristics and *M. conjunctivae* strains resulted in different epidemiological scenarios in chamois, ranging from the fading out of the mycoplasma to the epidemic and endemic long-term persistence. These findings highlight the capacity of *M. conjunctivae* to establish diverse interactions and persist in host populations, also with different transmission conditions.

Introduction

Complex systems involving several hosts suppose a challenge for disease ecology studies (Fenton and Pedersen, 2005; Haydon et al., 2002). In the absence of genetics, patterns of incidence and pathogen prevalence are often used to assess the epidemiological roles of host populations, but they have a number of limitations that may infer wrong functional roles (Viana et al., 2014). Even with genetics and the evidence of cross-species transmission, low resolution of spatiotemporal data may not provide a comprehensive insight of the host-pathogen system (Serrano et al., 2011). Furthermore, the identification of reservoir host populations can be especially critical in pathogens that can establish diverse interactions with its hosts, resulting in different clinical outcomes and epidemiological scenarios (Citti and Blanchard, 2013).

An illustrative example are infections by *Mycoplasma conjunctivae* at the wildlife-livestock interface in alpine ecosystems, where there is no clear consensus of the functional roles of wild and domestic hosts (Giacometti et al., 2002a; Ryser-Degiorgis et al., 2009). *Mycoplasma conjunctivae* is the causative agent of infectious keratoconjunctivitis (IKC), which is a highly contagious ocular disease that severely affects Caprinae (Giacometti et al., 2002a). Historical records of IKC in wild mountain ungulates date back to the beginning of the XXth century in the European Alps (Gauthier, 1991). Since then, outbreaks of IKC have been documented in wild populations from almost all European mountain ranges (Arnal et al., 2013a; Marco et al., 2009a; Tschopp et al., 2005), and is considered one of the main diseases of mountain ungulates (Giacometti et al., 2002a). Infectious keratoconjunctivitis outbreaks are locally perceived as problematic because of the visual impressiveness of the disease and the economic impact in hunting revenues, which usually decrease due to hunting restrictions (Arnal et al., 2013a). Infectious keratoconjunctivitis has been described in several wild hosts, chamois (*Rupicapra* spp.), Alpine ibex (*Capra ibex*), Iberian ibex (*Capra pyrenaica*), mouflon (*Ovis aries musimon*) and bighorn sheep (*Ovis canadensis*) (Arnal et al., 2009; Giacometti et al., 2002a; Jansen et al., 2006). Among them, chamois are the most common and widespread species in Europe and have suffered frequent and severe IKC outbreaks (Arnal et al., 2013a; Degiorgis et al., 2000b; Gelormini et al., 2016;

Giacometti et al., 2002b; Tschopp et al., 2005). Infectious Keratoconjunctivitis is therefore considered one of the main diseases of chamois and can exert strong influence on their population dynamics (Arnal et al., 2013a; Loison et al., 1996; Serrano et al., 2015). Domestic Caprinae, i.e. sheep and goats, also undergo spontaneous IKC outbreaks, although in general the health impact is comparatively lower than in their wild counterparts (Study I and II) (Janovsky et al., 2001). Clinical signs of IKC are associated with ocular damage and inflammation, which causes visual impairment and blindness (Mayer et al., 1997). Disease is usually transient and spontaneous clinical recovery is a common course of the infection (Giacometti et al., 1998; Ter Laak et al., 1988a). However, clinical signs may progress to staphyloma and perforation of the cornea. Mortality in wild hosts, which can range locally from 5 to 27%, is therefore derived from blindness of the animals that either starve or die because of traumatic accidents (Degiorgis et al., 2000b; Loison et al., 1996).

In alpine ecosystems, several Caprinae species that are competent hosts for *M. conjunctivae* dwell around meadows (Nicolet and Freundt, 1975). Domestic caprines seasonally (May-November) graze free-range in mountain pastures and interactions among wild and domestic ruminants have been broadly documented (Ryser-Degiorgis et al., 2002). Yet the same *M. conjunctivae* strains were found in sheep and chamois suffering from IKC in the same area, indicating cross-transmission between these species (Belloy et al., 2003a). This finding suggested that epidemiological cycles involving wild and domestic hosts may occur in alpine ecosystems. However, the epidemiology of *M. conjunctivae* may vary considerably among host species. While *M. conjunctivae* mostly occurs endemically and at high prevalence in sheep flocks (Study I and II)(Janovsky et al., 2001), wild host populations suffer from IKC outbreak events (Degiorgis et al., 2000b; Grattarola et al., 1999; Tschopp et al., 2005), and *M. conjunctivae* have been reported not to persist in chamois populations from Eastern Switzerland (Giacometti et al., 2002b). The health significance of *M. conjunctivae* infection is also different and a higher proportion of asymptomatic infections occurs in domestic sheep as compared to wild hosts (Study I and II)(Mavrot et al., 2012a), which in turn suffer from more severe clinical signs (Arnal et al., 2013a; Mayer et al., 1997). Altogether, these differences suggest

domestic sheep as an ideal candidate for being a reservoir host to the more susceptible wild hosts (Giacometti et al., 2002a), and a key species for the *M. conjunctivae* maintenance in the alpine ecosystems. Long-term studies that simultaneously consider all competent hosts are needed to yield a comprehensive epidemiological perspective of *M. conjunctivae* cycles and the relative epidemiological roles.

In this study, an integrative approach consisting in the assessment of long-term infection dynamics and molecular subtyping of *M. conjunctivae* strains is performed in host-pathogen alpine systems within the Southern chamois (*R. pyrenaica*) distribution range, in order to elucidate the relative functional roles of the ungulate species in IKC epidemiology and specifically assess the *M. conjunctive* persistence in wild host populations. The clinical outcome of the *M. conjunctivae* infection is also evaluated in all the ungulate community.

Materials and methods

Study areas

This study was performed within the distribution area of Pyrenean chamois (*Rupicapra p. pyrenaica*) and Cantabrian chamois (*Rupicapra p. parva*) in the two main mountain ranges from Northern Spain, the Pyrenees and the Cantabrian Mountains, respectively. Within these ranges, seven different geographical units were considered for the study, six from the Eastern and Central Pyrenees (Catalonia, NE Spain): Freser-Setcases National Game Reserve (PyFS), Cadí National Game Reserve (PyC), Cerdanya-Alt Urgell National Game Reserve (PyCAU), Boumort National Game Reserve (PyB), Alt Pallars National Game Reserve (PyAP) and Vall Aran Game Reserve (PyVA); and one in the Eastern Cantabrian Mountains (León, N Spain), the Natural Protected Area of Picos de Europa (CmPE) (Figure 4.3.1). In each study area, ungulate populations are managed independently along with hunting plans.
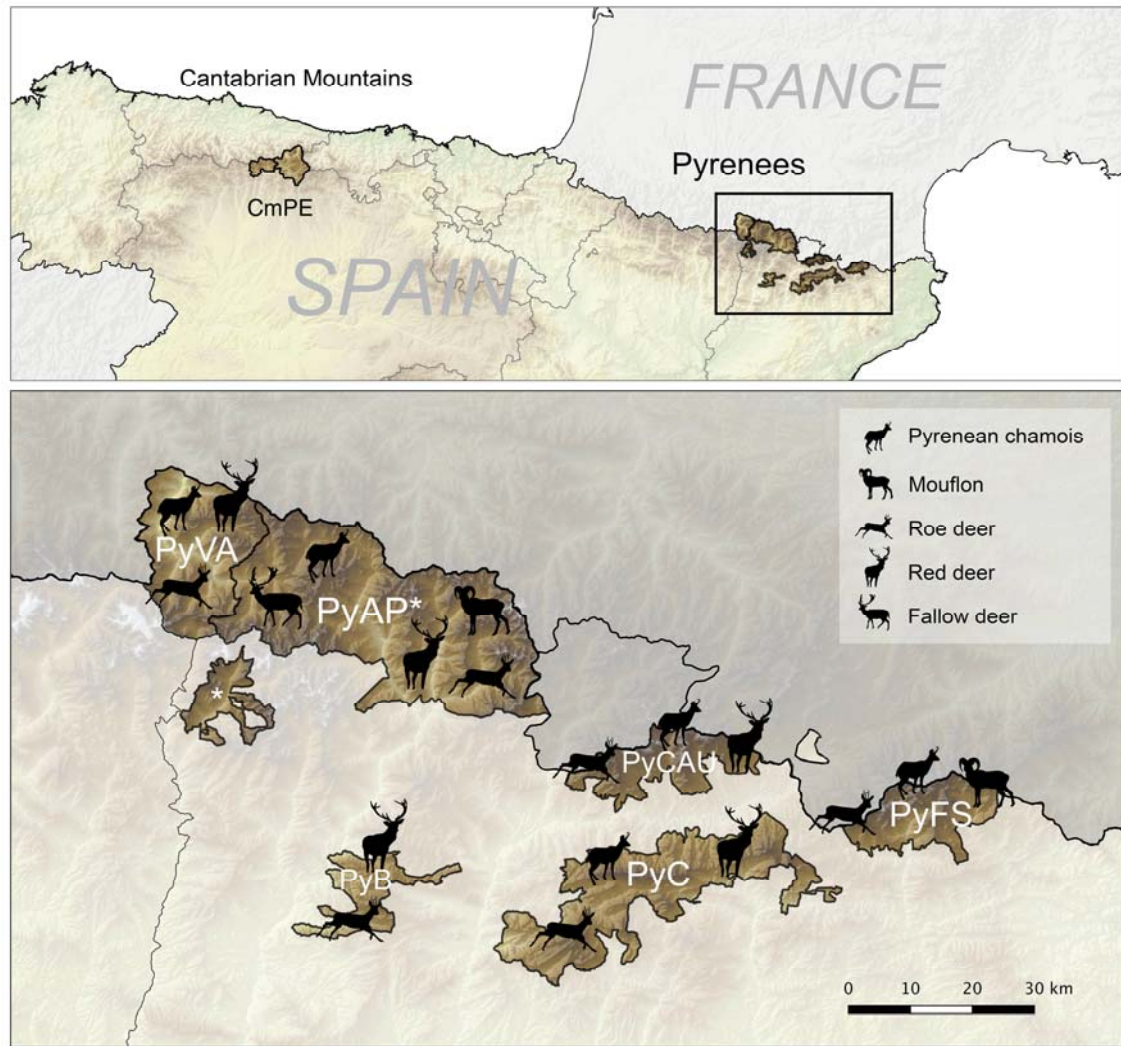
Figure 4.3.1 Maps of the study areas in Cantabrian Mountains (Picos de Europa - CmPE) and Eastern and Central Spanish Pyrenees. Ruminant species composition by study area from the Pyrenees is showed in detail in the image of the bottom: Vall Aran (PyVA), NGR Alt Pallars (PyAP), NGR Boumort (PyB), NGR Cerdanya-Alt Urgell (PyCAU), NGR Cadí (PyC) and NGR Freser-Setcases (PyFS).

These areas are high mountain habitats mostly composed by alpine or subalpine ecosystems with strong seasonal influence, with the exception of PyB which have a dryer climate with a higher Mediterranean and continental influence. Altitude ranges approximately from 800 in the bottom of the valleys to 3100 meters high in the Pyrenees and from 1100 meters to 2600 meters high in the Cantabrian Mountains. Chamois is the most abundant ungulate species in most of the study areas except in PyB, where red deer (*Cervus elaphus*) is the predominant wild ruminant (Figure 4.3.1). Chamois population size is estimated

yearly with linear transects performed by the rangers and is calculated to be about 7,500 in the six study areas from the Pyrenees and 3,800 in the study area from the Cantabrian Mountains. The chamois density varies among study areas as a result of differential pestivirus die-offs in the Pyrenees (Fernández-Sirera et al., 2012; Marco et al., 2009b). Mean minimum chamois abundances per square Km during the study period, calculated as estimated chamois population/area, were: PyFS, 14.7; PyC, 3.3; PyCAU, 2.7; PyAP, 1.8; PyVA, 1.8; CmPE 4.0. Other wild ungulates that cohabit with chamois include roe deer (*Capreolus capreolus*), red deer, mouflon, fallow deer (*Dama dama*) and wild boar (*Sus scrofa*), but with a different ruminant community composition among study areas from the Pyrenees, shown in Figure 4.3.1. Wild boars are present in all study areas and fallow deer and mouflon are not present in Cantabrian Mountains. Domestic ruminants (i.e. cattle, sheep, and goats) and domestic horses also share these habitats with the wild species during the grazing period (May-November) in all of the study areas.

Infectious keratoconjunctivitis was first described in Pyrenean chamois from the Central and Eastern Spanish Pyrenees in mid XX century (Sánchez-Belda and Martínez-Ferrando, 1985). As reported in sheep with the introduction of infected animals in naïve herds (Naglić et al., 2000), massive IKC outbreaks occurred in Pyrenean chamois spreading throughout all the study areas since 1981 in successive events (Gauthier, 1991; Marco et al., 1991). Conversely, no similar IKC outbreaks but only sporadic ocular clinical signs were described in 1979 in the Cantabrian chamois (Sánchez-Belda and Martínez-Ferrando, 1985).

Sampling method

A long-term cross-sectional sampling design was performed on alpine wild ungulates hunted during the regular hunting seasons from 2009 to 2015 in the Pyrenees (n=1556) and from 2010 to 2013 in the Cantabrian Mountains (n=132). Samples were collected from recently hunted ungulates in proportion to the total number of animals hunted in each study area (Table 4.3.1). Four sheep flocks from the Pyrenees that graze in the alpine meadows of three of the study areas (PyVA, PyAP and PyFS) were also sampled in 2014 (n=112; Table 4.3.1).
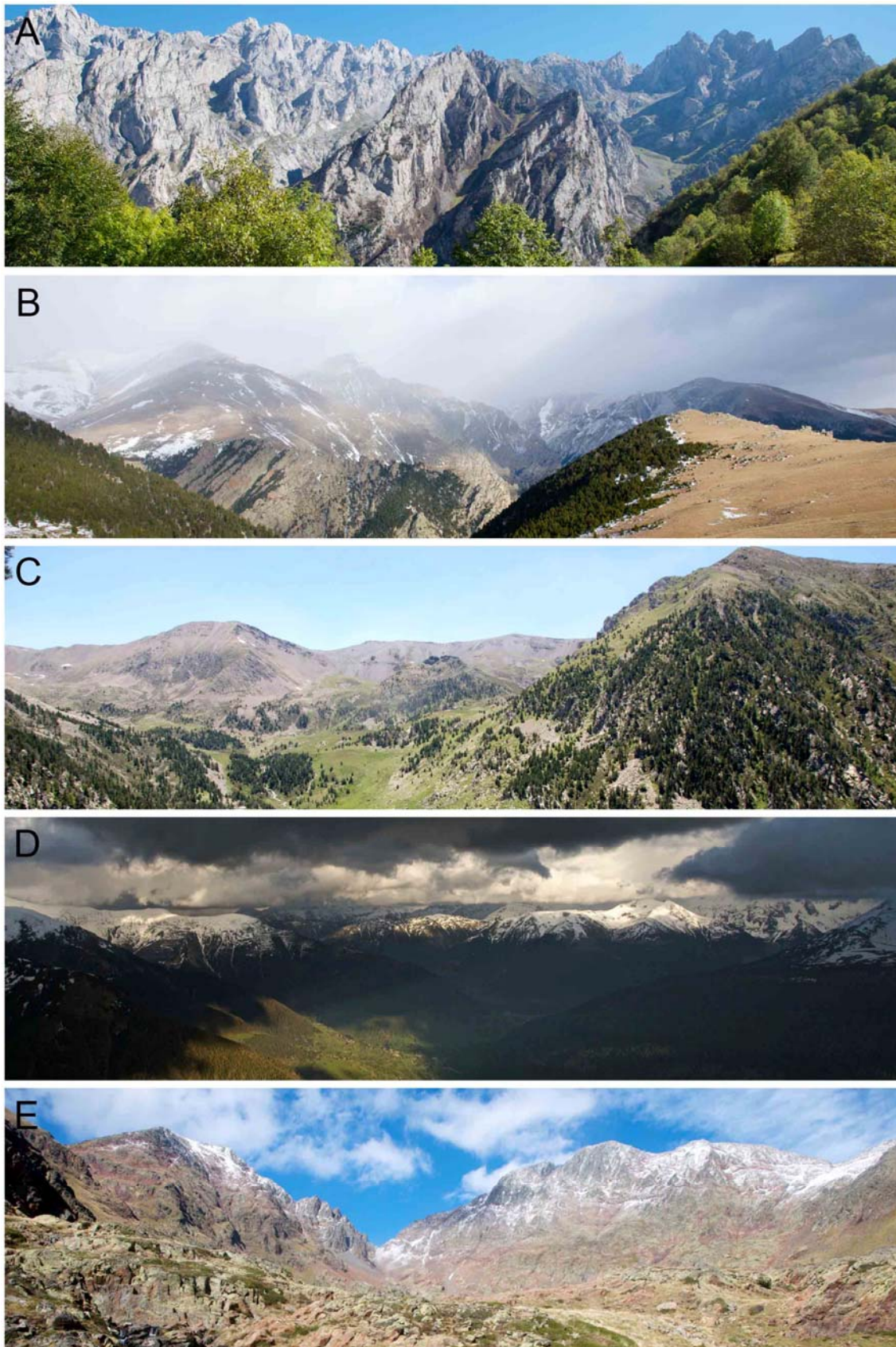
Figure 4.3.2 Images of study areas included in this work. A) Picos de Europa; B) NGR Freser-Setcases; C) NGR Cerdanya-Alt Urgell; D) Vall Aran; E) NGR Alt Pallars.

Table 4.3.1 Distribution of animals sampled by study areas and species.

Study Area	Chamois	Mouflon	Red deer	Roe deer	Fallow deer	Wild boar	Sheep	TOTAL
Vall Aran (PyVA)	125	-	26	25	-	3	30	209
NGR Alt Pallars (PyAP)	122	7	7	22	14	3	59*	234
NGR Boumort (PyB)	-	-	34	-	-	8	-	42
NGR Cerdanya-Alt Urgell (PyCAU)	24	-	3	10	-	2	-	39
NGR Cadí (PyC)	343	-	40	17	-	11	-	411
NGR Freser-Setcases (PyFS)	592	80	-	33	-	1	23	729
Picos de Europa (CmPE)	88	-	25	18	-	1	-	132
Total	1294	87	135	125	14	33	112	1800

*Two sheep flocks.

Samples were taken between the third eyelid and the palpebral conjunctiva with sterile cotton swabs without medium from each eye separately and frozen at -20°C within 24 hours from collection. Basic information of the individuals was also registered, including ocular signs, sex, age based on the annual horn segments for chamois and mouflon (Corlatti et al., 2015b), date and location. Geographic coordinates were also recorded in PyFS from 2012 to 2015. Age was classified in four categories in chamois according to social behaviour and aging process, kids (<1 year), yearling (1-2 years), juvenile (2-3 years) and adults (>3 years).

This study accomplish with current guidelines for ethical use of animals in research following the European (2010/63/EU) and Spanish (R.D. 53/2013) legislations. The approval of an ethic committee was not needed since management and sacrifice of animals were not performed for research purposes. Ungulate wild species studied are not endangered, and its abundant populations are managed along hunting plans, regulated by the competent public administrations. Samples were obtained by the rangers from hunted-harvested wild animals during the regular hunting plans from National Game Reserves and Hunting Reserves that belong to public administrations. Both samplings of wild animals and domestic livestock were performed in the frame of health surveillance programs approved by the Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural - Generalitat de Catalunya (DARPAMN, the Regional authority in charge of livestock and wildlife management).

Detection of Mycoplasma conjunctivae

Eye swabs were thawed, cut and mixed during one minute with 0.5 lysis buffer (100 mM Tris–HCl, pH 8.5, 0.05% Tween 20, 0.24 mg/mL proteinase K) in sterile tubes. The lysates of the cells were obtained by incubating the tubes at 60ºC for 60 minutes. Proteinase K was then inactivated at 97ºC for 15 minutes (Vilei et al., 2007). The resulting lysates were directly used as test samples for the molecular detection of *M. conjunctivae*.

The presence of *M. conjunctivae* DNA in the samples was assessed with a previously described TaqMan real time PCR (qPCR) using primers LPPS-TM-L, LPPS-TM-R, and probe LPPS-TM-FT (Vilei et al., 2007) (Annex II). Briefly, 2.5 μL of the sample lysates, 900nM of each forward and reverse primer, 300 nM of the probe, 12.5 μL TaqMan®2x Universal PCR MasterMix (Applied Biosystems, Warrington, UK) and an exogenous internal positive control (IPC; Applied Biosystems, Warrington, UK) were introduced in each reaction well and nuclease-free water up to a total volume of 25 μL. Cycling conditions were set for 40 cycles at 95°C for 15 s and 60°C for one min, with pre-cycling steps of 50 °C for 2 min and 95 °C for 10 min. The threshold cycle (Ct) of each sample was defined as the number of cycle at which the fluorescent signal of the reaction crossed the threshold that was set to 0.05.

Samples were analyzed per duplicate and were considered valid only if difference between the replicates was less than one Ct. All PCR reactions were run on Applied Biosystems® 7500 Fast Real-time PCR system (Applied Biosystems, Warrington, UK).

Mycoplasma conjunctivae subtyping and cluster analyses

The *lppS* gene of *M. conjunctivae* encodes for a membrane lipoprotein that is involved in adhesion (Belloy et al., 2003b), which variable domain can be used for *M. conjunctivae* subtyping and to perform molecular epidemiology analyses (Belloy et al., 2003a). For cluster analyses, samples from this study with Ct values lower than 33 at the qPCR and available sheep samples from a previous study carried out in the same study areas and in the same period were included (Study I). To obtain *lppS* gene sequences of these samples, a nested PCR was first performed as described with minor modifications of the primers (Belloy et al., 2003a; Mavrot et al., 2012a) (S1 Table). PCR products were then purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Rotkreuz, Switzerland). The sequences were determined with the sequencing primers Ser_start2, Ser_start0 and Ser_end0 (S1 table) using the BigDye termination cycle sequencing kit (Applied Biosystems, Forster City, CA, USA). The resulting sequences were trimmed to contain the region that comprises the nucleotide

positions 3935-5035 of the *lppS* gene from *M. conjunctivae* type strain HRC/581 (GenBank acc. number AJ318939), which corresponds to the variable *lppS* domain and flanking regions. Alignment and edition of the sequences were performed with the BioEdit software. A phylogenetic analysis of the sequences was then performed by the generation of cluster analyses trees built by the UPGMA statistical method and performing 1000 bootstrap replications (Sneath and Sokal, 1973). The generation of the phylogenetic tree was performed using MEGA software (Kumar et al., 2016).

For the tree construction, sequences of *M. conjunctivae* strains described in previous works from the same study areas were included for comparison (three chamois from PyAP and one mouflon from PyFS) (Marco et al., 2009a), covering a temporal period from 2006 to 2015 (S2 Table). Sequences from other areas (n=6) and the sequence of the type strain HRC/581 were also included in the tree (S2 Table).

Data management and statistical analyses

Each individual was considered "infected" if the qPCR was positive in one or both eye swabs. When appropriate, database was organized as recommended for proportion data (Crawley, 2013). *Mycoplasma conjunctivae* apparent prevalence was analyzed to assess 1) the relation between ocular clinical signs and the presence of *M. conjunctivae* and 2) the trend of *M. conjunctivae* infection probability during the study period in each study area. For the first analyses, a two-sided Chi-squared test for independence was performed. In the second analysis, generalized additive models (GAMs) were fitted with *M. conjunctivae* infection as response variable with a binomial distribution and the interaction of year with study area as predictor variables (Wood, 2006). GAMs can be used to model trends as a nonlinear function of time and provide a framework for testing statistical significance of changes in the response variable frequencies (Fewster et al., 2000). Known risk factors for *M. conjunctivae* infection, such as sex and age category (Giacometti et al., 2002a), were previously tested with Fisher's exact tests to be equally represented in all the years for each study area. The absence of residual patterns and other general

assumptions were confirmed to validate the model once it was fitted (Zuur et al., 2007). Statistical significance was set at p<0.05 for all the tests. The interval confidence of apparent prevalences were calculated with the "EpiR" package, the graphics were performed with the "ggplot2" package and the GAMs were implemented in the "mgcv" statistical package, all from the R statistical software (R Development Core Team 3.1.3, 2015). The spatial data representation and mapping was made with the software QGIS 2.14 Essen (QGIS Development Team, 2016).

Results

Prevalence and dynamics of Mycoplasma conjunctivae infections

Mycoplasma conjunctivae was detected in eye swabs from Pyrenean chamois in three of the study areas (PyFS, PyAP and PyVA). No *M. conjunctivae* was detected in Cantabrian chamois (CmPE), as well as in Pyrenean chamois from the rest of study areas from Pyrenees (PyCAU and PyC) (Table 4.3.2). Prevalence in Pyrenean chamois population ranged from 2.0% (95% CI 0.1-10.7) to 40.0% (95% CI 19.8-64.2) in years when it was detected (Table 4.3.2). The GAM model showed significant differences of *M. conjunctivae* infection by year in PyVA (p=0.025) and PyAP (p<0.001), but not in PyFS (8.12 edf; adjusted R^2 of 70.4 %). Thus, whereas infection indices followed a rather constant trend in PyFS, it changed throughout the study period both in PyAP and more pronouncedly in PyVA, where prevalence peaked in 2013 (Figure 4.3.3). No specific *M. conjunctivae* distribution pattern was found in Pyrenean chamois from PyFS during 2012-2015 as those described in IKC outbreaks (Degiorgis et al., 2000b; Gelormini et al., 2016), and infection cases were found distributed in all seasons and months and in throughout the reserve (Figure 4.3.4). Infection cases detected in PyAP and PyVA were localized in some geographic units within the study areas.

Table 4.3.2 Temporal trends of *M. conjunctivae* prevalence in Pyrenean chamois from three study areas where it was detected.

	2009	2010	2011	2012	2013	2014	2015	Total
Vall Aran (PyVA)								
Prev % (Pos/Tot)	0 (0/10)	0 (0/12)	2.0 (1/49)	0 (0/28)	40.0 (6/15)	0 (0/10)	0 (0/1)	5.6 (7/125)
CI 95%	0.0 – 27.8	0.0 – 24.2	0.1 – 10.7	0.0 – 12.1	19.8 – 64.2	0.0 – 27.7	0.0 – 94.9	2.7 – 11.1
NGR Alt Pallars (PyAP)								
Prev % (Pos/Tot)	20.0 (1/5)	2.5 (1/40)	0 (0/14)	0 (0/6)	0 (0/21)	0 (0/22)	14.3 (2/14)	3.3 (4/122)
CI 95%	1.1 – 62.4	0.1 – 12.9	0.0 - 21.5	0.0 - 39.0	0.0 - 15.5	0.0 – 14.9	4.1 - 39.9	1.3 – 8.1
NGR Freser-Setcases (PyFS)								
Prev % (Pos/Tot)	3.1 (1/32)	0 (0/8)	0 (0/87)	7.6 (8/105)	5.0 (5/100)	7.3 (10/137)	4.1 (5/123)	4.9 (29/592)
CI 95%	1.8 – 15.8	0.0 – 32.4	0.0 – 4.2	3.9 – 14.3	2.1 – 11.2	4.0 – 12.9	1.7 – 9.2	3.4 – 6.9

Prev, prevalence; Pos, positive; Neg, negative; Tot, Total animals analyzed; CI, confidence interval.
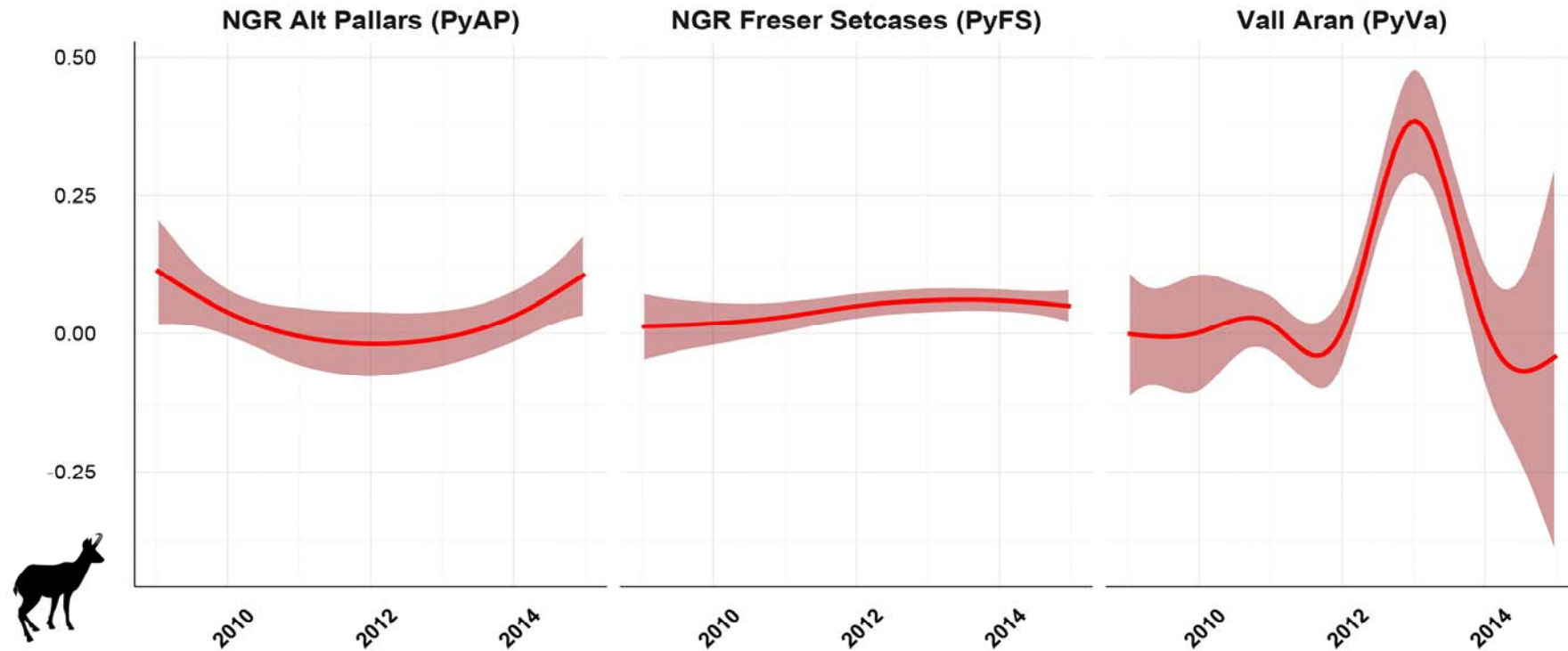
Figure 4.3.3 Modeled trend curves of *Mycoplasma conjunctivae* infection in Pyrenean chamois by generalized additive models. Infection indices show evident curve differences by study area, in which the interaction of year with the study area resulted statistically significant in NGR Alt Pallars and Vall Aran, but not in NGR Freser-Setcases.
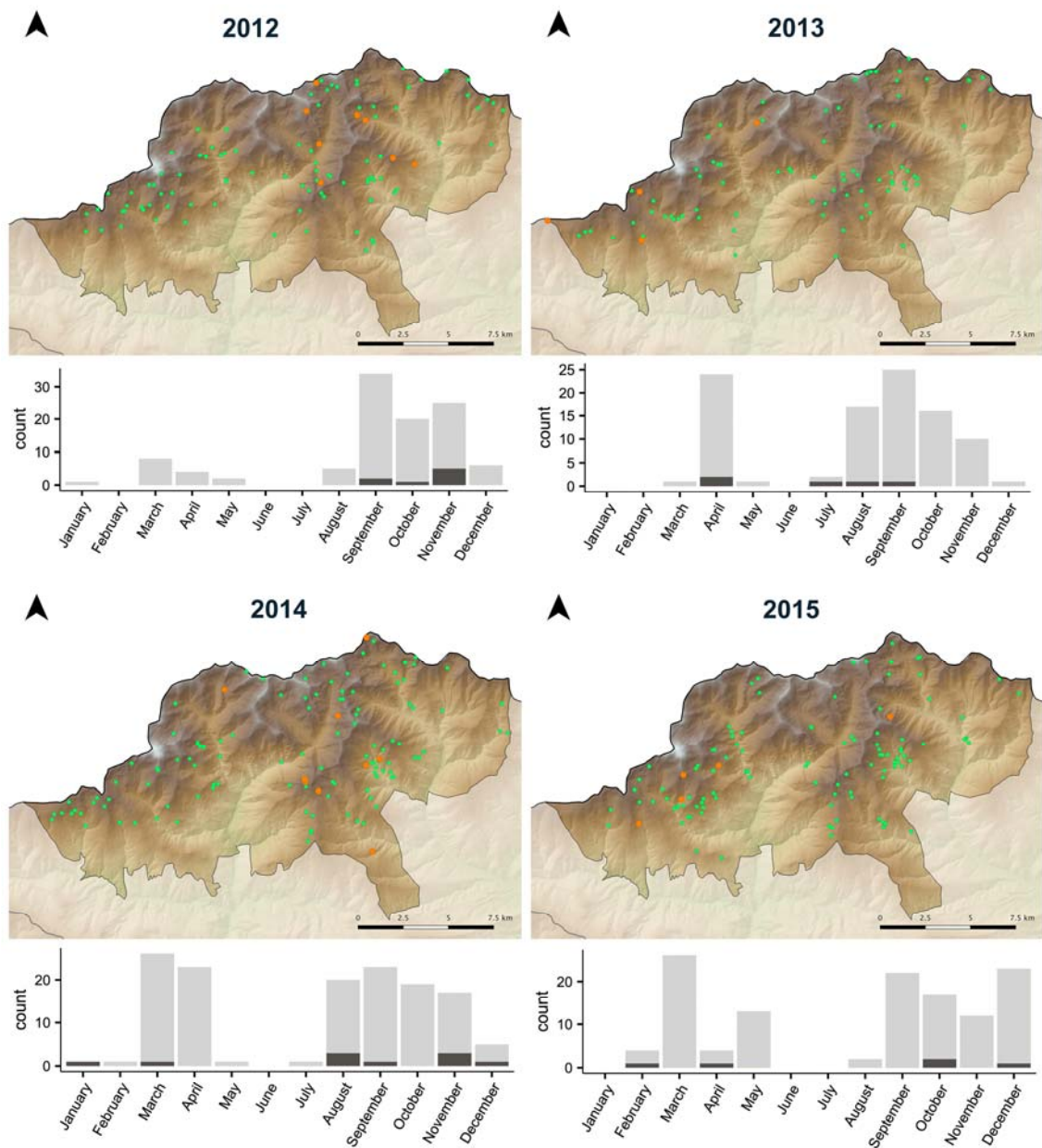
Figure 4.3.4 Spatio-temporal distribution of *Mycoplasma conjunctivae* in Pyrenean chamois from NGR Freser-Setcases (PyFS). Orange dots are *M. conjunctivae* qPCR-positive chamois and green dots are qPCR-negative chamois. The bar graph at the bottom of each map shows the number of qPCR-positive chamois (dark grey) in total sampled chamois by month that year.

Infection of *M. conjunctivae* in mouflon was detected only in 2014 in PyFS with a prevalence of 22.2% (95% CI 6.3-54.7). *Mycoplasma conjunctivae* was confirmed in three out of four sheep flocks sampled with an overall prevalence of 17.0% (95% CI 11.1-25.0%) and a within-flock prevalence that ranged from 6.9% (95% CI 1.9-22.0%) to 33.3% (95% CI 19.2-51.2%).

*C*luster analyses of *Mycoplasma conjunctivae* strains

A total of 81 *M. conjunctivae* qPCR-positive samples were sequenced from 65 ruminants, including sheep (n=48; 14 flocks that graze in PyFS, PyAP, PyVA and CmPE), chamois (n=16; PyFS, PyAP and PyVA) and mouflon (n=1; PyFS) (S2 Table). In order to simplify the cluster analyses tree, only *M. conjunctivae* strains that were different between both eyes of the same individual were considered for the tree construction (n=67). The tree revealed several clusters of *M. conjunctivae* strains, following a host species and geographic pattern (Figure 4.3.5). Two main clusters were identified in wild host populations, one cluster formed by chamois and mouflon strains detected during a nine-year period in PyFS, and another cluster with chamois strains detected during a six-year period in PyAP and PyVA altogether. Sheep showed higher strain diversity than chamois but also clustering among flocks and study areas. Main strain clusters of wild hosts and domestic livestock were neither similar nor divergent by study area. However, similar strains infecting mouflon and chamois from PyFS and sheep and chamois from PyAP and PyFS, respectively, were found (Figure 4.3.5).
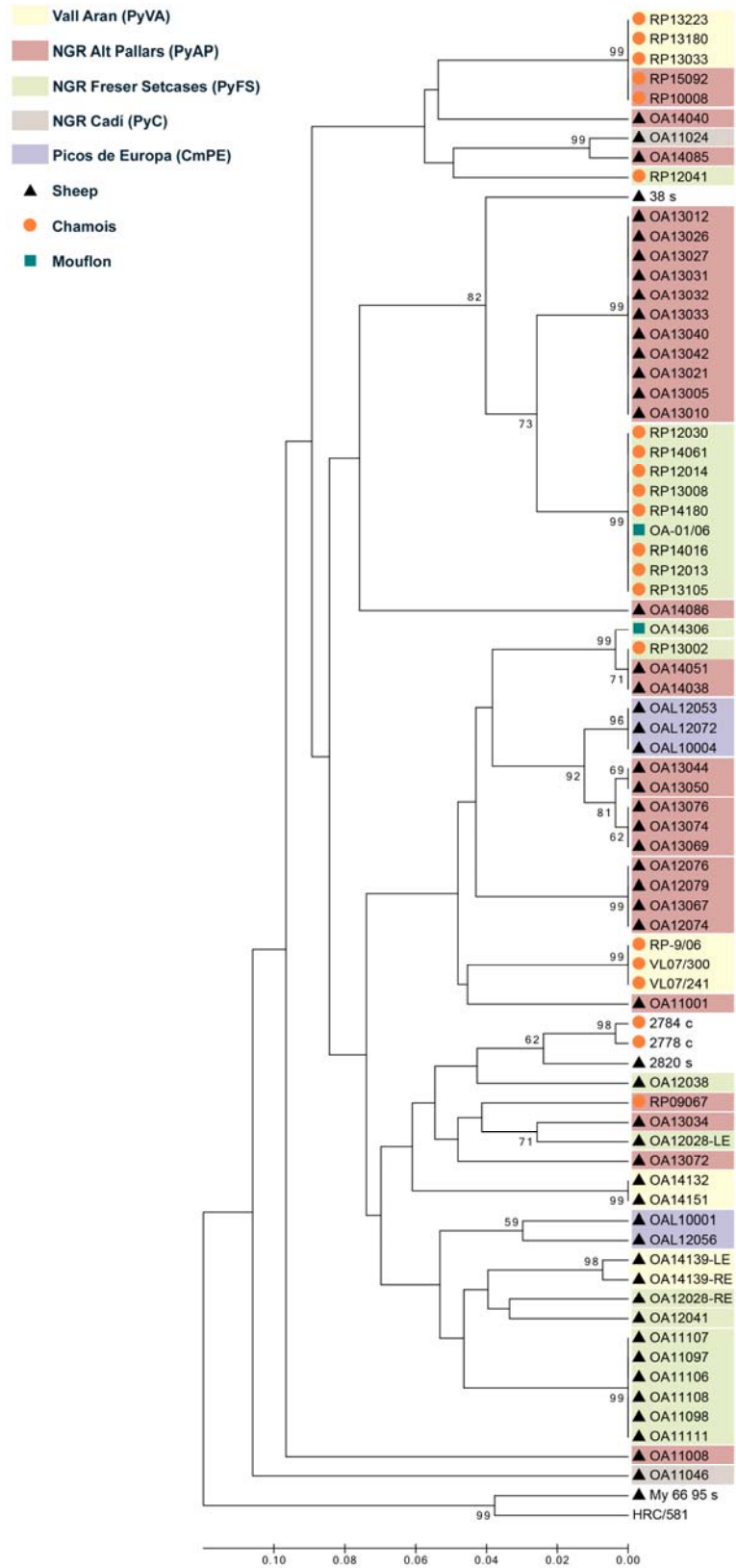
Figure 4.3.5 Cluster analyses tree inferred using the UPGMA method and including Mycoplasma conjunctivae strains identified in chamois, sheep and mouflon during a

ten-year period in the Pyrenees and the Cantabrian Mountains. The percentage of replicate trees in which the associated strains clustered together in the bootstrap test (1000 replicates) are shown newt to the branches. Chamois strains mainly clustered by geographic origin in NGR Freser-Setcases **(B)** and NGR Alt Pallars with Vall Aran as a single epidemiological unit **(A)**. Shared strain clusters among different host species is observed between chamois and mouflon **(B)** and chamois and sheep **(C)**. Sequences from other geographic regions that were included for comparison are showed without background colour. The two first digits of the strain ID indicate the year when was detected. Information associate to each strain is provided in Annex II.

Clinical outcome of M. conjunctivae infections

Ocular clinical signs were detected in 40/1294 chamois, 3/112 sheep, 1/87 mouflon, 1/125 roe deer, 1/135 red deer, 0/14 fallow deer and 0/33 wild boar, ranging from mild ocular discharge to perforation of the cornea (Figure 4.3.6). *Mycoplasma conjunctivae* was significantly ($X^2=488.5$, df=1, p<0.001) associated with ocular clinical signs in chamois, but not in the other ungulate species (Table 4.3.3). Among the *M. conjunctivae* infected chamois, 25.6% (95%CI 14.6-41.1) were asymptomatic, distributed differently among the study areas (PyAP 0/4; PyFS 9/29; PyVA 1/7).

Table 4.3.3 Detection of *Mycoplasma conjunctivae* by species and ocular clinical signs. Note that clinical signs correspond to any abnormality in the ocular structures or ocular discharge. Species in which *M. conjunctivae* was not detected are not shown in the table.

	Positive	Total	Prevalence % (CI 95%)
Chamois			
Clinical signs	30	57	52.6 (39.9-65.0)
No clinical signs	10	1238	0.8 (0.4-1.5)
Total	40	1294	3.1 (2.3-4.2)
Sheep			
Clinical signs	0	3	0.0 (0.0-56.1)
No clinical signs	19	109	17.4 (11.4-25.6)

Total	19	112	17.0 (11.1-25.0)
Mouflon			
Clinical signs	0	1	0.0 (0.0-94.9)
No clinical signs	2	86	2.3 (0.6-8.1)
Total	2	87	2.3 (0.6-8.0)

CI, Confidence Interval.

Figure 4.3.6 Cases of infectious keratoconjunctivitis (IKC) associated with *M. conjunctivae* detection in Pyrenean chamois from this study. A) Initial IKC stage, clear

cornea and purulent ocular discharge. B) Chronic IKC stage, diffuse corneal opacity, neovascularisation with central corneal defect/ulceration. C and D) Irreversible ocular lesions with corneal central perforation and staphyloma E and F) Resolving IKC stages, suppurative ocular discharge has stopped, but there is a sequela, an infraorbital alopecia and hyperpigmentation.

Discussion

Epidemiological cycles and M. conjunctivae persistence in host populations

With the aim to better assess the relative functional roles in IKC epidemiology, the long-term infection dynamics and molecular subtyping of *M. conjunctivae* was investigated on all potential host species from alpine ecosystems of North Spain, which constitutes a first approach with these characteristics. The results further support the specificity of *M. conjunctivae* for caprine and ovine hosts (Giacometti et al., 2002a), being consistently detected in Pyrenean chamois, domestic sheep and occasionally in mouflon. Based on *M. conjunctivae* subtyping, the low numbers of related strains shared between competent hosts disclosed domestic and sylvatic cycles, with domestic sheep and Pyrenean chamois as the key host species for each cycle. The overall prevalence of *M. conjunctivae* in domestic sheep was relatively high, as it was expected from previous studies performed in the Spanish Pyrenees (Study I) (Arnal et al., 2013a) and in the European Alps (Janovsky et al., 2001). Its detection in most of the sheep flocks sampled reinforce sheep as a proper host for the endemic *M. conjunctivae* maintenance (Study I and II) (Janovsky et al., 2001). Specific *M. conjunctivae* strain clusters were also recurrently detected in chamois populations in two independent epidemiological areas (PyFS and PyAP-PyVA), indicating that *M. conjunctivae* circulated within the sylvatic system at least along the temporal extent of the strains identified (up to nine years).

Long-term detection of *M. conjunctivae* clusters has also been demonstrated in wild Caprinae from the Alps, suggesting that *M. conjunctivae* persistence may also occur in other alpine systems (Gelormini et al., 2016; Ryser-Degiorgis et al., 2009). Contrary to this study, host community of sympatric Caprinae seems to be more important for *M. conjunctivae* maintenance in the ecosystems from the Alps, in which a same cluster of strains has been commonly detected in more than one wild host species (i.e. Alpine chamois and ibex)(Mavrot et al.,

2012a; Ryser-Degiorgis et al., 2009), as well as both in domestic and wild hosts (i.e. sheep, Alpine chamois and ibex)(Belloy et al., 2003a; Zimmermann et al., 2008). Population characteristics and allocation/abundance of resources that mainly drive cross-species interactions in natural systems can widely vary between populations and may explain these differences (Richomme et al., 2006; Ryser-Degiorgis et al., 2002). The results of this study indicate that *M. conjunctivae* can long-term persist in Pyrenean chamois populations without the significant contribution of other hosts, either domestic or wild, and dismantle the general though of domestic sheep as the sole reservoir of *M. conjunctivae* in the alpine host-pathogen systems. However, *M. conjunctivae* might have also faded out in some chamois populations studied (PyCAU and PyC) where historical IKC outbreaks have been documented (Gauthier, 1991). Chamois populations from these areas (PyCAU and PyC) have suffered from prior outstanding pestivirus die-offs (Marco et al., 2009b), which may have drop the population under a critical community size for *M. conjunctivae* maintenance (Swinton et al., 2009). The fading out of *M. conjunctivae* after an IKC outbreak has been also reported in Alpine chamois from some parts of the European Alps (Giacometti et al., 2002b; Loison et al., 1996). Altogether indicate that population characteristics can drive infection dynamics to result either in long-term persistence or fading out of *M. conjunctivae* in Pyrenean chamois, and reinforce similar observations in wild host communities from the Alps (i.e. Alpine chamois and ibex) (Gelormini et al., 2016; Giacometti et al., 2002b). Previous controversial hypothesis about reservoir hosts inferred by prevalence estimations and molecular epidemiology in a low spatio-temporal frame or studies performed on partial samplings of the host community should be critically revised (Viana et al., 2014).

The finding of a related strain in chamois and mouflon (PyFS) and in an allopatric sheep more than 100 Km away (PyAP) indicates a wide geographic dispersion of this single cluster. Despite *M. conjunctivae* epizootics can cover long distances (Arnal et al., 2013a; Gelormini et al., 2016), it is unlikely to be caused by a natural spread of *M. conjunctivae* under the non-epizootic conditions associated (Mavrot et al., 2012a). The limited dispersion movements of chamois (Loison et al., 1999) suggest that livestock trade and/or long-

distance movements of livestock in the Pyrenees may favour the introduction of *M. conjunctivae* strains between distant areas (Study II). Since both chamois and sheep can maintain *M. conjunctivae* as indicated in the present study, cross-transmission between them may have occurred in different past events in the Pyrenees (Kamath et al., 2016). Accordingly, *M. conjunctivae* was not detected in Cantabrian chamois, where most of the sympatric sheep flocks were free of *M. conjunctivae* (Study I). Similar correspondences between the epidemiological scenarios in sympatric sheep and chamois have been also reported for other pathogens in the study area (Fernández-Aguilar et al., 2016, 2014), highlighting the significance of spillover events among competent hosts at the wildlife-livestock interface, even if its occurrence is rare (Cassirer and Sinclair, 2007; Luzzago et al., 2016). Despite independent sylvatic cycles accounted for most of the IKC cases in wild hosts from the Pyrenees, the higher prevalence and diversity of *M. conjunctivae* strains in sheep suggest that sheep cannot be ruled out as a source of IKC outbreaks in chamois/wild hosts from alpine ecosystems, owing to cross-species transmission of highly virulent strains (Belloy et al., 2003a).

Mycoplasma conjunctivae infection dynamics in wild host populations

The different patterns of the *M. conjunctivae* infection indices observed in chamois populations (Figure 4.3.2) indicates that *M. conjunctivae* persistence entailed different infection dynamics including both epidemic and non-epidemic transmissions. Thus, the low but regular *M. conjunctivae* detection in PyFS along with the dispersed location of the infection cases agrees with an endemic maintenance of *M. conjunctivae* (Holzwarth et al., 2011; Mavrot et al., 2012a). Conversely, the prevalence in PyAP and PyVA fluctuated reaching higher incidence in some years followed by the absence of detection, suggesting a sporadic and localized epizootic spread of *M. conjunctivae* (Giacometti et al., 2002b). Similar IKC peaks have been observed every three to eight years in chamois populations from neighbouring areas in the Eastern and Central Pyrenees (Crampe, 1992; Pañella et al., 2010), as well as in the Alps (Gauthier, 1991; Tschopp et al., 2005), but without leading to the high proportion of mortality that occur at the first disease emergence (Degiorgis et al., 2000b). The

dramatic drop of some chamois populations from the Eastern and Central Pyrenees since the pestivirus arose may have also prevented from extensive IKC outbreaks, as occurred in 1981-1983 in these areas (Gauthier, 1991), or in other parts of the Pyrenees still not affected by the demographic effects of pestivirus (Arnal et al., 2013a; Serrano et al., 2015).

Virulence is positively related with mycoplasma transmission in ocular disease (Williams et al., 2014), which could suggest that the strain clusters that circulate in the two epidemiological areas identified in chamois (PyFS and PyVA-PyAP) differ in virulence. Population density, which is clearly different in both areas, can be also determinant for transmission by influencing connectivity among individuals, groups and subpopulations (Boots et al., 2004; Swinton et al., 2009). Since size and fusion rates of chamois groups increase with density (Pépin and Gerard, 2008), a nearly density-dependent transmission may occur if population density is high and groups/subpopulation units are highly connected (Manlove et al., 2014). The adaptive immune response of hosts does not always prevent from mycoplasma re-infection (Costa, 1986; Sydenstricker et al., 2005; Trotter et al., 1977), which may eventually be enhanced in denser populations and drive mycoplasma-host interaction to an endemic scenario, as observed in PyFS. Low population density in group-living ungulates can however shape pathogen transmission to be frequency-dependent if contacts between social groups or subpopulations are not common (Begon et al., 2002; Manlove et al., 2014). The scaling of *M. conjunctivae* transmission within and between subpopulations may underlay the temporally different disease peaks observed in PyAP and PyVA caused by the same strain cluster (Figure 4.3.5) (Loehle, 1995; Manlove et al., 2014). The detection of *M. conjunctivae* was not constant in these areas, suggesting that both could be part of a bigger epidemiological unit, probably including part of the French Pyrenees, which would enable the epidemic spread of these strains within a subpopulation and its recurrent detection after a temporal fading out (Gelormini et al., 2016).

Despite coexistence and spatial overlap of mouflon with chamois and sheep, and the close interaction that may occur among them (Darmon et al., 2012; Lauvergne et al., 1977), *M. conjunctivae* was detected only sporadically in

mouflon. Therefore, mouflon is probably a spill-over host in the systems studied with self-limiting *M. conjunctivae* infection in the population.

Ocular clinical signs and Mycoplasma conjunctivae

Mycoplasma conjunctivae infections were associated with ocular clinical signs only in chamois and was probably the main etiological cause of ocular disease in the Pyrenean chamois, as previously suggested (Marco et al., 2009a). These results further indicate a particular susceptibility of chamois to *M. conjunctivae* infection (Ryser-Degiorgis et al., 2009). Clinical signs compatible with IKC were not registered in Cantabrian chamois, which also agrees with the absence of *M. conjunctivae* detection in the area. Previous reports of keratoconjunctivitis in Cantabrian chamois have been reported in the absence of disease outbreaks, but they could not be attributed to *M. conjunctivae* nor an infectious origin was ascertained (Sánchez-Belda and Martínez-Ferrando, 1985). Asymptomatic infections were however detected in chamois in different proportions depending on the study areas, which may correspond with the different epidemiological scenarios (Mavrot et al., 2012a). Although sheep and mouflon may develop IKC due to *M. conjunctivae* infection (Baker et al., 2001; Marco et al., 2009a; Ter Laak et al., 1988b), both species showed a high rate of asymptomatic infections in this study, agreeing with the lack of statistical association between IKC and *M. conjunctivae* detection (Dagnall, 1994a). Altogether highlight differences of host susceptibility to the circulating *M. conjunctivae* strains (Gelormini et al., 2016; Ryser-Degiorgis et al., 2009). Other infectious agents may also have sporadically caused eye disease in negative qPCR-*M. conjunctivae* (Dagnall, 1994a; Wilsmore et al., 1990), although IKC healing stages in which *M. conjunctivae* is not present anymore but evident lesions are still observed probably accounted for most of Caprinae cases (Study IV).

In conclusion, independent *M. conjunctivae* sylvatic and domestic cycles occurred at the wildlife-livestock interface in alpine ecosystems from the Pyrenees, indicating that *M. conjunctivae* was maintained in some chamois populations without the substantial contribution of other hosts. Furthermore,

host population characteristics and *M. conjunctivae* strains resulted in different epidemiological scenarios in chamois, ranging from the fading out of the mycoplasma to the epidemic and endemic long-term persistence. Altogether, these findings highlight the capacity of *M. conjunctivae* to establish diverse interactions and persist in host populations, also with different transmission conditions. Population characteristics can therefore shape host-mycoplasma interaction and ultimately its functionality in the system. Host transitions of *M. conjunctivae* clusters in the alpine ecosystems were also occasionally observed, which indicates that cross-species transmission can be a source of IKC outbreaks.

4.4 Study IV:

Post epizootic persistence of asymptomatic *Mycoplasma conjunctivae* infections in Iberian ibex

Under review

Abstract

The susceptibility of Iberian ibex (*Capra pyrenaica*) to *Mycoplasma conjunctivae* ocular infection and the changes in their interaction over time were studied in terms of clinical outcome, molecular detection and IgG immune response in a captive population that underwent a severe infectious keratoconjunctivitis (IKC) outbreak.

Mycoplasma conjunctivae was detected for the first time in the Iberian ibexes coinciding with the IKC outbreak. Its prevalence had a decreasing trend in 2013 that was consistent with the clinical resolution (August 35.4%; September 8.7%; November 4.3%). Infections without clinical outcome were however still detected in the last handling in November. Sequencing and cluster analyses of the *M. conjunctivae* strains found one year later in the ibex population confirmed the persistence of the same strain lineage that caused the IKC outbreak, but with a high prevalence (75.3%) of mostly asymptomatic infections and with lower DNA load of *M. conjunctivae* in the eyes (qPCR Ct mean 36.1 vs. 20.3 in severe IKC). Significant age related differences of *M. conjunctivae* prevalence were only observed in IKC epizootic conditions. No substantial effect of systemic IgG on *M. conjunctivae* DNA in the eye was evidenced with a linear mixed models selection, which indicated that systemic IgG do not necessarily drive the resolution of *M. conjunctivae* infection and do not explain the epidemiological changes observed.

Results show how both epidemiological scenarios, severe IKC outbreak and mostly asymptomatic infections, can consecutively occur by entailing the mycoplasma persistence.

Introduction

Mycoplasma are small bacteria without cell wall that have strict parasitic life in association with their hosts, either as commensals or pathogens (Citti and Blanchard, 2013). *Mycoplasma* has several singular mechanisms for host adaptation and survival (Citti et al., 2010; Robinson et al., 2013; Rosengarten et al., 2000), which includes one of the highest nucleotide substitution rates among bacteria that provides chances for novel interactions with its hosts (Delaney et al., 2012; Woese et al., 1985). *Mycoplasma* infections can therefore involve diverse epidemiological scenarios, resulting either in the development of severe disease or in asymptomatic carriers that may further or not develop clinical symptoms (Citti and Blanchard, 2013; Osnas et al., 2015). To properly assess host-mycoplasma interaction dynamics a longitudinal sampling design is required. Unfortunately, such sampling conditions are usually unfeasible in wild host species.

The Infectious keratoconjunctivitis (IKC) is a contagious ocular disease caused by *Mycoplasma conjunctivae* that affects small domestic ruminants and more importantly wild Caprinae, in which mortality can reach 30% (Giacometti et al., 2002a). Despite being an old-known disease of wild mountain ungulates (Gauthier, 1991), several aspects of the IKC epidemiology in natural systems are not fully understood and apparent differences of susceptibility are associated with host species and its functional roles in alpine multi-host systems (Giacometti et al., 2002b; Mavrot et al., 2012a; Ryser-Degiorgis et al., 2009; Zimmermann et al., 2008) Clinical stages of IKC may evolve from conjunctivitis to several degrees of keratoconjunctivitis with clinical recovery as the predominant outcome of the disease (Giacometti et al., 1998; Janovsky et al., 2001). However, *M. conjunctivae* infection may still persist after clinical recovery up to six months in sheep (Janovsky et al., 2001) and evidences of temporary persistence and asymptomatic infections have also been reported in wild Caprinae (Giacometti et al., 1998; Mavrot et al., 2012a).

Whereas endemic and subclinical infections of *M. conjunctivae* are common among small domestic ruminants, mainly in sheep (Study I and II) (Janovsky et al., 2001), subclinical infections in wild mountain ungulates are less reported

and/or occur at lower prevalence (Mavrot et al., 2012a). The local fading out of clinical disease (IKC) and the more severe clinical signs typically exhibited by wild hosts, has lead to propose that *M. conjunctivae* cannot be maintained in wild host populations (Deutz et al., 2004; Giacometti et al., 2002b; Ryser-Degiorgis et al., 2009). *Mycoplasma conjunctivae* infection elicits strong immune IgG response as described for IKC outbreaks in wild Caprinae (Degiorgis et al., 2000a), and suggests that may be an important component of the immune response. However, field observations suggest that acquired immunity does not prevent from subsequent IKC episodes (Giacometti et al., 2002a). Therefore, maintenance of specific IgG may therefore be crucial to avoid *M. conjunctivae* persistence in the host population.

Susceptibility of Iberian ibex (*Capra pyrenaica*) to *M. conjunctivae* infection have been reported associated to few sporadic IKC cases in massifs from Spain but, to our knowledge, no IKC outbreaks have been described (Arnal et al., 2009; Cubero et al., 2002) . This medium size Caprinae is an endemic species of the Iberian Peninsula, adapted to rocky mountain ecosystems. It inhabits the Mediterranean mountain ranges of the Iberian Peninsula, where it is the most abundant mountain ungulate with over 50,000 individuals (Acevedo and Cassinello, 2009).

In this study an IKC outbreak in Iberian ibex is described for the first time, taking advantage of the opportunity to perform individual sequential sampling in a captive population of 60 individuals. The objectives of this study were (i) assess Iberian ibex susceptibility to *M. conjunctivae* infection in captive and free-ranging animals and describe the dynamics of the IKC outbreak, (ii) assess the presence of *M. conjunctivae* in the ibex population one year later, (iii) evaluate the influence of sex and age on *M. conjunctivae* infection and (iv) investigate the relationships between clinical signs, molecular detection, and IgG immune response against *M. conjunctivae* and possible changes over time.

Materials and methods

Study area and sampling procedure

This study was performed in a stock reservoir of 60 captive Iberian ibexes

located in Dílar (WGS84 37°03'N, 03°33'W), within the Sierra Nevada Natural Space (SNNS) (Southern Spain). The double-fence enclosure was build in 1993 to preserve the genetic diversity of the Iberian ibex population of SNNS from the massive die-offs that occurred because of sarcoptic mange (*Sarcoptes scabiei*) in the late eighties (León-Vizcaíno et al., 1999). This enclosure includes 30 hectares of Mediterranean forest composed by pine woods (*Pinus* spp.) and Mediterranean shrubs. Herd size can vary in about 15% over the year and is yearly handled for health surveillance. The captive population was historically free of ocular disease until the summer-autumn 2013 when an IKC outbreak occurred.

A longitudinal sampling was performed on the captive ibex population, yearly sampled between 2010 and 2014, except for 2012, when no samples were collected, and 2013, when the outbreak took place and up to three handlings were carried out (Table 4.4.1). For each handling, ocular swabs were taken of each eye between the third eyelid and the palpebral conjunctiva with sterile cotton swabs. Blood samples were also collected from jugular veins from 2013 onwards. From October 2013 to April 2014, ocular swabs and blood samples from 17 free-ranging ibexes captured in SNNS were also collected (Table 4.4.1). Captures were performed for other purposes by means of tele-anaesthesia (combination of xylazine 3 mg/Kg and ketamine 3 mg/Kg) (Casas-Díaz et al., 2011).

Blood samples were placed in sterile tubes, allowed to clot at room temperature and centrifuged at 1500 g to obtain the sera. Ocular swabs and the resulting sera were stored frozen at -20°C within 24 hours from sample collection. Sex determination was made by visual inspection of genitalia and age counting the annual horn segments (Fandos, 1991) and individuals were classified in five different age categories according to social segregation and aging process: Kids, less than six months; Yearlings, from six months to two years; Young, from two years to three; Prime age, from three to seven and Senescence from eight onwards (Acevedo and Cassinello, 2009; Gaillard et al., 2000; Ryser-Degiorgis et al., 2009).

Table 4.4.1 Summary of individuals sampled (n), ocular clinical signs (KC, current and evidence of past infections) and results of specific qPCR and IgG antibodies against *M. conjunctivae* with its respective confidence interval (95% CI) from captive and free-range Iberian ibexes. Ratios in the last three columns are positive ibex to each technique among ibexes that exhibited KC or were qPCR positive.

		KC*	q-PCR		ELISA		qPCR+/KC*	ELISA+/KC*	ELISA+/qPCR+
		Pos./n	Pos./n	Prevalence % (95% CI)	Pos./n	Prevalence % (95% CI)			
Captive ibexes									
2010	November	0/64	0/64	0 (0-5.7)	-	-	0/0	-	-
2011	November	0/51	0/51	0 (0-7.0)	-	-	0/0	-	-
2013	July	2/2	2/2	-	1/2	-	2/2	1/2	1/2
	August	12/48	17/48	35.4 (23.4-49.6)	33/48	68.7 (54.7-80.0)	6/12	10/12	14/17
	September	12/46	4/46	8.7 (3.4-20.3)	25/46	54.3 (40.2-67.8)	4/12	5/12	1/4
	November	8/46	2/46	4.3 (1.2-14.5)	20/43	46.5 (32.5-61.1)	1/8	2/8	1/2
2014	November	1/69	52/69	75.3 (64.0-84.0)	10/26	38.5 (23.4-59.3)	1/1	0/1	13/21
Free-ranging ibexes									
2013 - 2014	October - April	0/14	1/17	5.9 (0.3-27.0)	1/13	7.8 (0.4-33.3)	1/17	-	0/1

*Ocular clinical signs of current inflammation (IKC stages from I to III) and healing (IKC stage V) or past infections (IKC stages VI).

Handling procedures of the ibexes were designed to minimize stress and health risk for subjects according with the current guidelines for ethical use of animals in research (Kilkenny et al., 2010) and following the European (2010/63/EU) and Spanish (R.D. 53/2013) legislations. This study accomplished with all Andalusian, Spanish and European legal requirements and guidelines regarding animal welfare, and was approved by the Ethics on Animal Welfare Committee of the Universidad de Jaén and authorized by the Dirección General de Producción Agrícola y Ganadera of the Consejería de Agricultura, Pesca y Medio Ambiente of the Junta de Andalucía.

Clinical signs

Ocular clinical signs were macroscopically classified in seven categories based on severity and resolution course of the ocular disease (6, see Figure 4.4.1): 0, asymptomatic (no apparent clinical signs); I, mild signs (hyperaemia of the conjunctiva and moderate ocular discharge without evident corneal damage or inflammation); II, moderate signs (ocular discharge, moderate corneal opacity and neovascularization of peripheral cornea); III, severe signs (ocular discharge, widespread and severe corneal opacity and neovascularization of peripheral and central cornea); IV, ultimate severe signs (clinical signs of stage III might be present along with staphyloma and/or corneal perforation); V, evidence of clinical resolution (slight central opacity of the cornea; concentric pigmentation of the cornea and central neovascularization might be still present); VI, clinical resolution without current inflammatory features (invading pigmentation from the limbus to the vertex of the cornea).
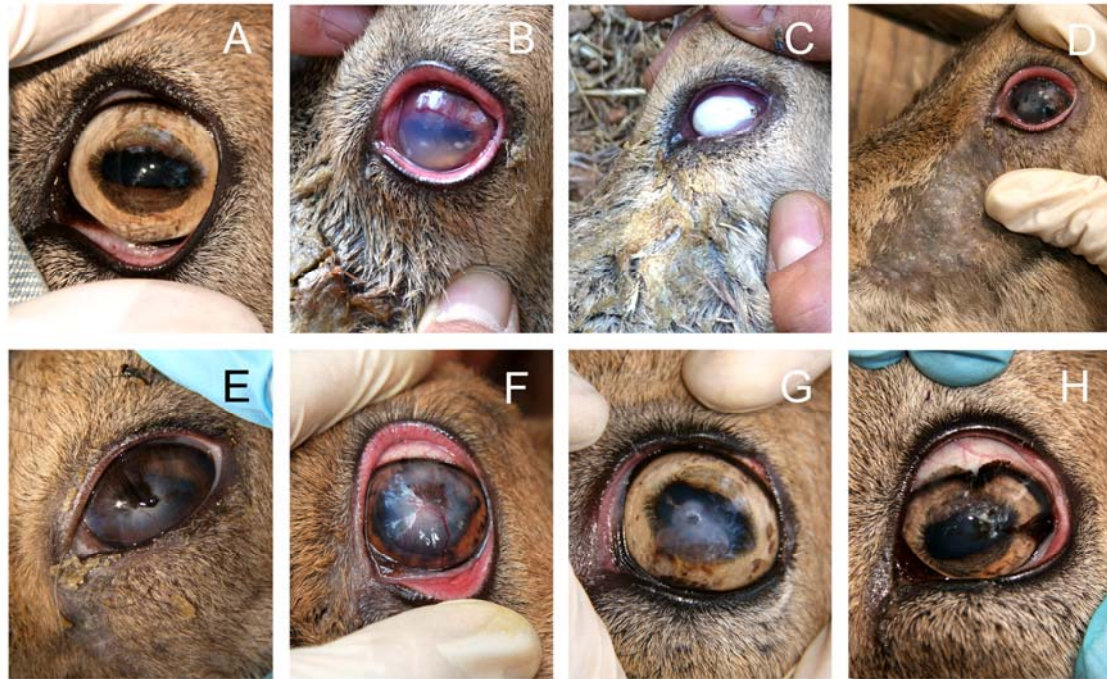
Figure 4.4.1 Ocular lesions in Iberian ibex corresponding to IKC stages of disease severity and resolution observed during the natural course of an IKC outbreak. A) Normal eye appearance of Iberian Ibex. B) IKC stage II showing moderate corneal opacity, neovascularization of the peripheral cornea and ocular discharge. C) IKC stage III with severe corneal opacity, neovascularisation of the peripheria of the cornea and ocular discharge. D) Chronic IKC stage III showing infraorbital alopecia. E), F) and G) Different IKC stage V corresponding to healing process with neovascularization of the vertex of the cornea, slight central corneal opacity and evidences of a central ulcer in F and G. H) IKC stage VI, pigmentation of the cornea.

Mycoplasma conjunctivae qPCR detection and sequencing

For *M. conjunctivae* detection, eye swabs were placed with 0.5 ml of lysis buffer (100 mM Tris–HCl, pH 8.5, 0.05% Tween 20, 0.24 mg/mL proteinase K) into sterile tubes and were mixed with a vortex. Lysis of the cells was performed at 60°C for 60 minutes, followed by the inactivation of the proteinase K at 97°C for 15 minutes.

Lysates were directly used as the test sample in a specific *M. conjunctivae* qPCR (Vilei et al., 2007). Each reaction consisted in 2.5 µl of the sample, 900 nM of LPPS-TM-L primer and LPPS-TM-R, 300 nM of LPPS-TM-FT probe (Annex II), 12.5 µl of TaqMan®2x Universal PCR MasterMix (Applied

Biosystems, Warrington, UK), an exogenous internal positive control (IPC; Applied Biosystems, Warrington, UK) and water up to 25 μl of volume. The cycle threshold was set at 0.05 and the PCR cycle when the sample fluorescence crossed the threshold was recorded as the Ct value, which inversely corresponds to the initial amount of target DNA in the test sample. All samples were analyzed per duplicate including positive and negative controls and positive-samples reactions were repeated if standard deviation between the replicates was more than one Ct.

For molecular epidemiological studies, a nested PCR that targets the 3' end of the gene encoding for the serine rich part of the membrane protein *lppS* of *M. conjunctivae* was performed on randomly selected samples with low Ct values at the q-PCR. Reactions were performed according to Belloy et al., 2003 (Belloy et al., 2003a) with minor modifications of the primers, using the Serstart3 and LppTA2 for the first PCR reaction, and Serstart2 and LppTA in a nested reaction if the first amplification was not enough.

For DNA sequence analyses, all products from the nested PCR were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Rotkreuz, Switzerland). The sequencing of the amplicons was performed using the primers Ser_start2, Ser_start0 and Ser_end0 (Annex II) using the BigDye termination cycle sequencing kit (Applied Biosystems, Forster City, CA, USA).

The resulting sequences were trimmed to obtain the variable part of the gene *lppS* corresponding to the nucleotides positions 3935 – 5035 of *lppS* of the *M. conjunctivae* type strain HRC/581 (accession number AJ318939). Alignment and editing of the sequence data were performed with the BioEdit software. Cluster analyses tree of the sequences was inferred using the UPGMA method with 1,000 bootstrap replications, performed with the MEGA 7 software (Kumar et al., 2016). DNA sequence data from the type strain HRC/581 (Genbank acc. number LT174654), and six strains more from different hosts and geographical placements were included in the tree for comparison (2880 s: accession LT174667; 38 s: accession LT174670; My 66 95 s: accession LT174668; My 7/96 g: accession LT174669; 2778 c: accession LT174666; 2784 c: accession LT174671). The partial *lppS* gene sequences from the ibexes were deposited in GenBank under accession shown in Annex II.

Enzyme-Linked Immunosorbent Assay (ELISA) for M. conjunctivae antibodies

The humoral immune response against the *M. conjunctivae* infection was assessed in sera with an indirect ELISA based on the Tween 20 membrane proteins extract of the *M. conjunctivae* strain HRC/581T (Belloy et al., 2001). This ELISA showed good sensitivity and specificity for *M. conjunctivae* antibodies in sheep and has also been validated for Alpine chamois (*Rupicapra rupicapra*) (Giacometti et al., 2002b; Janovsky et al., 2001). Cross board titrations where performed to determine the serum sample dilution, the concentration of Tween 20 extracts and the conjugated antibodies to obtain an optimal signal/noise ratio. All the samples were analyzed in duplicate and all sera from the same ibex were included in the same ELISA plate to have the same analytical conditions for each individual. Serum from a captive-born ibex negative to the *M. conjunctivae* specific qPCR in eye swabs and that never had ocular disease was used as the negative reference standard. On the other hand, serum from an ibex that showed severe IKC during the outbreak and with *M. conjunctivae* infection confirmed by qPCR was used as positive reference standard.

For the ELISA development, NUNC A/S Maxisorb® microtiter plates (NUNC, Roskilde, Denmark) were coated with the Tween 20 extracts at an optical concentration of 5 µg·mL–1 according to standard methods (Nicolet and Martel, 1996). Briefly, sera samples were applied to the plates diluted 1:50 with an ELISA diluent solution 5X (eBioscience, San Diego, USA) and incubated for 90 minutes at room temperature. Then the wells were washed and a monoclonal anti-Sheep/Goat IgG antibody conjugated to horseradish peroxidase (Sigma, St. Lois, USA) diluted 1:15,000 was added and incubated for 90 minutes at room temperature. Subsequently, the wells were washed and the chromogen solution 3,3',5,5' - tetramethylbenzidine (Sigma, St. Lois, USA) was added and incubated for 15 minutes following the producers' instructions, when 0.16 M sulphuric acid was applied to stop the reaction. The values of the samples were calculated as the mean optical density of the duplicates measured at a wavelength of 405 nm (OD_{450}), and were expressed as the percentage of the positive reference standard considering the negative reference standard value

as zero, according to accepted methods (Nicolet and Martel, 1996).

The optimal cut-off point was obtained with the Youden index, which is determined where the maximum sensitivity and specificity is achieved based on values of both diseased and healthy individuals (Yin and Tian, 2014). Sera samples from Iberian ibexes from two populations in Catalonia (NE-Spain) where ocular disease has not been described were used as negative controls for the cut-off determination (n = 71). On the other hand, sera from ibexes from the reported outbreak that exhibited ocular disease and were positive to the qPCR were used as positive controls for the cut-off determination (n = 33).

Statistical analyses

Data were analyzed separately for 2013 (epidemic IKC) and 2014 (non epidemic IKC). *Mycoplasma conjunctivae* infection was treated as a categorical variable with two levels (positive and negative), in which ibexes were considered positive if the qPCR resulted positive in any of the three samplings from 2013. Differences in *M. conjunctivae* prevalence related to age categories and sex were assessed with a Fischer's exact test.

To assess the importance of the IgG humoral response in the *M. conjunctivae* clearance we fit a set of linear mixed effects models (LMM) in which *M. conjunctivae* abundance in the eye (Ct values of the qPCR) was explained by the fixed effects of IgG concentration (relative ELISA OD_{450}), age class and their two-way interactions. Since at least two blood samplings (one by year) were taken from each individual, the ibex was considered as a random term in the LMM following a repeated measure-fixed block design. Then, the best random structure was selected following Zuur et al 2011 (Zuur et al., 2009). For the purpose of this analysis and in order to increase sample size per category, the Young, Prime age and Senescence categories were merged. Because of the short-term persistence of the IgG antibodies, only ibexes that were positive to the qPCR were considered. The ELISA results were square root transformed to reduce residual variability and the effects of outliers (Zuur et al., 2007). A theoretic information approach based on the Akaike's Information Criterion corrected for small sample sizes (AICc) was then followed to select the best

model (Johnson and Omland, 2004). Briefly, competing models were ranked according to the difference between their Akaike scores and the score of the model with the lowest AICc. The models that substantially explain the observed variability have an i < 2 units in comparison with the model with the lowest AICc. Then the Akaike weight (wi) was calculated to know the relative probability that a given model is the best of those being compared. For the best models, the lack of residuals patterns was checked and to obtain a general measure of goodness of fit the marginal (variance explained by the fixed terms) and conditional (by the fixed and random terms) variance were also calculated following the protocol reported by Nakagawa and Schielzeth (Nakagawa and Schielzeth, 2013).

All the statistical analyses were performed with the R software (R Development Core Team 3.1.3, 2015) setting α at 0.05 when appropriate. The linear mixed models were fitted with the R package "lme4" (Bates et al., 2016). The R package of "OpticalCutpoints" was used to determine the cut-off point of the indirect ELISA and the derived estimates of the sensitivity and specificity with a 95% CI (López-Ratón et al., 2014).

Results

Clinical follow-up and course of the IKC outbreak

Ocular clinical signs were not observed in ibexes sampled in 2010 and 2011. First evidence of ocular disease appeared on July 1st 2013, when two Iberian ibexes showing severe ocular clinical signs were captured by hand due to visual impairment and total blindness. Both ibexes suffered from bilateral keratoconjunctivitis with a widespread corneal opacity and abundant neovascularization in the peripheral cornea accompanied with severe mucopurulent effusion, corresponding with IKC stage III (Figure 4.4.1). From the first handling in August 2013 to November 2013 the ocular clinical signs progressed from predominantly severe clinical signs (IKC stage II and III) to chronic and healing stages V and VI (Figure 4.4.1; Figure 4.4.2 A). Clinical signs were bilateral mainly in severe IKC stages II (1/2) and III (7/9) and less common in initial IKC stage I (0/1) or healing stages V (2/14) and VI (3/9).

Bilateral infections were different between both eyes only in two cases, one showing IKC stage V and VI and the other one showing IKC stage II and III. Among age categories, younger ibexes and specially kids showed a higher proportion of severe clinical signs (IKC stage II and III) than older animals (Figure 4.4.4). No IKC stage IV (ultimate severe IKC signs) or direct mortality was attributable to *M. conjunctivae* infection during the IKC outbreak. Only one ibex showed unilateral IKC stage V in November 2014. Although ocular clinical signs were observed in free-ranging ibexes from the SNNS in 2013, they were not present in none of the 17 ibexes captured and sampled outside the enclosure.
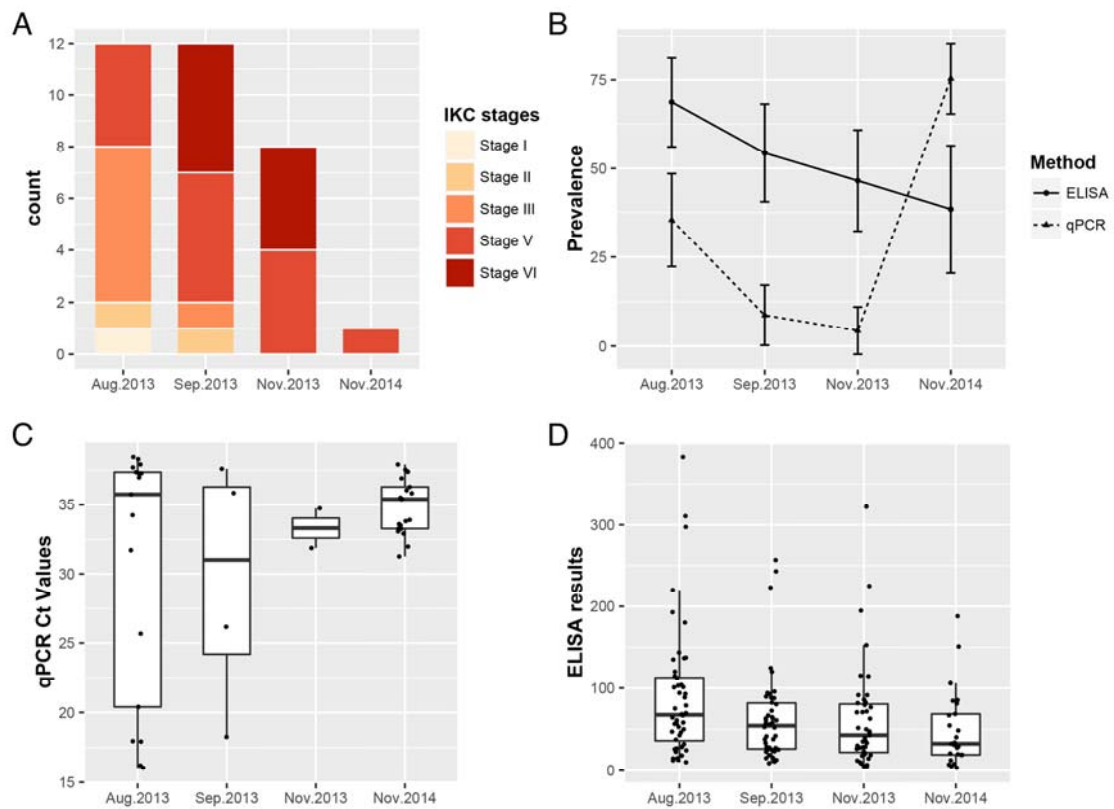
Figure 4.4.2 Clinical, serological and molecular trends of *M. conjunctivae* infection in the Iberian ibex population. Results showed by handlings that correspond with two epidemiological scenarios, epizootic IKC disease in 2013 and mostly asymptomatic infections in 2014. **A)** Number of IKC cases by clinical stages. **B)** Prevalence of *M. conjunctivae* seropositive ibexes assessed by an indirect ELISA and prevalence of *M. conjunctivae* detection in eye swabs by qPCR. Confidence intervals 95% are

represented with error bars. **C)** Distribution of Ct values of the *M. conjunctivae*-qPCR. Note that boxplots of Ct values from July and November 2013 are build with only two observations. **D)** Absorbance relative values of the indirect ELISA that recognize specific IgG for *M. conjunctivae*.

Mycoplasma conjunctivae qPCR detection and sequencing

Mycoplasma conjunctivae was firstly detected in ibexes showing IKC in July 2013. The prevalence showed a decreasing trend from August 2013 until November 2013, in concordance with the course of the clinical signs (Table 4.4.1; Figure 4.4.2 A and 4.4.2 B). Two ibexes that were qPCR-positive to the *M. conjunctivae* and/or showed IKC in August 2013, were qPCR-negative by September 2013, and turned qPCR-positive in November 2013 (Figure 4.4.3; 501 and ALP-276). Prevalence of *M. conjunctivae* rose again in November 2014 to significantly higher values as compared to all 2013 handlings (August: X^2=17.1, df=1, p<0.001; September: X^2=46.5, df=1, p<0.001; November: X^2=53.1, df=1, p<0.001), in the absence of clinical signs in most of the ibexes (Table 4.4.1; Figure 4.4.2 B). One out of the 17 free-ranging Iberian ibexes sampled was positive to *M. conjunctivae* by the qPCR (Table 4.4.1).
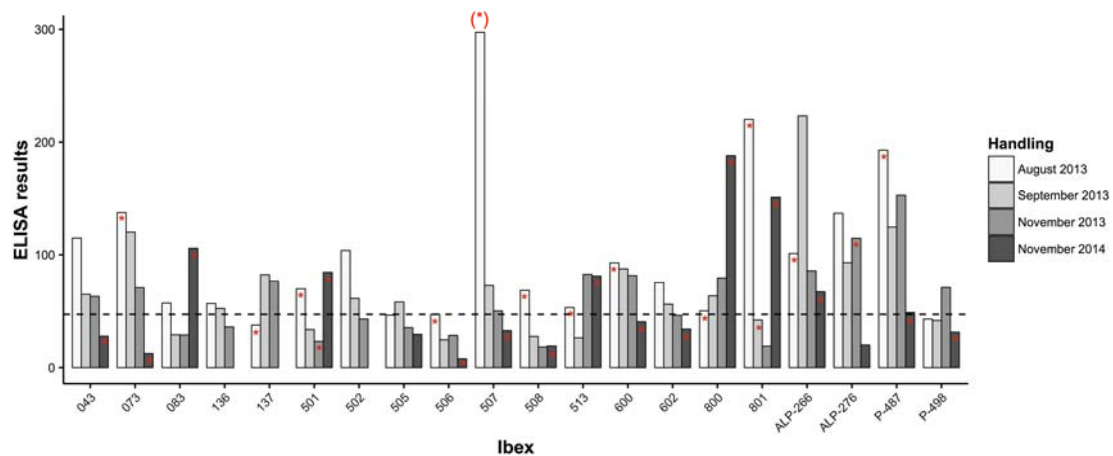
Figure 4.4.3 Bar graph showing individual trends of the ELISA results from a random selection of ibexes that were sampled in most of the handlings in 2013 during the IKC outbreak and in 2014 with mostly asymptomatic infections. Numbers in the horizontal axis are the individual ibex reference and ELISA results of the vertical axis correspond to the relative percentage from the positive reference standard. The detection of *M.*

conjunctivae in eye swabs is showed with a red asterisk in the top of the bars and the cut-off for seropositivity is shown with a horizontal dashed line.

Asymptomatic infections were detected in 11 out of 17 (64.7%) qPCR-positive ibexes in August 2013, none out of four (0.0%) in September 2013, and one out of two (50.0%) in November 2013. Fifty-one out of the 52 (98.1%) qPCR-positive ibexes in November 2014 were asymptomatic as well as the free-ranging positive ibex. With regard to the whole population, asymptomatic infections were higher in August 2013 (22.9%) during the IKC outbreak and in November 2014 (73.9%) as compared to September 2013 (0.0%) and November 2013 (2.2%).

Among the *M. conjunctivae*-infected ibexes, qPCR-positive eyes were bilateral in July 2013 (2/2), 47.1% in August 2013 (8/17), 50.0% in September 2013 (2/4), none in November 2013 (0/2), and 46.1% in November 2014 (24/52). The infection of both eyes was associated with severe IKC signs (stages II and III) in 2013 (10 out of 12 bilateral infections) but not in 2014, when no ibexes showed severe IKC signs.

Only two ibex kids with severe IKC were qPCR-positive in two consecutive samplings in 2013, which implies that were probably infected for at least seven weeks. The prevalence of *M. conjunctivae* was inversely related to age class during the outbreak of IKC in 2013 (X^2=19.24, df=4, p<0.001), but not in 2014 (X^2=7.73, df=4, p=0.102) (Figure 4.4.4).
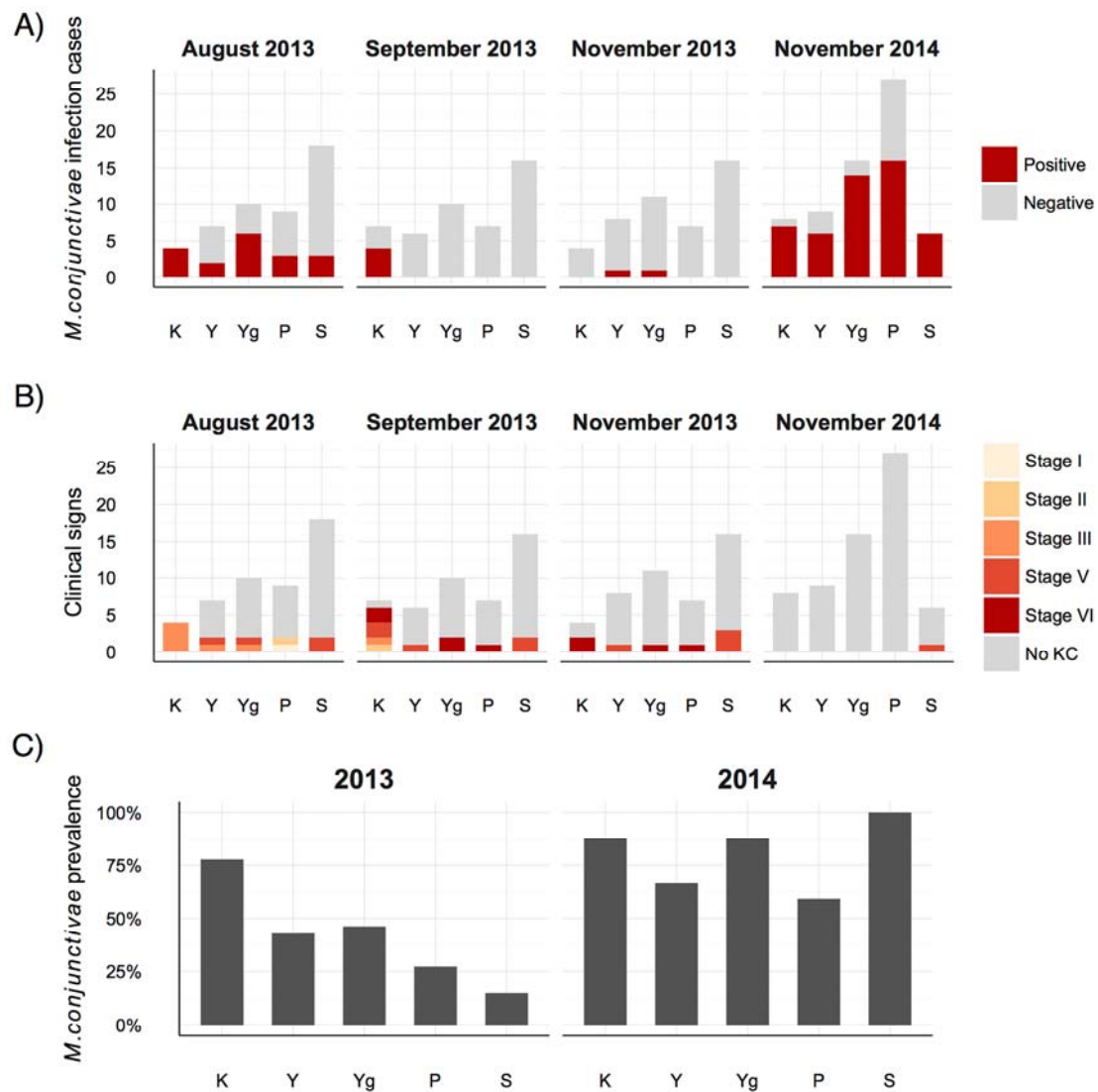
Figure 4.4.4 *Mycoplasma conjunctivae* infection cases (A), clinical signs (KC; B) and prevalence (C) showed by age class (horizontal axis, K=Kids; Y=Yearlings; Yg=Youngs; P=Prime age; S=Senescence) in the Iberian ibex population in two different epidemiological scenarios, an IKC outbreak in 2013 and mostly asymptomatic infections in 2014. Higher *M. conjunctivae* prevalence and more severe clinical signs are observed in kids only during the IKC outbreak

Detection of *M. conjunctivae* by qPCR was consistent in IKC stages with evidence of current inflammation, IKC stage I (1/1), IKC stage II (1/2) and stage III (8/9), but not in healing IKC stage V (4/14) or with evidences of past inflammatory processes in IKC stage VI (0/9). The mean Ct values of the qPCR were significantly lower in the severe IKC stages II and III (20.3) as compared to stages V (IKC resolution, 35.1; p=0.045) and asymptomatic ibexes (36.1;

p<0.001). The only IKC stage I observed had high Ct values (38.3, see Figure 4.4.5). Mean Ct values of the positive qPCR samples increased progressively in the consecutive handlings from September 2013 onwards in accordance with the fading away of the clinical signs throughout the outbreak of 2013 and 2014 (Figure 4.4.2 C).
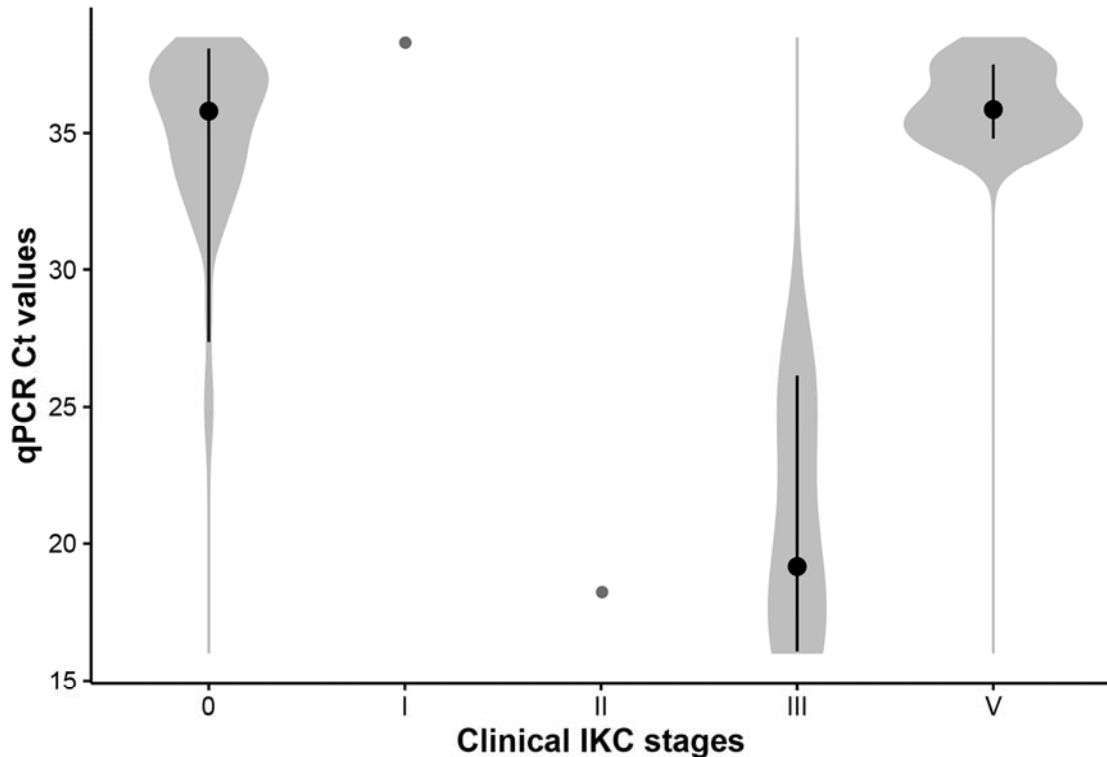
Figure 4.4.5 Violin plot showing the distribution of the Ct values in eye swabs in IKC clinical stages that were qPCR positive. Ct values were significantly lower in severe IKC stages (II and III) than initial (I) and healing stages (V) and asymptomatic infections (0). Black bars show the interquartile range and black dots indicate the median of the Ct values per each group.

Eighteen PCR amplicons of approximately 700 bp that corresponded to the variable domain of the *M. conjunctivae lppS* gene were sequenced from nine ibexes sampled in 2013 (two from July C_507 and C_686; five from August C_506, C_508, C_800, C_801 and C_695; two from September C_2298, C_5125) and four ibexes sampled in November 2014 (C_501, C_801, 575N and ALP321). The *M. conjunctivae* strains causing the IKC outbreak in 2013

and those associated with asymptomatic infections from 2014 belonged to a same cluster clearly separated from the rest of the sequences included in the tree, suggesting a phylogenetic relationship and a common origin (Figure 4.4.6).
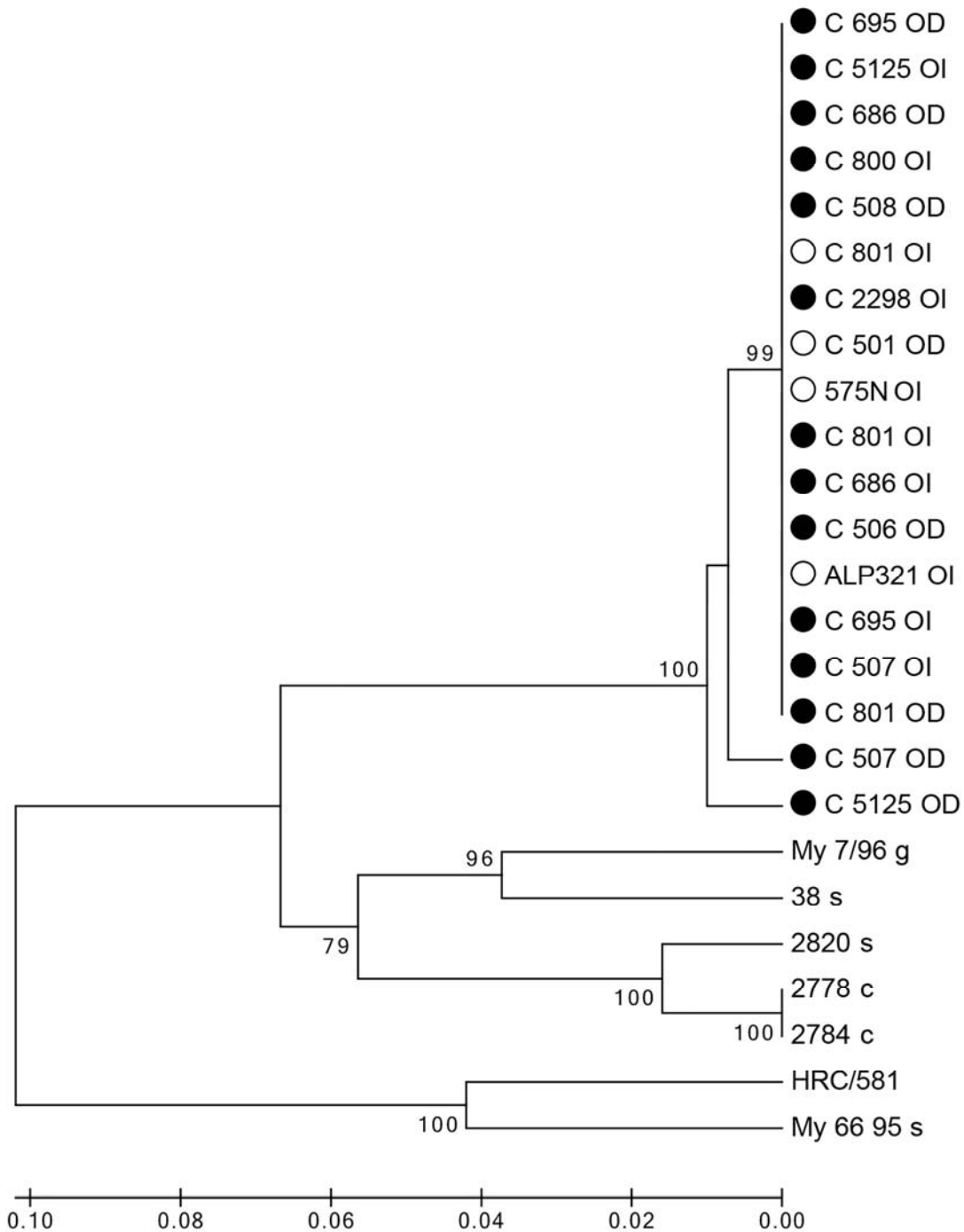
Figure 4.4.6 Cluster analyses of the *M. conjunctivae* strains detected in this study based on the variable domain of the *lppS* gene. Name of sequences indicates the individual reference followed by right (OD) or left (OI) eye from which was detected.

The year of sampling is showed with black or white dots in 2013 and 2014 respectively. For comparison, sequences from isolates in other hosts are included: HRC/581 type strain, sequences obtained in sheep infections in Eastern Swiss Alps (38 s and 2820 s) and Croatia (My66 95), from a goat in Croatia (My 7/96) and from chamois in the Austrian Alps (2778 c and 2784 c). A separate clade of the ibex strains, all with a common origin, is observed in the tree.

ELISA for M. conjunctivae antibodies

The cut-off determined by the Youden index resulted in a sensitivity of 93.9% and a specificity of 84.3% for the ELISA test. The seroprevalence was higher in the first handling (August 2013) and decreased steadily during all the consecutive samplings (Table 4.4.1; Figure 4.4.2 B). Among ibexes that were negative to the ELISA test in August 2013, 33% (4/12) turned positive in September 2013. Among ibexes that were negative to the ELISA test in September 2013, 33% (4/12) turned positive in November 2013. Both the mean and individual ELISA values also showed a similar decreasing trend throughout the 2013 and 2014 handlings (Figure 4.4.2 D, Figure 4.4.3).

The number of qPCR-positive ibexes that resulted also positive to the ELISA test decreased along sampling periods. In August 2013 82.4% (14/17) of the qPCR-positive individuals showed evidence of a systemic IgG response. In September, however, this proportion decreased to 25% (1/4), slightly rising in November 2013 (50%, 1/2) and 2014 (61.9%, 9/21). The ELISA value of each individual declined steadily after the first *M. conjunctivae* detection in the eye swabs in most of the ibexes (Figure 4.4.3). ELISA values were not clearly related with the presence or the severity of the clinical signs, and three ibexes with severe IKC did not seroconvert during or after the *M. conjunctivae* infection (e.g. Figure 4.4.3, 506).

None of the selected linear mixed models included the optical density of the ELISA test as explanatory fixed effect (Table 4.4.2), which indicated that no substantial variability of the Ct values was explained by the ELISA results (proxy for IgG). The best model only included the age of ibexes, which explained 68.35% of the observed variability of Ct values (Wi $_{Age}$ = 0.80, F=17.36). A small proportion in the Ct response was due to the Ibex random

term (17.05%) (Table 4.4.2).

Table 4.4.2 Linear Mixed Model selection performed to assess the effect of ELISA results (proxy for IgG response) on Ct values obtained by a specific *M. conjunctivae*-qPCR (proxy for *M. conjunctivae* loads) in eye swabs of Iberian ibexes. Nested models for the random terms were fitted with the individual ibex and the year (corresponding to two epidemiological scenarios) as random effects. K = number of parameters, AICc = Akaike Information Criterion corrected for small sample sizes, Δi = difference of AICc with respect to the best model, wi = Akaike weight, Marginal R^2 = observed variability in the response variable explained by the fixed factors, Conditional R^2 = observed variability in the response variable explained by both the fixed and random factors. The model with substantial support is highlighted in bold.

Biological models	K	AICc	Δi	wi	Marginal R^2	Conditional R^2
Age	**5**	**38.47**	**0.00**	**0.80**	**0.61856109**	**0.68359573**
Age + Year	6	43.26	4.79	0.07	0.61088941	0.67602488
Age + ELISA	6	48.54	10.07	0.01	0.61074212	0.70032295
Year	4	49.86	11.39	0.00	0.16534722	0.29751655
Mo	3	50.08	11.61	0.00	0.00000000	0.00652695
ELISA	4	57.87	19.40	0.00	0.03221345	0.03221350
ELISA + Year	5	58.69	20.22	0.00	0.15863676	0.28898391
Age * ELISA	8	67.30	28.83	0.00	0.58927052	0.60548979

Discussion

Iberian ibex susceptibility to *M. conjunctivae* infection has been clearly confirmed by this study, which can suffer from IKC outbreaks but also can maintain *M. conjunctivae* at high prevalence with mostly asymptomatic infections. Both epidemiological situations (i.e., IKC outbreak and endemic asymptomatic infections) have been described in other domestic (Janovsky et al., 2001; Naglić et al., 2000) and wild free-ranging hosts (Arnal et al., 2013a; Degiorgis et al., 2000b; Mavrot et al., 2012a). The results of the present study

show how both epidemiological situations can occur consecutively in a host population, depending on *M. conjunctivae* circulation, its maintenance in the population and the progression of the host-pathogen interactions.

Infectious keratoconjunctivitis outbreak

There are few reports of IKC in Iberian ibex and to the authors' knowledge this is the first IKC outbreak described. The consistent detection of *M. conjunctivae* in severe IKC stages (II and III) and the correlation of Ct values with IKC stages confirm that it was the primary agent of the outbreak. Ibexes that showed IKC stage II and III in which *M. conjunctivae* was not detected were seropositive and most probably correspond to false negative results of the detection method (sampling/qPCR), or less probably be caused by other infectious agents (Andrews et al., 1987; Rodríguez et al., 1996). The absence of *M. conjunctivae* in advanced and healing IKC stages V and VI agrees with the resolution of the infection and with previous research (Surman, 1973). Since *M. conjunctivae* was not detected in the captive ibex population before 2013 and was also confirmed in free-ranging ibexes from the surrounding SNNS (clinical signs compatible with IKC were also observed, data not shown), *M. conjunctivae* was probably introduced along with asymptomatic carriers or transported by eye-frequenting insects from outside the enclosure (Degiorgis et al., 1999). Previous research in the Iberian ibex population from SNNS failed to detect *M. conjunctivae* (González-Candela et al., 2006), which suggests a likely recent introduction of this *Mycoplasma* in the population.

Clinical signs during the IKC outbreak in 2013 were severe as those reported in other IKC outbreaks in wild Caprinae (Arnal et al., 2013a; Tschopp et al., 2005), or in naïve domestic sheep flocks (Motha et al., 2003; Naglić et al., 2000). According to the progression of the clinical signs and the molecular and serological results, the IKC outbreak probably started before July 2013 and clinical resolution (no severe IKC stages II and III) was observed by November 2013. However, both seroconversion and *M. conjunctivae* detection in the last 2013 handling indicate that there was still transmission of *M. conjunctivae* among ibexes almost five months after the identification of the first IKC cases.

Asymptomatic post-epizootic M. conjunctivae persistence

Sequences obtained from asymptomatic infections in 2014 were very similar to sequences from the IKC epizootic in 2013 indicating that the same *M. conjunctivae* lineage persisted after the initial disease outbreak. Whether *M. conjunctivae* persisted throughout the whole study period in the captive population or was reintroduced is not known, but persistence in host populations through singular mechanisms of host immune evasion is a common feature of mycoplasma infections (Arfi et al., 2016; Baranowski et al., 2014; Rosengarten et al., 2000). It is unlikely that a rare event as the introduction of similar *M. conjunctivae* strains from outside the double-fence enclosure occurred consecutively two years. On the other hand, the known clinical outcome of the introduction of *M. conjunctivae* in a host population does not correspond with the epidemiological scenario found in 2014 (Baas et al., 1977; Naglić et al., 2000).

Within-host persistence of *M. conjunctivae* after clinical recovery have been reported from three to six months in sheep (Janovsky et al., 2001) and, at least, up to two months in Alpine ibex (*Capra ibex*) (Giacometti et al., 1998). In the Iberian ibex, the maximum period of infection demonstrated in this study has been seven weeks in 2013. Given that mycoplasma virulence and clinical signs is positively related with transmission in ocular diseases (Williams et al., 2014), the high *M. conjunctivae* prevalence detected in 2014 was presumably achieved by chronic persistence in the eyes (Janovsky et al., 2001). Altogether suggest that *M. conjunctivae* probably became endemic in the Iberian ibex population with apparently low health implications as typically reported in domestic sheep flocks (Study II).

Age and sex effect

The higher prevalence of *M. conjunctivae* in younger Iberian ibexes during the IKC outbreak concurs with previous studies that reported higher *M. conjunctivae* prevalence in yearling ibexes (Mavrot et al., 2012a) and lambs (Study II). A specific role of lambs for *M. conjunctivae* persistence in sheep flocks have been

also suggested based on this higher prevalence and milder clinical signs (Hosie, 2007; Janovsky et al., 2001). However, there were no age-class differences of *M. conjunctivae* in the ibexes in 2014 suggesting that this potential age factor may be dynamic and dependent on the established interaction between the hosts and the circulating mycoplasma strains. In contrast with previous reports of IKC epizootics in sheep and chamois (Arnal et al., 2013a; Crampe, 2008; Hosie, 2007; Jones et al., 1976), more severe clinical signs and higher mycoplasma DNA loads were exhibited by kids than adults in this study, which suggest that in this case ocular tissue damage was apparently directly caused by *M. conjunctivae* and not by an exacerbated immune response of adult individuals (Rosengarten et al., 2000; Rottem and Naot, 1998). Captive conditions may have influenced on these results and in the lack of the female-biased IKC incidence described in disease outbreaks (Degiorgis et al., 2000b; Tschopp et al., 2005).

Immune IgG reactions

Strong immune reactions against *M. conjunctivae* as described in natural IKC outbreaks were observed in the ibexes (Figure 4.4.3). However, *M. conjunctivae* detection was not always consistent with a subsequent increase of IgG in sera as previously reported (Baas et al., 1977; Trotter et al., 1977), and suggests that a systemic involvement of the immune system is not always triggered by the ocular infection. Moreover, mixed-models indicated that systemic antibodies had a limited role in recovery from infection, which is not rare in mycoplasmas (Gourlay and Howard, 1979; Noormohammadi et al., 2002; Schieck et al., 2014; Simecka et al., 1993). Acquired immunity may be therefore based on exocrine production of IgG, other immunoglobulins and/or local immune mechanisms that occur in mucosal and local infections (Avakian and Ley, 1993; Simecka et al., 1993).

Mycoplasmas typically cause chronic and persistent infections and systemic IgG titres can be maintained stable in sera for long-time periods through continuous stimulations of the immune system (Schieck et al., 2014). Systemic IgG in the Ibexes were however short-lived which agree with some previous reports of single natural infections (Lambert, 1987; Waites et al., 2009). These

results further suggest that systemic IgG does not probably play an important role for the acquired immunity against *M. conjunctivae*.

Since the immune responses of the ibexes were assessed by an antigen-based diagnostic technique, changes in the antigenic profile of *M. conjunctivae* during the infection can explain part of the individual variability observed (Baranowski et al., 2014). Although the indirect ELISA may not be a good diagnostic tool for *M. conjunctivae* infection in case studies, it has been demonstrated to be effective for *M. conjunctivae* surveillance in Iberian ibex populations. Further research is needed to elucidate the immune mechanisms developed by ruminant hosts to face and overcome *M. conjunctivae* infection.

Host-pathogen interactions

Two different epidemiological scenarios were found in the ibex population suggesting different *Mycoplasma*-host interaction if we consider the prevalence, the *M. conjunctivae* DNA abundance and the clinical outcome of the infection. The clearance of *M. conjunctivae* from the eyes in most of the Iberian ibex that suffered from severe IKC (outbreak 2013) indicated that the infection triggered a host reaction directed to eliminate ocular infections. Subsequent higher prevalence of asymptomatic infections in 2014 however suggests a longer low-DNA *M. conjunctivae* (proxy for bacterial load) persistence in the eyes owing to changes in the host-*M. conjunctivae* interaction. Similar to these results, low mycoplasma loads have been associated with minimal direct damage to the epithelium and persistent infections (McGowin et al., 2012), and a correlation between disease severity and *M. conjunctivae* loads has been also reported in free-ranging chamois and Alpine ibex independently of the infecting strain (Mavrot et al., 2012a; Ryser-Degiorgis et al., 2009). The transition between the two scenarios observed is consistent with transmission-recovery trade-offs of pathogen virulence, in which gained virulence favours persistence by reducing the up regulation of the immune response of the host with a lower replication rate (loads) and tissue damage (Alizon, 2008; Frank and Schmid-Hempel, 2008).

Evidence of one past ocular inflammatory process in 2014 (IKC stage V) in at

least one Iberian ibex however suggests that the infection may still be pathogenic if influenced by other factors (Study II) (Mavrot et al., 2012a, 2012b).

This study allows a better understanding of the transition between two different *M. conjunctivae* epidemiological scenarios described and highlights the ability of *M. conjunctivae* to establish diverse interactions with its hosts. The outcome of the *M. conjunctivae* infection can be variable and more dependent on a specific host-mycoplasma interaction rather than intrinsic host-species traits (i.e. susceptibility). Protective immune reactions against *M. conjunctivae* are not based on a systemic IgG response, which is probably neither relevant to prevent from repeated infections in the Iberian ibex.

Infectious diseases have been identified as potential threats for wild Iberian ibex populations (Acevedo and Cassinello, 2009). Surveillance of *M. conjunctivae* infection and the potential impact in free-ranging Iberian ibex populations may therefore be considered from this study on in the management of this species. The potential role of Iberian ibex as a reservoir host for other susceptible species should be also considered in animal health policies.

4.5 Study V:

New insights on *Mycoplasma conjunctivae* transmission and within-host maintenance in wild and domestic hosts

Abstract

Mycoplasma conjunctivae is an obligate microparasite without cell wall that does not long resist environmental conditions and causes Infectious Keratoconjunctivitis (IKC) in Caprinae. Despite direct contact is considered the main way of IKC transmission, alternative routes and locations other than the eyes have not been thoroughly studied. In this study, the presence of *M. conjunctivae* has been assessed in non-ocular locations and vectors that could be involved in transmission and within-host persistence.

Mycoplasma conjunctivae was detected by qPCR in auricular and nose swabs of 7.3 % (CI 95% 4.6-11.6) and 8.2 % (CI 95% 5.1-12.9) of the ruminants sampled, respectively (Pyrenean chamois *Rupicapra pyrenaica*=108; Iberian ibex *Capra pyrenaica*=46, domestic sheep *Ovis aries* =75, Mouflon *Ovis aries musimon* =four). No differences among the species sampled were found. Its detection in nasal cavity was always associated with ocular infection. However, only 50.0% (8/16; CI 95% 28.0-72.0) of the *M. conjunctivae* detection in ear canals was associated with ocular infection. Among the eye-positive ruminants, 66.7% (CI 95% 46.7-82.0) were positive in nose swabs and 34.8% (CI 95% 18.8-55.1) in auricular swabs. Ct values of the qPCR were significantly higher in the ear than the nose and eyes and higher in the nose than the eyes. *Mycoplasma conjunctivae* was also detected in 7.1 % (CI 95% 2.0-22.6) of *Musca* sp. (n= 28) captured in an epidemic IKC scenario but not in an endemic IKC scenario (n= 86).

M. conjunctivae transmission probably occurs by direct contact from eye or nasal secretions and/or indirectly through flies, particularly from clinically affected individuals during IKC outbreaks. Ear canals may act as a relevant location for within-host *M. conjunctivae* persistence, which would have both epidemiological and clinical consequences.

Introduction

Infectious keratoconjunctivitis (IKC) is a highly contagious ocular disease that affects wild and domestic Caprinae (Giacometti et al., 2002a). It is typically caused by *Mycoplasma conjunctivae,* an obligate microparasite without cell wall that does not resist environmental conditions out of the host (Razin et al., 1998). *Mycoplasma conjunctivae* invades ocular structures and may cause severe clinical signs which may hamper visual performance or cause irreversible corneal perforation (Mayer et al., 1997). It is considered to exclusively affect ocular tissues since it has been consistently isolated from IKC-affected eyes and no lesions or health dysfunctions in other organs have been demonstrated in IKC outbreaks (Arnal et al., 2013a; Mayer et al., 1997). Hypermetric and circling movements occasionally described in severely IKC affected wild Caprinae are considered to be secondary to blindness and disorientation (Giacometti et al., 2002a). Within-host persistence of *M. conjunctivae* has been therefore only assessed in eyes but not in other anatomical locations (Giacometti et al., 1998; Janovsky et al., 2001).

However, other mycoplasmas can also be found in other body locations, such as ear canals, without causing clinical signs (Gómez-Martín et al., 2012; González-Candela et al., 2007). Auricular carriers of *M. mycoides* and *M. capricolum* are considered epidemiologically important for persistence and re-emergence of contagious agalaxia in domestic sheep and goat herds (Bergonier et al., 1997; DaMassa and Brooks, 1991). *Mycoplasma bovis* is also a major pathogen in cattle, causing otitis media and media-interna which may course with neurological clinical signs (Bertone et al., 2015; Francoz et al., 2004). Therefore, ear canals are important body locations for mycoplasma persistence in ruminants. However, the presence of *M. conjunctivae* in ear canals has not been assessed still in wild and domestic ruminants.

Within-host persistence and excretion of *M. conjunctivae* may influence transmission and infection dynamics in host populations (Swinton et al., 2009). Infectious keratoconjunctivitis epizootics are characterized by a rapid spread of the disease within a herd that largely affects most of the animals (Baas et al., 1977; Naglić et al., 2000). Experimental infections with *M. conjunctivae* demonstrated the delayed contagion of control sheep, chamois and ibex by

direct contact with the infected animals (Blancou et al., 1985; Degiorgis et al., 2000a; Giacometti et al., 1998). The isolation of *M. conjunctivae* from the nasopharynx and nasal secretions suggested the nose as an alternative excretion route apart from eye discharge (Baas et al., 1977; Dagnall, 1993). Direct contact among competent hosts is therefore considered the main interaction leading to *M. conjunctivae* transmission (Baas et al., 1977). However, IKC outbreaks in wild mountain ungulates may spread through several consecutive massifs at an average speed of 15 Km/year with peaks of incidence in spring and summer (Arnal et al., 2013a; Degiorgis et al., 2000b; Tschopp et al., 2005). These spatio-temporal patterns of IKC epidemics suggest that transmission of *M. conjunctivae* does not rely only on direct transmission between competent hosts (Rossi et al., 2007). Since IKC is an ocular disease that induces extensive ocular discharge, eye-frequenting flies have been proposed to have a role in *M. conjunctivae* transmission (Degiorgis et al., 1999). Flies stay in large numbers around outer mucosa and body secretions in livestock and wild ruminants, frequently feeding from lachrymal discharge (Hammer, 1941; Thomas and Jespersen, 1994). In fact, mechanical vectors are important for ocular disease contagion in humans (Forsey and Darougar, 1981) as well as in cattle (Glass and Gerhardt, 1984; Thomas and Jespersen, 1994). Given that direct contact between different host species is rare in alpine pastures (Richomme et al., 2006; Ryser-Degiorgis et al., 2002), flies can have a major role in cross-species transmission of *M. conjunctivae* and cause IKC outbreaks at the wildlife-livestock interface in alpine areas (Belloy et al., 2003a). Insights on the diversity of eye-frequenting insects in wild and domestic ruminants from alpine ecosystems have been already performed indicating that up to four genera of Muscidae (*Hydrotaea*, *Musca*, *Morellia* and *Polietes*) could be involved in IKC epidemiology (Degiorgis et al., 1999). However, *M. conjunctivae* have never been detected in eye-frequenting insects and its relative importance in the different IKC epidemiological scenarios has neither been assessed.

With the aim to investigate the potential within-host persistence in non-ocular locations and transmission routes, in this study the presence of *M. conjunctivae* is assessed in ear canals, nasal cavity and flies occurring around different host

species associated to different IKC epidemiological scenarios. The implications in IKC epidemiology and potential clinical alterations other than ocular disease are discussed.

Materials and Methods

Sample collection

Swabs without medium were introduced into both ear canals of 218 ruminants and into both nostrils in 195 of these ruminants (Table 4.5.1). Additionally, samples were obtained from each eye of the same ruminants by introducing swabs without medium beneath the third eyelid. The wild ruminants sampled were associated to an endemic IKC incidence area in the National Game Reserve (NGR) of Freser-Setcases (north-eastern Spain; Pyrenean chamois, *Rupicapra p. pyrenaica*, n= 108 and European mouflon, *Ovis aries musimon*, n= 2) and to severe IKC outbreaks in NGR Muela de Cortes (Eastern Spain, European mouflon, n=2) and from a captive population in Sierra Nevada (southern Spain; Iberian ibex, *Capra pyrenaica*, n= 46). The samples were obtained from recently hunted wild ruminants during the regular hunting season in the NGR of Freser-Setcases (Study III) and during routine handling procedures in the captive Iberian ibex population (Study IV). Four domestic sheep flocks with an endemic status of mostly asymptomatic *M. conjunctivae* infections were also sampled in Bellaterra, la Garriga, Puiggròs and Térmens (n=75) (northeastern Spain). All the swabs were frozen and stored at -20ºC less than 12 hours after collection until analyzed.

Flies in one of the sheep farms sampled and in the Iberian ibex enclosure were randomly captured with a butterfly net and introduced into individual sterile containers (Figure 4.5.1; Table 4.5.2). The flies were directly stunned in the freezer and were stored at -20ºC until analyzed. Previous to laboratory procedures, the fly species was determined for each specimen (D'Assis-Fonseca, 1968).
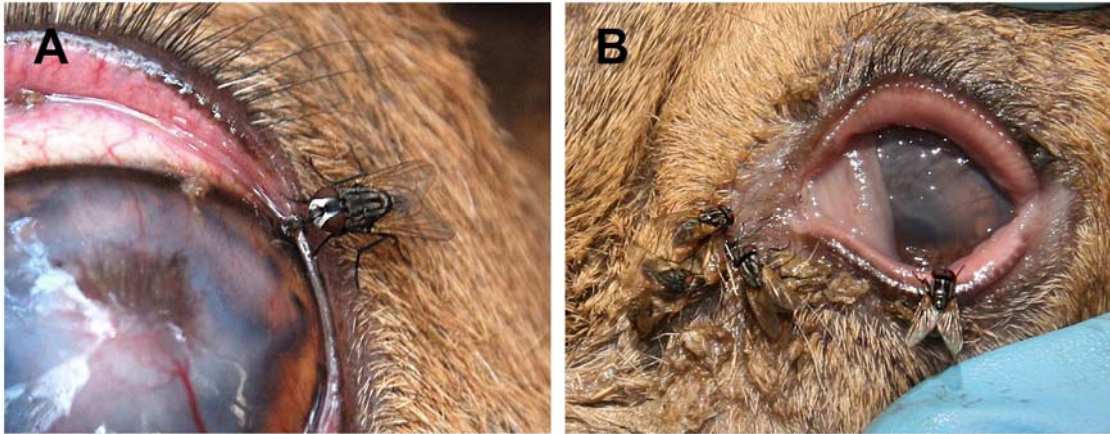
Figure 4.5.1 A) Female of *Musca* sp. feeding from an IKC affected eye of an Iberian ibex (*Capra pyrenaica*), spontaneously placed in the eye while the picture of the eyes lesions were taken. B) High numbers of flies are attracted by the induced eye discharge of IKC-affected Iberian ibex.

Sample preparation and detection of M. conjunctivae

The eye swabs were thawed and introduced in sterile tubes with 500 µl of lysis buffer (100 mM Tris–HCl, pH 8.5, 0.05 Tween 20, 0.24 mg/mL proteinase K). The eye swab samples were then mixed with a vortex during one minute, cell lysed for 60 minutes at 60ºC and then heated to 97ºC for 15 minutes to inactivate proteinase K.

The flies were introduced individually in sterile tubes with 300 µl of phosphate-buffered saline (PBS). Homogenates were then mechanically obtained with a tissue grinder pestle and subsequently vortexed for one minute.

The nucleic acids from the ear and nose swabs and the fly homogenates were purified with a commercial kit based on magnetic-particle technology (MagAttract® 96 *cador* ®Pathogen Kit, Qiagen Inc.) and the workstation 96 Biosprint (Qiagen Inc.). The detection of *M. conjunctivae* DNA was performed on the eye swab lysates and the purified DNA from ear, nose and flies homogenates with a qPCR as previously described (Vilei et al., 2007) (Annex II). Briefly, each reaction consisted in 2.5 µl of the sample, 900 nM of LPPS-TM-L primer and LPPS-TM-R, 300 nM of LPPS-TM-FT probe, 12.5 µl of TaqMan®2x Universal PCR MasterMix (Applied Biosystems, Warrington, UK), an exogenous internal positive control (IPC; Applied Biosystems, Warrington,

UK) and water up to 25 µl of volume. The threshold was set at 0.05 and the cycle number when the fluorescence crossed the threshold was recorded as the Ct value. The detection limit was established at a Ct value of 39 to detect low concentrations of the target DNA.

Data analyses

Differences among species and anatomic locations were assessed with two-sided chi-squared test for independence. Differences of Ct values of the *M. conjunctivae*-qPCR by anatomic locations were pairwise compared with a Wilcoxon signed-rank test for non-parametric distributions using the Bonferroni correction (Wilcoxon, 1945). Significance was set at 0.05 for all tests. The confidence intervals (CI) of the apparent prevalences were calculated with the "EpiR" package, and the graphics were performed with the "ggplot2" package, all from the R statistical software (R Development Core Team 3.1.3, 2015).

Results

Mycoplasma conjunctivae was detected in the eyes, ear canals and nose cavity in all the ruminant species tested (Table 4.5.1). The total prevalence of *M. conjunctivae* in the nose swabs was 8.2% (16/195; CI 95% 5.1-12.9) for all the species, and nose-positive swabs were only found when *M. conjunctivae* was also detected in the eyes. Among the eye-positive ruminants, 66.7% (16/24; CI 95% 46.7-82.0) were also positive in nose swabs. This percentage increased up to 78.9% (15/19; CI 95% 56.7-91.5) in the ruminants with Ct values lower than 35 in the eyes. *Mycoplasma conjunctivae* was detected in the ears in 7.3 % (16/218; CI 95% 4.6-11.6) of the ruminants sampled, but conversely to the nose swabs only 50.0% (8/16; CI 95% 28.0-72.0) of the ear-positive animals were also positive in the eyes. Among the eye-positive ruminants, 34.8% (8/23; CI 95% 18.8-55.1) were also positive in the auricular swabs. No substantial increase of this prevalence was observed, 38.9% (7/18; CI 95% 20.3-61.4), in ruminants with Ct values lower than 35 in the eyes. The detection of *M.*

conjunctivae by anatomic location and ruminant species is shown in Table 4.5.1.

Table 4.5.1 Samples and detection of *M. conjunctivae* in ear and nose swabs showed by species and by detection of *M. conjunctivae* in the eyes. Ruminants were considered positive in eyes if *M. conjunctivae* was detected in at least one eye.

	Ear		Nose	
	Pos./Tot.	**Prev. % (CI 95%)**	**Pos./Tot.**	**Prev. % (CI 95%)**
Sheep				
Positive in eyes	1/9	11.1 (0.6-43.5)	5/8	62.5 (30.6-86.3)
Negative in eyes	4/64	6.2 (2.4-15.0)	0/61	0.0 (0.0-5.9)
Total	5/73	6.8 (3.0-15.0)	5/69	7.2 (3.1-15.9)
Chamois				
Positive in eyes	4/8	50.0 (21.5-78.5)	8/11	72.2 (43.4-90.2)
Negative in eyes	3/87	3.4 (1.2-9.6)	0/83	0.0 (0.0-4.4)
Total	7/95	7.4 (3.6-14.4)	8/94	8.5 (4.4-15.9)
Iberian ibex				
Positive in eyes	2/4	50.0 (15.0-85.0)	1/3	33.3 (1.7-79.2)
Negative in eyes	1/42	2.4 (0.1-12.3)	0/25	0.0 (0.0-13.3)
Total	3/46	6.5 (2.2-17.5)	1/28	3.6 (0.2-17.7)
Mouflon				
Positive in eyes	1/2	50.0 (2.6-97.4)	2/2	100 (34.2-100.0)
Negative in eyes	0/2	0.0 (0.0-65.8)	0/2	0.0 (0.0-65.8)
Total	1/4	25.0 (1.3-70.0)	2/4	50.0 (15.0-85.0)
All species				
Positive in eyes	8/23	34.8 (18.8-55.1)	16/24	66.7 (46.7-82.0)
Negative in eyes	8/195	4.1 (2.1-7.9)	0/171	0.0 (0.0-2.2)
Total	16/218	7.3 (4.6-11.6)	16/195	8.2 (5.1-12.9)

Pos.=Positive; Prev.= Prevalence; Tot.= Total; CI = Confidence Interval

Mycoplasma conjunctivae prevalence was not statistically different among the anatomical sites studied. However, the median Ct values and its range were significantly ($p<0.01$) higher in the ears (38.2; 29.7-38.7) as compared to the eyes (27.6; 17-5-38.4) and the nose (33.0; 28.9-37.4), and significantly higher ($p<0.05$) in the nose as compared to the eyes (Figure 4.5.2).
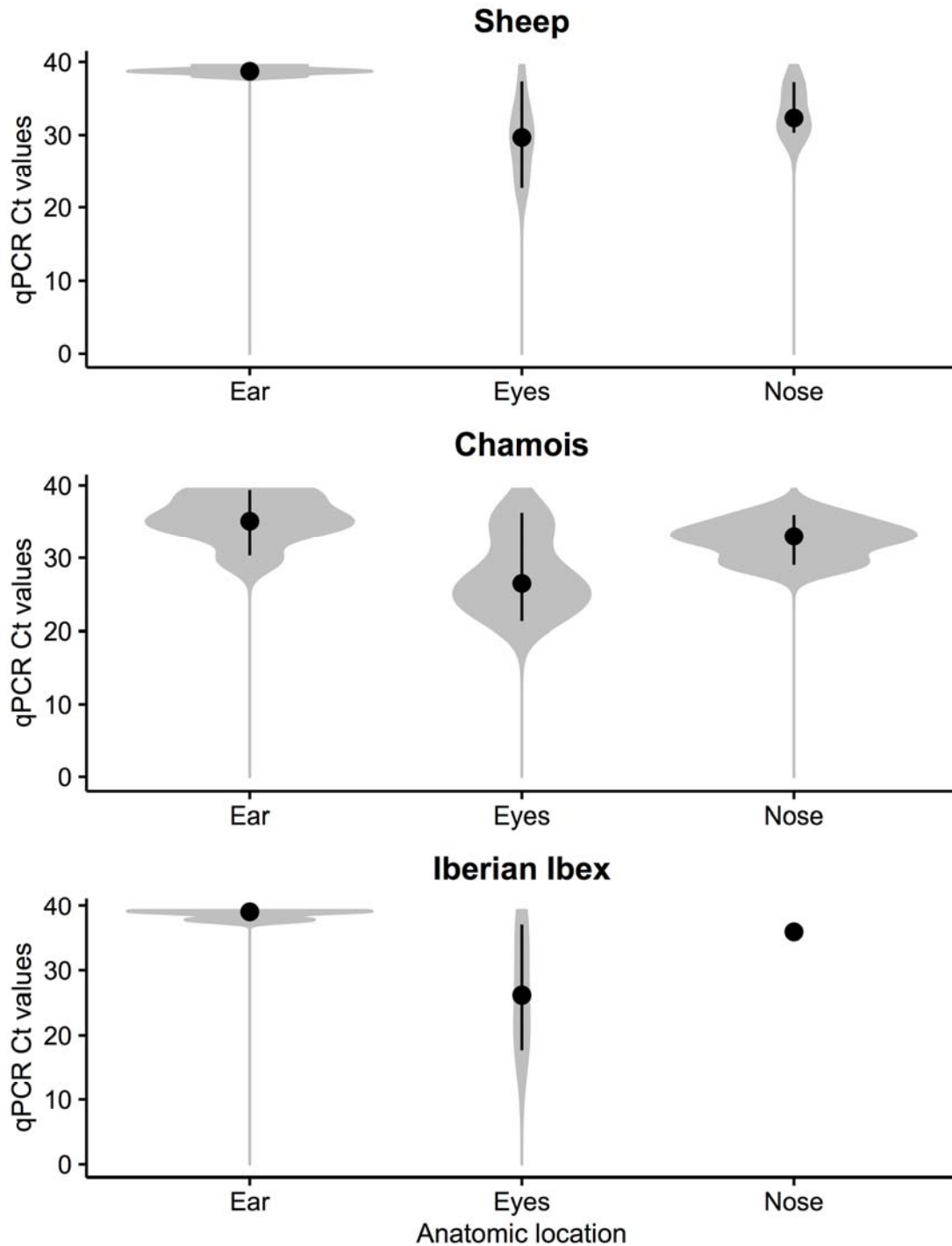
Figure 4.5.2 Distribution of Ct values of *M. conjunctivae* qPCR positive samples in violin plots by anatomic location and by ruminant species. Black dots indicate the median of the distribution and the bars shows the range of the second and third quartile.

The flies captured belonged to the genera *Musca* (Table 4.5.2). *Mycoplasma conjunctivae* was detected only in flies from the genera *Musca*, in 7.1 % (CI

95% 2.0-22.6) of the flies associated with the IKC outbreak in Iberian ibex (Table 4.5.2). The Ct values obtained in the two positive-qPCR flies were 35.82 and 38.9.

Table 4.5.2 Results of *Mycoplasma conjunctivae* on eye-frequenting insects captured meanwhile ruminant hosts were sampled.

	Herd Prev.*	Pos.	Total	Prev. (CI 95%)	Date
Sheep flock 1 (IKC endemic)					
Musca sp.	6.6% (1.8-21.3)	0	86	0.0 % (0.0-4.3)	10/08/2016
Iberian ibex (IKC epidemic)					
Musca sp.	8.7 (3.4-20.3) ‡	2	28	7.1 % (2.0-22.6)	26/09/2014

Pos.=Positive; Prev.=Prevalence; Tot.=Total
* Prevalence obtained by ocular detection in any of both eyes.
‡ Described in the Study IV of this thesis.

Discussion

This study provides new insights on *M. conjunctivae* transmission and within-host persistence by assessing its presence in non ocular locations. Despite there were several evidences from observational IKC studies that *M. conjunctivae* did not rely only on direct contact between competent hosts, to the authors knowledge, this is the first time that *M. conjunctivae* is detected in flies. The quantified DNA detection in eyes, nose cavity and flies unveils the transmission mechanisms of *M. conjunctivae*, which may occur by direct or close contact through ocular or nasal secretions of competent hosts, and/or potentially by indirect interactions at larger distances by flies. Both kinds of interactions occur among ruminants at the wildlife-livestock interface in alpine and subalpine ecosystems (Richomme et al., 2006; Ryser-Degiorgis et al., 2002). Additionally, the finding of *M. conjunctivae* in the ear canals, particularly in animals where it was not detected in the eyes, indicates that *M. conjunctivae*

can be maintained in the ears, which probably has both epidemiological and clinical implications.

Despite IKC is considered a specific ocular disease, *M. conjunctivae* was previously reported in nasal smears (Surman, 1973), as well as isolated from the nasopharynx (Baas et al., 1977; Dagnall, 1993). Yet, up to 10^7 cfu/ml of *M. conjunctivae* have been reported in nasal secretions of experimentally infected sheep indicating that it may be a major source of *M. conjunctivae* transmission (Dagnall, 1993). In the present study, the slightly higher Ct values in nose swabs than eye swabs indicate that similar loads of mycoplasma cells can be present in eye and nose secretions. Since *M. conjunctivae* has been detected in the nose only when it was also detected in the eyes, and it is not commonly detected in nasal swabs from sheep (Brogden et al., 1988), the nasal cavity is probably an excretion route but not a maintenance site for *M. conjunctivae*. Despite an early report describes the *M. conjunctivae* isolation from the lungs of an IKC-affected chamois (Nicolet and Freundt, 1975), the detection of *M. conjunctivae* is not consistent with respiratory tract lesions in most of the literature and it is not typically considered to be able to infect the oropharynx, trachea or lungs (Giacometti et al., 2002a).

Apart from direct contacts between hosts, *M. conjunctivae* transmission is probably enhanced by flies during the vector season, which may explain the peaks of disease incidence commonly observed in spring and summer during IKC epidemics (Arnal et al., 2013a; Giacometti et al., 2002b; Tschopp et al., 2005). Transmission of *M. conjunctivae* by flies could be either mechanical by superficial contact with infectious secretions of the host or by regurgitations of the crop of the flies in the eyes, as demonstrated with *Moraxella bovis* and ocular disease in cattle (Glass and Gerhardt, 1984). The *M. conjunctivae* detection in *Musca sp.*, which commonly feeds around the eyes and the nose of ruminants (Thomas and Jespersen, 1994), indicate that flies from this genera may be relevant vectors for IKC. However, given the high diversity of fly species found in natural systems (Hammer, 1941) with different life cycles and seasonal dynamics, many other insect species may probably be involved in IKC epidemiology (Degiorgis et al., 1999).

Prevalence of *M. conjunctivae* in eye-frequenting insects and its role in IKC transmission is probably dependent on the infectious status of the frequented hosts and on its own abundance and dynamics. The detection of *M. conjunctivae* in flies only in the IKC epidemic scenario (Iberian ibex) suggests a more relevant role of flies in *M. conjunctivae* transmission during epizootics, which are associated with high *M. conjunctivae* loads and severe IKC, lachrymation and ocular secretions (Mavrot et al., 2012a; Mayer et al., 1997). Conversely, the lower *M. conjunctivae* eye loads and the absence of ocular discharge in asymptomatic infections probably reduce the probability of transmission by flies in endemic scenarios (Study I and II) (Mavrot et al., 2012a).

In contrast to the nose, the presence of *M. conjunctivae* in the ear canals of sheep, Pyrenean chamois, Iberian ibex and mouflon was not necessarily related with its presence in the eyes, which supports that *M. conjunctivae* can persist in ear canals post ocular infection. Other mycoplasmas have been previously found in ear canals of ruminants with a prevalence of 8% for *M. mycoides* subsp. *mycoides* large colony in asymptomatic goat herds (Tardy et al., 2007) or 12.7% for *M. agalactiae* in free-ranging Iberian ibex (González-Candela et al., 2007). In fact, ear canals are considered a good ecological niche with a lower immune pressure of the host than other anatomical locations (Bergonier et al., 1997). Auricular carriers have therefore relevant epidemiological implications for the emergence and re-emergence of mycoplasmosis within host herds (Gómez-Martín et al., 2012; Tardy et al., 2007), and may contribute to the *M. conjunctivae* maintenance in wild Caprinae populations with a steady low incidence of infections (as observed in NGR Freser-Setcases, Study III). However, the *M. conjunctivae* prevalence in the ear canals was in general lower, either in IKC epidemics (Iberian ibex) or endemic scenarios (sheep and chamois), than the previously reported for other mycoplasmas. Whether the ear canals are a good body site for *M. conjunctivae* individual persistence would probably need a longitudinal sampling to be elucidated.

The detection of *M. conjunctivae* in the ear canals could also have clinical consequences. *Mycoplasma bovis* is the main cause of otitis media and otitis

media-interna in cattle, which may show neurological signs, such as head tilt, gait abnormalities and vestibular ataxia (Bertone et al., 2015; Francoz et al., 2004; Van Biervliet et al., 2004). The neurological clinical signs observed in wild Caprinae affected by IKC are also consistent with a peripheral vestibular syndrome (Figure 4.5.3). However, investigations on these behavioural abnormalities have been only conducted on the central nervous system without pathological findings (Arnal et al., 2013a; Mayer et al., 1997), but not in the internal and middle ear. Therefore, the neurological signs associated with IKC and attributed up to now to blindness and disorientation (Degiorgis et al., 2000b; Giacometti et al., 2002a), could be originated by the colonization of the medium or inner ear by *M. conjunctivae*.

Figure 4.5.3 Still frames of a recoded circling behaviour in a severely IKC affected Pyrenean chamois (*Rupicapra pyrenaica*) from the National Game Reserve Alt Pallars (Eastern Pyrenees), in 2015. Pathological nystagmus to the same circling side is also possible to observe in the original video (author: Jaume Montané).

In conclusion, *M. conjunctivae* transmission probably occurs by direct contact from eye or nasal secretions and/or indirectly through flies, particularly from clinically affected individuals during IKC outbreaks. Transmission of *M. conjunctivae* by flies probably increases the spread of IKC, enables the

contagion in low density host populations and/or in spatially grouped hosts such as wild Caprinae (Manlove et al., 2014), and is probably a major factor in cross-species transmission. Ear canals may act as a relevant location for within-host persistence of *M. conjunctivae*, which would have both epidemiological and clinical consequences. However, further research on this issue is required in order to ascertain this.

5. GENERAL DISCUSSION

The interaction of *Mycoplasma conjunctivae* with its hosts and the outcome of infectious keratoconjunctivitis (IKC) have been investigated in this thesis in the frame of host-pathogen systems at the wildlife-livestock interface from different countries. Despite IKC is a common and old-known disease, the wide range of clinical outcomes, epidemiological scenarios and contrasted observations of *M. conjunctivae* persistence and fading out had complicated investigations on host functional roles in alpine systems. These are indeed common epidemiological features of mycoplasmas, which are bacteria able to modulate its interaction with the host, resulting in eventual asymptomatic infections but with a high potential for virulent disease emergence and re-emergence (Citti and Blanchard, 2013; Hawley et al., 2013). Mycoplasmas have one of the highest mutation rate among bacteria and evolve fast to adapt to different host environments (Delaney et al., 2012; Woese et al., 1985). Moreover, phase and antigenic membrane variation in mycoplasmas provides the means to overcome host acquired immunity, establish chronic infections and enable re-infections (Baranowski et al., 2014; Citti et al., 2010). Host-mycoplasma interactions, that rely on the adaptive response of mycoplasmas and the host immunity pressure, are dynamic and may change and evolve over time as long as mycoplasmas can circulate within a host population and chances for novel interactions are provided (Hawley et al., 2010; Osnas et al., 2015; Williams et al., 2014).

Mycoplasmas are therefore particularly problematic pathogens causing human and animal disease that may involve complex and dynamic interactions in host-pathogen systems (Rosengarten et al., 2000). From this perspective it is deduced that general assumptions based on local systems would probably not be consistent with the wide range of the mycoplasma-host interactions that can be established elsewhere.

5.1 Domestic sheep and *Mycoplasma conjunctivae*

Mycoplasma conjunctivae is commonly found in domestic sheep, either causing IKC outbreaks (Baker et al., 2001; Naglić et al., 2000) or asymptomatic infections (Jones et al., 1976; Ter Laak et al., 1988b). It was demonstrated that *M. conjunctivae* is endemic in almost 90% of the sheep flocks from Switzerland (Janovsky et al., 2001) and endemic infections probably occur widespread in other European countries (Akerstedt and Hofshagen, 2004; Baker et al., 2001; Ter Laak et al., 1988b; Van Halderen et al., 1994). Infectious Keratoconjunctivitis outbreaks have also been described in sheep flocks elsewhere in the world (Lysnyansky et al., 2007; Motha et al., 2003; Van Halderen et al., 1994). However, epidemiological data of targeted surveillance on *M. conjunctivae* was in general lacking in small domestic ruminants, especially by using other than culture-based techniques. In this thesis, endemic infections have been found in most of the sheep flocks sampled in the Pyrenees (Spain) and in remote villages from the Central Karakoram (Pakistan). These endemic scenarios are moreover associated with highly prevalent (19% - 26%) and mostly asymptomatic infections (87% - 88%). In the Study I and II, ocular disease in endemic sheep and goat mixed flocks has been found to be sporadic and in general milder than in IKC epizootics. Altogether highlights a common epidemiological scenario in sheep flocks worldwide where *M. conjunctivae* is maintained with a reduced host response and fitness cost. Sheep is therefore a good host for the endemic and mostly asymptomatic maintenance of *M. conjunctivae*, and it has been probably linked to the natural history and the ecology of this mycoplasma. It would also explain the worldwide distribution of *M. conjunctivae* despite being a specific ocular pathogen that only infects Caprinae (Surman, 1973). Sheep could be considered the natural host that has been probably commonly involved in the introduction of *M. conjunctivae* at the wildlife-livestock interface (Belloy et al., 2003a; Marco et al., 1991; Zimmermann et al., 2008). Consequently, the low rate of *M. conjunctivae* detection in sheep flocks from the Cantabrian Mountains was consistent with the absence of IKC and *M. conjunctivae* detection in the sympatric chamois (Study I). Accordingly, the first IKC epizootics in chamois from the Pyrenees appeared for the first time in 1980, when chamois populations were abundant and have recovered from its

scarcity in mid XIXth century (Gortazar et al., 1998), suggesting that IKC in chamois was originated by a spillover event from a reservoir host (Annex I). The correspondence of the epidemiological scenario between sheep and chamois in the alpine ecosystems from Northern Spain has been also described for other infectious diseases (Fernández-Aguilar et al., 2016). Therefore, despite transitions of *M. conjunctivae* among different host species do not seem to be common in the Pyrenees (Study III), it can have important epidemiological implications.

5.2 *Mycoplasma conjunctivae* dynamics and persistence in wild host populations

Infectious keratoconjunctivitis has been extensively investigated in mountain ungulates. However, investigations have been mainly based on syndromic surveillance (clinical signs) or in short-term studies focused on *M. conjunctivae* but without a comprehensive sampling representatively including the susceptible host community. Throughout the studies reported in wild hosts from the Alps and the Pyrenees, persistence of *M. conjunctivae* in a particular host species or community has been controversial because of the commonly observed disappearance of ocular disease after IKC epizootics (Arnal et al., 2013a; Deutz et al., 2004; Giacometti et al., 2002a, 2002b; Tschopp et al., 2005). However, sporadic IKC may eventually occur after IKC outbreaks in wild Caprinae, and persistence in these wild host populations has been discussed based on the detection of asymptomatic infections (Mavrot et al., 2012a; Ryser-Degiorgis et al., 2009). In the different studies of this thesis, it has been shown that asymptomatic infections can be relatively high in IKC epidemics (higher re-infections and initial and recovery IKC stages) as well as in non-epidemic IKC (milder infection processes). This is further supported by previous reports of *M. conjunctivae* asymptomatic infections in diverse hosts and epidemiological scenarios (Giacometti et al., 1998; Janovsky et al., 2001; Mavrot et al., 2012a). However, asymptomatic infections do not provide relevant information on persistence if they are not integrated with the dynamics of the pathogen in the

population (i.e. total prevalence and temporal trends). An integrative approach combining large spatial and temporal data of *M. conjunctivae* prevalence and molecular epidemiology from all competent hosts is therefore needed to yield conclusive information on the functionality of host populations in the systems (Viana et al., 2014).

In the Study III, the consistent detection of specific *M. conjunctivae* genetic clusters during nine-year and six-year periods in wild Caprinae (i.e. Pyrenean chamois and European mouflon) indicates that *M. conjunctivae* may eventually persist in sylvatic cycles and IKC cases are not necessarily originated by contemporary strains of domestic hosts. The consistent detection of *M. conjunctivae* clusters in wild Caprinae has also been described in the Alps, but without information of the strains circulating in sympatric domestic sheep (Gelormini et al., 2016). In fact, shared *M. conjunctivae* strains between domestic and wild hosts have been previously reported in the Alps (Belloy et al., 2003a; Zimmermann et al., 2008). Therefore, *M. conjunctivae* persistence in Alpine areas may rely on complex multi-host systems (Haydon et al., 2002). The seasonal contribution of domestic sheep for the maintenance and circulation of *M. conjunctivae* strains in natural systems from the Alps should be still better assessed. The present thesis provides for the first time comprehensive epidemiological data on *M. conjunctivae* long-term persistence in wild hosts from the Pyrenees. The fading out of *M. conjunctivae* in the study areas were past IKC outbreaks have occurred (Study III; Annex I) also highlight the diverse dynamics that *M. conjunctivae* can establish in free-ranging host populations, and that both persistence and fading out may occur. This is consistent with previous studies that concurred with sporadic IKC clinical cases after epizootics (Arnal et al., 2013a; Gauthier, 1991) and the disappearance of the disease (Arnal et al., 2013a; Giacometti et al., 2002b; Tschopp et al., 2005).

Infectious keratoconjunctivitis epizootics may however extend through large areas (Arnal et al., 2013a; Gauthier, 1991; Gelormini et al., 2016), and may combine recurrent patterns of local peaks of IKC incidence followed by temporal fading out, as observed in the Study III. Epidemiological areas for *M. conjunctivae* maintenance can be huge and involve different countries because high altitude mountains are common barriers that determine country borders

(Gauthier, 1991; Gelormini et al., 2016). This complicates the assessment of *M. conjunctivae* dynamics and persistence in free-living hosts, which needs long time periods and large areas of surveillance beyond country borders. Movement of hosts, which determine interactions within and among species, populations and subpopulations, can be influenced by landscape features and connection of favourable habitats (Loison et al., 1999; Marchand et al., 2017), and probably determine the epidemiological units where *M. conjunctivae* strains can be maintained (Study III).

5.3 Host-mycoplasma interaction and within-host persistence

Diverse host-mycoplasma interactions can be inferred from the epidemiological data obtained in the studies of this thesis. On the one hand, the high prevalence of mostly asymptomatic *M. conjunctivae* infections in domestic sheep flocks and in the post-epizootic persistence in Iberian ibex (Studies I, II, III and IV), suggests a balance between the immune response of the hosts and the invasiveness of the mycoplasma, causing minimal tissue damage and clinical signs (McGowin et al., 2012). Lower virulence and lighter ocular clinical signs reduce mycoplasma excretion and consequently its transmission (Williams et al., 2014). Therefore, the higher *M. conjunctivae* prevalence detected in these endemic scenarios, associated with mostly asymptomatic infections, is presumably achieved by a chronic persistence in the eyes rather than to a higher transmission rate (Janovsky et al., 2001; Ter Laak et al., 1988a).

On the other hand, the severe clinical signs observed in the IKC outbreak from the Study IV were mostly associated with the clearance of *M. conjunctivae* from the eyes once the clinical signs were resolved. This is in contrast with the results obtained in the endemic scenarios described (Studies I, II and post-epizootic persistence of Study IV) and with the *M. conjunctivae* ocular persistence reported in Alpine ibex and domestic sheep after mild IKC (Giacometti et al., 1998; Janovsky et al., 2001). In wild Caprinae, severity of IKC clinical signs is related with mycoplasma loads (Mavrot et al., 2012a; Ryser-Degiorgis et al., 2009) (Study IV). Higher within-host rate of pathogen replication (loads) can induce greater tissue damage and inflammation, which

may stimulates the up-regulation of the immune response of the hosts and enhance the clearance of the pathogen (Alizon, 2008; Frank and Schmid-Hempel, 2008). Persistence of *M. conjunctivae* in the eyes after clinical recovery may therefore be negatively related with the exhibited clinical signs. This is consistent with the findings of prevalence and clinical signs in the two consecutive epidemiological scenarios observed in the Study IV.

The IgG systemic response of the IKC-affected Iberian ibexes showed that neither *M. conjunctivae* ocular infection nor clinical signs necessarily induce a systemic response of this immunoglobulin. Because *M. conjunctivae* mainly causes ocular infection, local immunoglobulins and/or cellular immunity are probably the main host drivers of the infection and determinants for the *M. conjunctivae* persistence in the eyes (Avakian and Ley, 1993; Burns et al., 1982). The location of *M. conjunctivae* in ear canals without ocular infections described in the Study V may have important implications for *M. conjunctivae* within-host persistence, which warrant further research.

5.4 Epidemic and non-epidemic *Mycoplasma conjunctivae* infections

Pathogen virulence evolves with transmission and is shaped by trade-offs associated with the infectious period of the host (Alizon et al., 2009; Frank, 1996; May and Anderson, 1983). Waning virulence is therefore a common long-term trend observed in epizootics of infectious diseases and is also suggested for mycoplasmosis (Hawley et al., 2013; Williams et al., 2014). Considering disease-induced mortality as an indicator of virulence (Ebert and Herre, 1996), the emergence of highly virulent IKC epidemics in free-ranging hosts may also evolve to a reduced virulence along with its geographical spread, as indicated by the mortality patterns reported in different epidemic events (Figure 5.1).
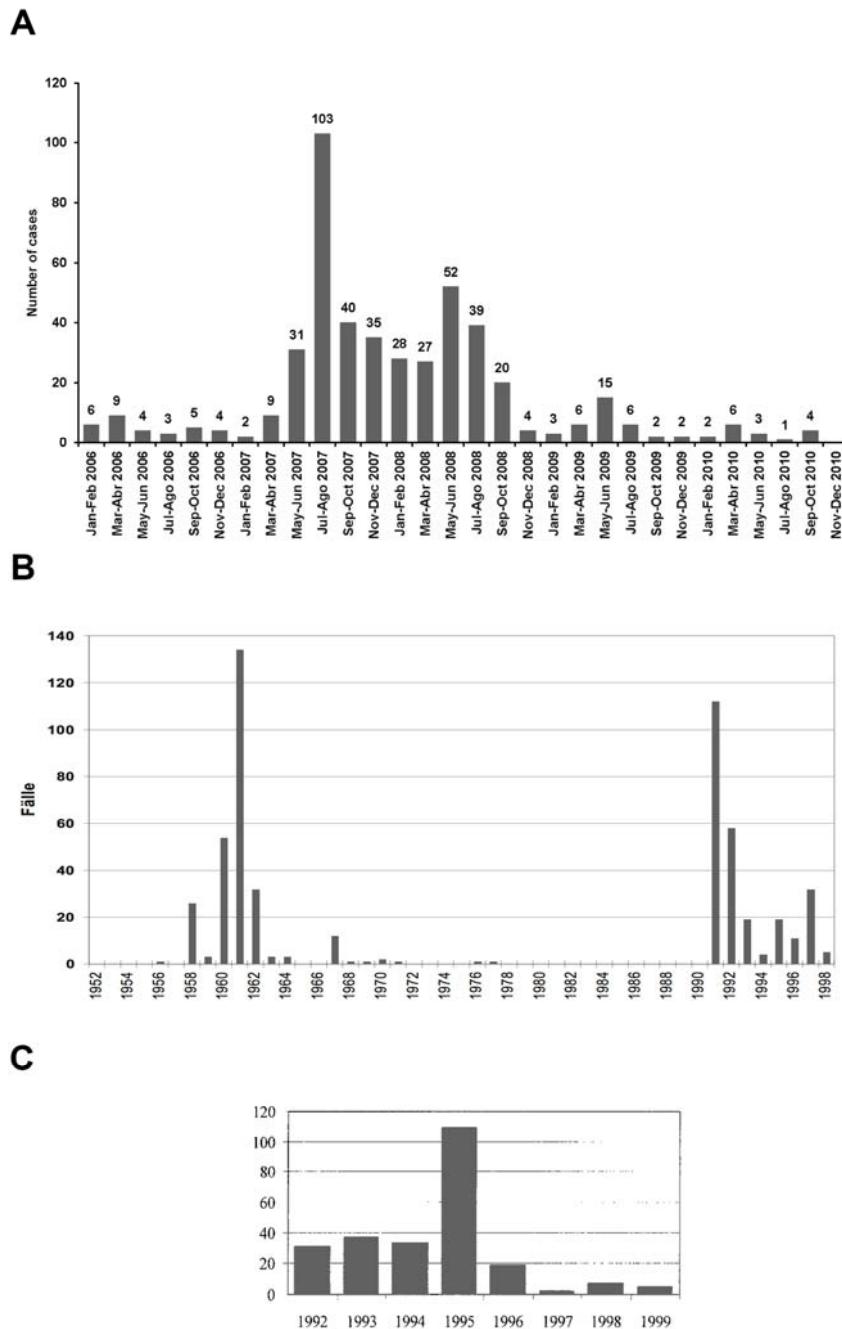
Figure 5.1 Temporal patterns of virulence of IKC epidemics, based in disease-induced mortality (Ebert and Herre, 1996), in different outbreaks described in chamois species. A) Total mortality of the Pyrenean chamois in Aragón, North-East Spain, by months during an IKC outbreak 2007-2009 (Arnal et al., 2013a). B) Historical IKC cases by year registered in the Austrian Alps (Deutz et al., 2004). C) Incidence of IKC-specific mortality cases in Grisons, Switzerland (Giacometti et al., 2002b).

However, the emergence of highly virulent strain variants may be favoured in the short-term and pathogens would not necessarily evolve to the lowest possible level of virulence (Hawley et al., 2013), especially in structured and sparse host populations (Boots et al., 2004).

These two virulence evolution patterns (virulence decrease and maintenance of high virulence) could be inferred associated with *M. conjunctivae* persistence in the host populations studied in this thesis. The Study IV provided an opportunity to follow-up the interaction of *M. conjunctivae* with a highly dense population of a wild host (under captive conditions) whose epidemiological scenario drastically changed from a severe IKC outbreak to high prevalence of asymptomatic infections one year after. This corresponds with the classical theoretical framework of virulence evolution in highly connected host populations (Alizon et al., 2009; Boots et al., 2004). Similar interaction processes may have occurred in other high-density host populations studied in this thesis, where *M. conjunctivae* persistence was related to lower health impact, as observed in a free-ranging Pyrenean chamois population (National Game Reserve Freser-Setcases; Study III) and in domestic sheep flocks (Studies I and II). Acquired immunity may influence the clinical outcome and transmission by limiting the mycoplasma loads in the eyes.

An opposite situation was observed in the low-density free-ranging chamois populations studied (i.e. National Game Reserve Alt Pallars and Vall Aran; PyAP and PyVA; Study III), where *M. conjunctivae* persistence was associated with an epidemic spread causing localized outbreaks. The long-term epidemic spread of specific *M. conjunctivae* strain clusters has been also reported in wild host populations from the Alps (Gelormini et al., 2016), and it is also consistent with historical records of IKC epizootics (Gauthier, 1991). Group-living behaviour and structured host populations may favour the maintenance of epizootic disease, which may leap between successive groups and subpopulation units (Boots et al., 2004; Deredec and Courchamp, 2003; Manlove et al., 2014).

Eye-frequenting insects may enhance *M. conjunctivae* transmission and can be especially important in host populations with low interactions between groups or

subpopulations (Crampe et al., 2007; Pépin and Gerard, 2008; Tschopp et al., 2005).

5.5 Host species differences in IKC susceptibility

Early reports already suggested different susceptibility of Alpine chamois and Alpine ibex to IKC (Couturier, 1962), which was later reinforced in other studies (Ryser-Degiorgis et al., 2009). In the Study III performed in the Pyrenees, the sporadic detection of *M. conjunctivae* in mouflon only one year and only associated with asymptomatic infections also suggests differences of host susceptibility to the circulating *M. conjunctivae* strains as compared to the sympatric Pyrenean chamois. However, these differences are generally difficult to assess only with observational studies in which the preceding host-pathogen interaction is not usually known. Moreover, the interactions between *M. conjunctivae* and its hosts can be highly dynamic, and the conditions of a specific population (e.g. aggregation) may significantly influence the outcome of the infection. For example, the epidemiological scenario of *M. conjunctivae* persistence described in the Iberian ibex (Study IV) was likely not exclusively related to the species traits, but also to the captive conditions that maintained higher density and aggregation than free-ranging populations (Acevedo and Cassinello, 2009).

In spite of such population-related factors, the susceptibility of domestic and wild hosts to *M. conjunctivae* may however be different as indicated by the different relationships between *M. conjunctivae* Ct values in the qPCR (proxy of bacterial loads) and the severity of ocular clinical signs observed in the different studies of this thesis. Whereas high loads of *M. conjunctivae* are commonly detected in asymptomatic domestic sheep (Study II), *M. conjunctivae* loads are positively correlated with clinical signs in wild Caprinae, and asymptomatic infections typically have low bacterial loads in Iberian ibex (Study IV), Alpine ibex and Alpine chamois (Mavrot et al., 2012a; Ryser-Degiorgis et al., 2009). Given that domestic sheep can also suffer from severe IKC outbreaks (Baker et al., 2001; Naglić et al., 2000), high loads of *M. conjunctivae* in asymptomatic infections most probably are derived from a long-term host-pathogen adaptation

process of the circulating strains. A reduced promotion of lesions and inflammatory responses caused by *M. conjunctivae* strains that persist in endemic sheep flocks may also explain these differences (Ley, 2008). However, this interaction (i.e. common cases of high loads of *M. conjunctivae* in asymptomatic infections) has never been described in wild hosts. Further research would be needed to clarify whether it responds to specific traits of domestic sheep or it is derived from a long-term adaptation process in a highly dense and aggregated host population.

5.6 Closing remarks, IKC management and research at the wildlife-livestock interface

Mycoplasma conjunctivae is the unequivocal main agent causing infectious keratoconjunctivitis in wild and domestic Caprinae in Alpine ecosystems and mountain areas from Europe and probably in other mountain ranges worldwide. It is further identified as one of the most common pathogens found in domestic sheep worldwide. In co-infection, other infectious agents such as *Chlamydiaceae* may influence the expression of clinical signs, especially in non-epidemic *M. conjunctivae* infections.

Mycoplasma conjunctivae generally results in clinical disease in free-ranging wild Caprinae, and its influence on their population dynamics is far more important than in domestic hosts. However, efficient control measures of IKC in wild Caprinae are not feasible or known at the moment, and preventing contact with the infectious agent is the only management recommendation (Loison et al., 1996). Healthy carriers in domestic livestock may favour the introduction of *M. conjunctivae* in alpine ecosystems and be a source of IKC outbreaks for wild ruminants (Egwu et al., 1995; Naglić et al., 2000). However, highly virulent strains that may arise sporadically in endemic livestock flocks and that can exert a strong impact in wildlife may probably be detected by syndromic surveillance (Baas et al., 1977; Baker et al., 2001). Livestock showing ocular clinical signs should be treated with antibiotics before grazing in mountain areas where endangered or locally scarce wild Caprinae inhabit.

The adaptability of *M. conjunctivae* to different host environments promotes a range of interactions that entail different epidemiological scenarios. Thus, the functional roles of each host species may differ in complex natural systems and long-term dynamics depend on multiple factors that are not necessarily directly related with mycoplasma and/or the host species. Population characteristics, such as density, and landscape-derived factors in free-ranging populations may modulate disease transmission rate (Swinton et al., 2009), the infection dynamics and ultimately the evolution of pathogen virulence (Boots et al., 2004; Marchand et al., 2017). High density of hosts probably provides favourable conditions for *M. conjunctivae* re-infection and circulation (Study IV), which in turn may drive the host-pathogen interaction and the infection dynamics. Further research is needed to better understand the influence of factors shaping *M. conjunctivae* dynamics in free-ranging host populations.

A collection of *M. conjunctivae* strains isolated from different hosts and epidemiological scenarios would help to identify the molecular mechanisms and genes related with disease virulence, as well as their evolution in relation with host population characteristics. The conditions associated with highly virulent strains may also provide information on the potential population and/or environmental factors that can predispose the emergence of virulent strains at the wildlife-livestock interface. Strain characterization should ideally involve several conserved genes or full genome sequencing to be able to perform more precise phylogenetic relationships between isolates, which would also help to predict historic and contemporary host transitions in the systems studied (Kamath et al., 2016).

6. CONCLUSIONS

CONCLUSIONS

1. Among the ruminant species sampled, only Caprinae are competent hosts for *Mycoplasma conjunctivae*, including the Iberian ibex where IKC epizootics have been described for the first time.

2. *Mycoplasma conjunctivae* is commonly endemic with high prevalence and a low biological cost (i.e. clinical signs and body index) in small domestic ruminant flocks that seasonally graze in alpine meadows.

3. Domestic sheep flocks are proper host populations to maintain *M. conjunctivae* and a common reservoir in multi-host alpine systems.

4. Independent domestic and sylvatic cycles occur in the Central and Eastern Pyrenees along with sporadic cross-species transmission.

5. *Mycoplasma conjunctivae* dynamics in Pyrenean chamois vary depending on population characteristics and may range from fading out to the endemic or epidemic long-term persistence without the substantial contribution of other hosts.

6. The role of European mouflon in IKC epidemiology in the Eastern Pyrenees is negligible.

7. The clinical expression of *M. conjunctivae* infection is not intrinsically related to host-species but to a specific host-pathogen interaction, which in turn influences infection dynamics in the host population.

8. High host densities favour the eventual endemic *M. conjunctivae* persistence in wild host captive and free-ranging populations.

9. Eye-frequenting flies, nasal and ocular secretions are direct and indirect *M. conjunctivae* transmission mechanisms. The role of vectors is probably positively related to the severity of the clinical signs.

10. *Mycoplasma conjunctivae* can be located in the ear canals independently of concurrent ocular infections, which may enhance *M. conjunctivae* persistence within the host population.

7. ACKNOWLEDGEMENTS

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9. ANNEXES

9.1 ANNEX I: Historical records of IKC outbreaks in the Pyrenees

Records of IKC outbreaks in Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) until 2008 are subsequently reported based on available data of research articles, internal reports and other non published information. Data of morbidity and mortality is an approximate estimation in most of the reports.

Year	Zone	Area	Population affected	Morbidity	Mortality	References
1980-1981	Ordesa y Monte perdido NP Los Circos NGR Benasque NGR Viñamala NGR Los Valles NGR Pyrénées NP Moudang Reserve	Central Pyrenees	All chamois from Central Pyrenees	High	10-15%	Catusse, 1982; Müller, 1984
1981-1983	Benasque NGR Vall Aran Alt Pallars NGR Aigüestortes NP Biros, Haut Salat, Vicdessos, Aston, Aute Ariège	Central and Eastern Pyrenees	Unknown	High	10-15%	Catusse, 1982; Müller, 1984
1983-1984	Isolated cases same previous	Central and Eastern	Unknown	Unknown	Unknown	Pluye, 1986

	areas	Pyrenees				
1987-1988	Pyrénées NP (Cauterets, Arrens, Luz)	Central Pyrenees	800-950	80%	1-3% (11 carcasses detected)	Crampe, 1992
1990	Aigüestortes NP Alta Ribagorça	Eastern Pyrenees	900	50-60%	10%	Marco et al., 1991; Marco and Gonzalo, 1991
1991	Cadí-Moixeró NGR	Pre-Pyrenees (Eastern)	400	40-50%	10%	Viñas et al., 1991
1993	Aigüestortes NP	Eastern Pyrenees	150	40-50%	5-10%	Internal Report ‡
1995	Cadí-Moixeró NGR	Pre-Pyrenees (Eastern)	1600	40-50%	1-3%	Internal Report ‡
1995	Fresser- Setcases NGR*	Eastern Pyrenees	2000	80-90%	5%	Internal Report ‡
1996-1997	Aigüestortes NP	Eastern Pyrenees	Unknown	Unknown	Unknown	Pañella et al., 2010
2004-2005	Aigüestortes NP	Eastern Pyrenees	Unknown	Unknown	Unknown	Pañella et al., 2010
2006-2007	Alt Pallars Vall Aran	Eastern Pyrenees	Unknown	Unknown	Unknown	Marco et al., 2009a

| 2006-2008 | Ordesa y Monte perdido NP
Los Circos NGR
Benasque NGR
Viñamala NGR
Los Valles NGR | Central Pyrenees | All chamois from Central Pyrenees (15,000 in Spanish side) | 15% | Arnal et al., 2013a; Crampe, 2008 |

NGR = National Game Reserve; NP = National Park.
* Since this first IKC outbreak, sporadic IKC cases occur yearly in this area.
‡ Servei d'Ecopatologia de Fauna Salvatge (SEFaS), Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona.

9.2 ANNEX II: Primers and probes

Primers and probes used in this thesis for real-time PCR (qPCR), PCR, nested PCR and Sanger DNA sequence analysis.

Primer	Target gene	Sequence (5'-3')	Use	Reference
Mycoplasma conjunctivae				
LPPS-TM-L	_LppS_	CAGCTGGTGTAGCACTTTTTGC	qPCR	Vilei et al., 2007
LPPS-TM-R	_LppS_	TTAACACCTATGCTCTCGTCTTTGA	qPCR	Vilei et al., 2007
LPPS-TM-FT	_LppS_	6FAM-TGCTTCGACTACCAAATA TGATGGTGATCCTCT-TAMRA	TaqMan probe	Vilei et al., 2007
Serstart3	_LppS_	TTTAGTAGACTCCACTTCACC	PCR	Belloy et al., 2003
Serstart2	_LppS_	CACTATACTTAACAGATAGTCC	Nested PCR, Sequencing	Mavrot et al., 2012
Serstart0	_LppS_	ATACTCAAAGTGGAAATAATGGAA	Sequencing	This thesis
Serend0	_LppS_	GCAACAACAATAGTAAGAGCAG	Sequencing	This thesis
lppTA2	_LppS_	TTTGATCTCTCCACCTTCAGC	PCR	Mavrot et al., 2012
lppTA	_LppS_	GGCACTAATAGTGCGTAATTC	Nested PCR	Mavrot et al., 2012

Mycoplasma agalactiae

MAP40127F	*p40*	TCATTTACAGCAGTGCCTTTATTAG	qPCR	Oravcová et al., 2009
MAP40235R	*p40*	CACCTAATGCTTGTTTTTCAACC	qPCR	Oravcová et al., 2009
MAP40160P	*p40*	FAM-TGTGATGATAAGAAC GAAAATTCACAAA-BHQ1	TaqMan probe	Oravcová et al., 2009

Chlamydiaceae

Chuni1-F	*23S rRNA*	GGGCTAGACACGTGAAACCTA	qPCR	Nordentoft et al., 2011
Chuni2-R	*23S rRNA*	CCATGCTTCAACCTGGTCATAA	qPCR	Nordentoft et al., 2011
16SIGF	*16S rRNA*	CGGCGTGGATGAGGCAT	Sequencing	Everett et al., 1999
16SIGR	*16S rRNA*	TCAGTCCCAGTGTTGGC	Sequencing	Everett et al., 1999

9.3 ANNEX III: Genbank numbers and information of *M. conjunctivae* sequences

Information of the *M. conjunctivae* strains included in the cluster analyses of this thesis. Sequences are shown by Study and belonged to strains detected in sheep (*Ovis aries*) and goats (*Capra hircus*) from Pakistan (Study II), sheep, Pyrenean chamois (*Rupicapra p. pyrenaica*) and mouflon (*Ovis aries musimon*) from the Pyrenees and Cantabrian Mountains (Study III) and Iberian Ibex (*Capra pyrenaica*) from a captive population located in Sierra Nevada (Study IV). Ocular clinical signs associated to the strain are registered in the "IKC" column. Shaded rows are strains from other areas included in the cluster analyses for comparison.

Study II

ID sequence	Species	Zone	Year	IKC	Herd status	GenBank	Reference
P 0G	Goat	Kanday Village, Gilgit district of Pakistan	2013	No	Sporadic IKC cases	LT174661	Study II
P 16	Goat	Kanday Village, Gilgit district of Pakistan	2013	No	Sporadic IKC cases	LT174664	Study II
P 73	Goat	Hushey Village, Gilgit district of Pakistan	2013	No	Sporadic IKC cases	LT174662	Study II
P 98	Sheep	Minapin Village, Gilgit district of Pakistan	2013	No	Sporadic IKC cases	LT174663	Study II
P 101	Sheep	Minapin Village, Gilgit district of Pakistan	2013	No	Sporadic IKC cases	LT174655	Study II
P 117	Sheep	Hoper Village, Gilgit district of Pakistan	2013	Yes	Sporadic IKC cases	LT174665	Study II

P 119	Goat	Hoper Village, Gilgit district of Pakistan	2013	No	Sporadic IKC cases	LT174656	Study II
P 124	Goat	Hoper Village, Gilgit district of Pakistan	2013	No	Sporadic IKC cases	LT174657	Study II
P 127	Goat	Hoper Village, Gilgit district of Pakistan	2013	No	Sporadic IKC cases	LT174658	Study II
P 132	Sheep	Hoper Village, Gilgit district of Pakistan	2013	No	Sporadic IKC cases	LT174659	Study II
P 136	Goat	Hoper Village, Gilgit district of Pakistan	2013	No	Sporadic IKC cases	LT174660	Study II
38 s	Sheep	Swiss Alps	2000	Yes	Unknown	LT708379	Belloy et al., 2003
2820 s	Sheep	Swiss Alps	2001	Yes	Unknown	LT708377	Belloy et al., 2003
2778 c	Alpine chamois	Austrian Alps	2000	Yes	Unknown	LT708376	Belloy et al., 2003
2784 c	Alpine chamois	Austrian Alps	2000	Yes	Unknown	LT708380	Belloy et al., 2003
My 66 95 s	Sheep	Croatia[a]	1995	Yes	Unknown	LT708378	Giacometti et al., 1999
My 7/96 g	Goat	Croatia[a]	1995	Yes	Unknown	LT708378	Giacometti et al., 1999
HRC/581	Type strain – Sheep	United States of America	1972	Yes	Unknown	LT708381	Barile et al., 1972

[a] Isolates from small domestic ruminants imported from Australia

Study III

ID sequence	Species	Zone	Year	IKC	Herd status	GenBank	Reference
RP-9/06	Pyrenean chamois	Vall Aran, Pyrenees, Spain	2006	Yes	IKC outbreak	LT708373	Marco et al., 2009
VL07/241	Pyrenean chamois	Vall Aran, Pyrenees, Spain	2007	Yes	IKC outbreak	LT708374	Marco et al., 2009
VL07/300	Pyrenean chamois	Vall Aran, Pyrenees, Spain	2007	Yes	IKC outbreak	LT708375	Marco et al., 2009
RP09067	Pyrenean chamois	NGR Alt Pallars, Pyrenees, Spain	2009	Yes	IKC outbreak	LT708305	Study III
RP10008	Pyrenean chamois	NGR Alt Pallars, Pyrenees, Spain	2010	Yes	IKC outbreak	LT708306	Study III
RP12013	Pyrenean chamois	NGR Freser-Setcases, Pyrenees, Spain	2012	Yes	Sporadic IKC cases	LT708307	Study III
RP12014	Pyrenean chamois	NGR Freser-Setcases, Pyrenees, Spain	2012	Yes	Sporadic IKC cases	LT708308	Study III
RP12030	Pyrenean chamois	NGR Freser-Setcases, Pyrenees, Spain	2012	Yes	Sporadic IKC cases	LT708309	Study III
RP12041	Pyrenean chamois	NGR Freser-Setcases, Pyrenees, Spain	2012	Yes	Sporadic IKC cases	LT708310	Study III
RP13002	Pyrenean chamois	NGR Freser-Setcases, Pyrenees, Spain	2013	Yes	Sporadic IKC cases	LT708311	Study III
RP13008	Pyrenean chamois	NGR Freser-Setcases, Pyrenees, Spain	2013	Yes	Sporadic IKC cases	LT708312	Study III
RP13033	Pyrenean chamois	Vall Aran, Pyrenees, Spain	2013	Yes	IKC outbreak	LT708313	Study III

RP13105	Pyrenean chamois	NGR Freser-Setcases, Pyrenees, Spain	2013	Yes	Sporadic IKC cases	LT708314	Study III
RP13180	Pyrenean chamois	Vall Aran, Pyrenees, Spain	2013	Yes	IKC outbreak	LT708315	Study III
RP13223	Pyrenean chamois	Vall Aran, Pyrenees, Spain	2013	Yes	IKC outbreak	LT708316	Study III
RP14016	Pyrenean chamois	NGR Freser-Setcases, Pyrenees, Spain	2014	Yes	Sporadic IKC cases	LT708317	Study III
RP14061	Pyrenean chamois	NGR Freser-Setcases, Pyrenees, Spain	2014	Yes	Sporadic IKC cases	LT708318	Study III
RP14180	Pyrenean chamois	NGR Freser-Setcases, Pyrenees, Spain	2014	Yes	Sporadic IKC cases	LT708319	Study III
RP15092	Pyrenean chamois	NGR Alt Pallars, Pyrenees, Spain	2015	Yes	IKC outbreak	LT708321	Study III
OA-01/06	Mouflon	NGR Freser-Setcases, Pyrenees, Spain	2006	Yes	No IKC	LT708372	Marco et al., 2009
OA14306	Mouflon	NGR Freser-Setcases, Pyrenees, Spain	2014	No	No IKC	LT708320	Study III
OAL10001	Sheep	Picos de Europa, Spain	2010	No	Sporadic IKC cases	LT708322	Study III
OAL10004	Sheep	Picos de Europa, Spain	2010	No	Sporadic IKC cases	LT708323	Study III
OA11001	Sheep	NGR Alt Pallars, Pyrenees, Spain	2011	No	Sporadic IKC cases	LT708324	Study III
OA11008	Sheep	NGR Alt Pallars, Pyrenees, Spain	2011	No	Sporadic IKC cases	LT708325	Study III
OA11024	Sheep	NGR Cadí, Pyrenees, Spain	2011	No	Sporadic IKC cases	LT708326	Study III
OA11046	Sheep	NGR Cadí, Pyrenees, Spain	2011	No	Sporadic IKC cases	LT708327	Study III

OA11097	Sheep	NGR Freser-Setcases, Pyrenees, Spain	2011	No	Sporadic IKC cases	LT708328	Study III
OA11098	Sheep	NGR Freser-Setcases, Pyrenees, Spain	2011	No	Sporadic IKC cases	LT708329	Study III
OA11106	Sheep	NGR Freser-Setcases, Pyrenees, Spain	2011	No	Sporadic IKC cases	LT708330	Study III
OA11107	Sheep	NGR Freser-Setcases, Pyrenees, Spain	2011	Yes	Sporadic IKC cases	LT708331	Study III
OA11108	Sheep	NGR Freser-Setcases, Pyrenees, Spain	2011	No	Sporadic IKC cases	LT708332	Study III
OA11111	Sheep	NGR Freser-Setcases, Pyrenees, Spain	2011	No	Sporadic IKC cases	LT708333	Study III
OAL12053	Sheep	Picos de Europa, Spain	2012	No	Sporadic IKC cases	LT708334	Study III
OAL12056	Sheep	Picos de Europa, Spain	2012	No	Sporadic IKC cases	LT708335	Study III
OAL12072	Sheep	Picos de Europa, Spain	2012	No	Sporadic IKC cases	LT708336	Study III
OA12028-LE*	Sheep	NGR Freser-Setcases, Pyrenees, Spain	2012	No	Sporadic IKC cases	LT708337	Study III
OA12028-RE*	Sheep	NGR Freser-Setcases, Pyrenees, Spain	2012	No	Sporadic IKC cases	LT708338	Study III
OA12038	Sheep	NGR Freser-Setcases, Pyrenees, Spain	2012	No	Sporadic IKC cases	LT708339	Study III
OA12041	Sheep	NGR Freser-Setcases, Pyrenees, Spain	2012	No	Sporadic IKC cases	LT708340	Study III
OA12074	Sheep	NGR Alt Pallars, Pyrenees, Spain	2012	No	Sporadic IKC cases	LT708341	Study III
OA12076	Sheep	NGR Alt Pallars, Pyrenees, Spain	2012	No	Sporadic IKC cases	LT708342	Study III

OA12079	Sheep	NGR Alt Pallars, Pyrenees, Spain	2012	No	Sporadic IKC cases	LT708343	Study III
OA13005	Sheep	NGR Alt Pallars, Pyrenees, Spain	2013	No	Sporadic IKC cases	LT708344	Study III
OA13010	Sheep	NGR Alt Pallars, Pyrenees, Spain	2013	No	Sporadic IKC cases	LT708345	Study III
OA13012	Sheep	NGR Alt Pallars, Pyrenees, Spain	2013	No	Sporadic IKC cases	LT708346	Study III
OA13021	Sheep	NGR Alt Pallars, Pyrenees, Spain	2013	No	Sporadic IKC cases	LT708347	Study III
OA13026	Sheep	NGR Alt Pallars, Pyrenees, Spain	2013	No	Sporadic IKC cases	LT708348	Study III
OA13027	Sheep	NGR Alt Pallars, Pyrenees, Spain	2013	No	Sporadic IKC cases	LT708349	Study III
OA13031	Sheep	NGR Alt Pallars, Pyrenees, Spain	2013	No	Sporadic IKC cases	LT708350	Study III
OA13032	Sheep	NGR Alt Pallars, Pyrenees, Spain	2013	No	Sporadic IKC cases	LT708351	Study III
OA13033	Sheep	NGR Alt Pallars, Pyrenees, Spain	2013	No	Sporadic IKC cases	LT708352	Study III
OA13034	Sheep	NGR Alt Pallars, Pyrenees, Spain	2013	No	Sporadic IKC cases	LT708353	Study III
OA13040	Sheep	NGR Alt Pallars, Pyrenees, Spain	2013	No	Sporadic IKC cases	LT708354	Study III
OA13042	Sheep	NGR Alt Pallars, Pyrenees, Spain	2013	Yes	Sporadic IKC cases	LT708355	Study III
OA13044	Sheep	NGR Alt Pallars, Pyrenees, Spain	2013	No	Sporadic IKC cases	LT708356	Study III
OA13050	Sheep	NGR Alt Pallars, Pyrenees, Spain	2013	No	Sporadic IKC cases	LT708357	Study III
OA13067	Sheep	NGR Alt Pallars, Pyrenees, Spain	2013	No	Sporadic IKC cases	LT708358	Study III

OA13069	Sheep	NGR Alt Pallars, Pyrenees, Spain	2013	Yes	Sporadic IKC cases	LT708359	Study III
OA13072	Sheep	NGR Alt Pallars, Pyrenees, Spain	2013	No	Sporadic IKC cases	LT708360	Study III
OA13074	Sheep	NGR Alt Pallars, Pyrenees, Spain	2013	No	Sporadic IKC cases	LT708361	Study III
OA13076	Sheep	NGR Alt Pallars, Pyrenees, Spain	2013	No	Sporadic IKC cases	LT708362	Study III
OA14038	Sheep	NGR Alt Pallars, Pyrenees, Spain	2014	No	Sporadic IKC cases	LT708363	Study III
OA14040	Sheep	NGR Alt Pallars, Pyrenees, Spain	2014	No	Sporadic IKC cases	LT708364	Study III
OA14051	Sheep	NGR Alt Pallars, Pyrenees, Spain	2014	No	Sporadic IKC cases	LT708365	Study III
OA14085	Sheep	NGR Alt Pallars, Pyrenees, Spain	2014	No	Sporadic IKC cases	LT708366	Study III
OA14086	Sheep	NGR Alt Pallars, Pyrenees, Spain	2014	No	Sporadic IKC cases	LT708367	Study III
OA14132	Sheep	Vall Aran, Pyrenees, Spain	2014	No	Sporadic IKC cases	LT708368	Study III
OA14139-LE*	Sheep	Vall Aran, Pyrenees, Spain	2014	No	Sporadic IKC cases	LT708369	Study III
OA14139-RE*	Sheep	Vall Aran, Pyrenees, Spain	2014	No	Sporadic IKC cases	LT708370	Study III
OA14151	Sheep	Vall Aran, Pyrenees, Spain	2014	No	Sporadic IKC cases	LT708371	Study III
38 s	Sheep	Swiss Alps	2000	Yes	Unknown	LT708379	Belloy et al., 2003
2820 s	Sheep	Swiss Alps	2001	Yes	Unknown	LT708377	Belloy et al., 2003
2778 c	Alpine chamois	Austrian Alps	2000	Yes	Unknown	LT708376	Belloy et al., 2003

2784 c	Alpine chamois	Austrian Alps	2000	Yes	Unknown	LT708380	Belloy et al., 2003
My 66 95 s	Sheep	Croatia[a]	1995	Yes	Unknown	LT708378	Giacometti et al., 1999
HRC/581	Type strain – Sheep	United States of America	1972	Yes	Unknown	LT708381	Barile et al., 1972

*Same animal with different strain in each eye.
[a] Isolates from small domestic ruminants imported from Australia

Study IV

ID sequence	Species	Zone	Year	IKC	Herd status	GenBank	Reference
C_507 OD	Iberian ibex	Sierra Nevada, Spain	2013	Yes	IKC outbreak	LT629279	Study IV
C_507 OI	Iberian ibex	Sierra Nevada, Spain	2013	Yes	IKC outbreak	LT629278	Study IV
C_686 OD	Iberian ibex	Sierra Nevada, Spain	2013	Yes	IKC outbreak	LT629281	Study IV
C_686 OI	Iberian ibex	Sierra Nevada, Spain	2013	Yes	IKC outbreak	LT629280	Study IV
C_506 OD	Iberian ibex	Sierra Nevada, Spain	2013	Yes	IKC outbreak	LT629282	Study IV
C_508 OD	Iberian ibex	Sierra Nevada, Spain	2013	Yes	IKC outbreak	LT629283	Study IV
C_695 OI	Iberian ibex	Sierra Nevada, Spain	2013	Yes	IKC outbreak	LT629284	Study IV
C_695 OD	Iberian ibex	Sierra Nevada, Spain	2013	Yes	IKC outbreak	LT629285	Study IV
C_2298 OI	Iberian ibex	Sierra Nevada, Spain	2013	Yes	IKC outbreak	LT629287	Study IV

C_5125 OD	Iberian ibex	Sierra Nevada, Spain	2013	Yes	IKC outbreak	LT629289	Study IV
C_5125 OI	Iberian ibex	Sierra Nevada, Spain	2013	Yes	IKC outbreak	LT629288	Study IV
C_800 OI	Iberian ibex	Sierra Nevada, Spain	2013	Yes	IKC outbreak	LT629286	Study IV
C_801 OD	Iberian ibex	Sierra Nevada, Spain	2013	Yes	IKC outbreak	LT629291	Study IV
C_801 OI	Iberian ibex	Sierra Nevada, Spain	2013	Yes	IKC outbreak	LT629290	Study IV
575N OI	Iberian ibex	Sierra Nevada, Spain	2014	No	Sporadic IKC cases	LT629292	Study IV
C_501 OD	Iberian ibex	Sierra Nevada, Spain	2014	No	Sporadic IKC cases	LT629293	Study IV
C_801 OI	Iberian ibex	Sierra Nevada, Spain	2014	No	Sporadic IKC cases	LT629294	Study IV
ALP321 OI	Iberian ibex	Sierra Nevada, Spain	2014	No	Sporadic IKC cases	LT629295	Study IV
38 s	Sheep	Swiss Alps	2000	Yes	Unknown	LT708379	Belloy et al., 2003
2820 s	Sheep	Swiss Alps	2001	Yes	Unknown	LT708377	Belloy et al., 2003
2778 c	Alpine chamois	Austrian Alps	2000	Yes	Unknown	LT708376	Belloy et al., 2003
2784 c	Alpine chamois	Austrian Alps	2000	Yes	Unknown	LT708380	Belloy et al., 2003
My 66 95 s	Sheep	Croatia[a]	1995	Yes	Unknown	LT708378	Giacometti et al., 1999
My 7/96 g	Goat	Croatia[a]	1995	Yes	Unknown	LT708378	Giacometti et al., 1999
HRC/581	Type strain – Sheep	United States of America	1972	Yes	Unknown	LT708381	Barile et al., 1972