Frequency of zoonotic enteric pathogens and antimicrobial resistance in wild boar (*Sus scrofa*) Iberian ibex (*Capra pyrenaica*) and sympatric free-ranging livestock in a natural environment (NE Spain)

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HACEN CONSTAR

que la memoria titulada "Frequency of zoonotic enteric pathogens and antimicrobial resistance in wild boar (*Sus scrofa*), Iberian ibex (*Capra pyrenaica*) and sympatric free-ranging livestock in a natural environment (NE Spain)" presentada por Nora Navarro González para la obtención del grado de Doctora en Veterinaria por la Universitat Autònoma de Barcelona, ha sido realizada bajo nuestra dirección y, considerándola satisfactoriamente finalizada, autorizamos su presentación para que sea juzgada por la comisión correspondiente.

Y para que conste a los efectos oportunos, firmamos el presente informe en Bellaterra, a siete de mayo de dos mil trece.

Firmado: Santiago Lavín González

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Firmado: Emmanuel Serrano Ferron

Firmado: Nora Navarro González

"Como un mar me presenté ante tí;
en parte agua y en parte sal.
Lo que no se puede desunir
es lo que nos habrá de separar..."

Nacho Vegas, 2011

"La gran broma final"

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Summary

The transmission of zoonotic pathogens from wildlife to livestock, and vice versa, is a cause of global concern since wild ungulates are becoming more abundant and widely distributed throughout Europe, even in urban areas. In parallel, in the European Union some food-borne zoonoses are increasing in frequency each year. To compound these factors, antimicrobial resistance is a global threat and should be monitored to prevent its spread.

The purpose of this Thesis was to investigate whether selected zoonotic pathogens are shared between free-ranging livestock and sympatric wild ungulates (wild boar – *Sus scrofa* - and Iberian ibex – *Capra pyrenaica*) in a natural environment in NE Spain. Additionally, we explored if antimicrobial resistance in pathogens and indicator bacteria is also distributed among those host species. Furthermore, the abovementioned pathogens and antimicrobial resistance were investigated in an urban wild boar population in Barcelona, Spain.

Salmonella prevalence was higher in wild boars co-habiting with cattle (35.67%, CI 95% 28.19 - 43.70) than in wild boar from cattle-free areas (17.54 %, CI 95% 8.74 - 29.91). Moreover, the probability of a wild boar being a *Salmonella* carrier increased with cattle herd size, and serotype richness was higher in wild boars co-habiting with cattle. The finding of a *S*. Enteritidis strain resistant to ciprofloxacin and nalidixic acid in wild boars is cause for public health concern. The different *Salmonella* prevalence in cattle (21.92%, 95% CI 13.10-33.14) and in Iberian ibex (0.96%, 95% CI 0.2-2.8) and the lack of shared serotypes suggest no association. Please see Study I and II for more details.

Prevalence of *E. coli* O157:H7 was low at 3.41% in wild boars (95% CI 0.94 - 8.52) and 1.88% in Iberian ibex (95% CI 0.39 - 5.38), and was not isolated from livestock faeces (see Study III). With regards to *Campylobacter* spp., faecal samples from Iberian ibex were negative. *C. jejuni* was carried by one wild boar (0.67%, 95% CI 0.02 – 3.66), while this was the predominant species in cattle at 9.09% (95% CI 3.02 – 19.95). *C. lanienae* was the most frequent species in wild boar at 12% (95% CI 7.27 – 18.3) and was concurrently isolated from one cow. These results (Study IV) suggest that Iberian ibex do not play an important role in the epidemiology of *Campylobacter,* and that despite wild boar and cattle having their own predominant *Campylobacter* species, there is a potential spill-over of *C. lanienae* and *C. jejuni*.

Both wild and domestic animals appeared to be low-significance reservoirs of antimicrobial resistance, as stated in Study III. The frequency of resistance in indicator *E. coli* was low, ranging from 0 to 7.9%. Remarkably, one isolate carried by wild boar showed resistance to a

third-generation cephalosporin and fluoroquinolone-resistance was detected in isolates from cattle and wild boar. We suggest the potential of wild boar as a sentinel for antimicrobial resistance in a broad range of environmental conditions.

Salmonella enterica was found in 5% (95% CI 0.61-16.91) of urban wild boars in Study V, and *Campylobacter coli* in 4.88% (95% CI 0.6-16.53). Other thermophilic *Campylobacter* were moderately prevalent (19.51%, 95% CI 8.82-34.87), while *C. jejuni* and *E. coli* O157:H7 were not found. Antimicrobial resistance was most frequent in *Enterococcus faecium* (95% of the isolates were resistant to at least one antimicrobial agent), followed by *Enterococcus faecalis* (50%) and *Escherichia coli* (10%). We report for the first time resistance to linezolid in wildlife. These results have implications for public health, and thus, further research is needed on wildlife in urban environments.

Resumen

La transmisión de patógenos zoonósicos desde la fauna salvaje al ganado doméstico, y vice-versa, es una problemática global ya que los ungulados salvajes aumentan en número y distribución en toda Europa, incluso en áreas urbanas. Al mismo tiempo, en la Unión Europea la frecuencia de ciertas zoonosis de transmisión alimentaria aumenta cada año. Además, la resistencia antimicrobiana es una amenaza mundial que urge ser monitorizada para evitar su diseminación.

El propósito de esta Tesis es investigar si ciertos patógenos zoonósicos se comparten entre el ganado doméstico en extensivo y los ungulados salvajes (el jabalí – *Sus scrofa* - y la cabra montés – *Capra pyrenaica*) de un entorno natural del noreste español. Adicionalmente, hemos analizado la presencia de resistencia antimicrobiana tanto en patógenos como en bacterias comensales de estos hospedadores. Por último, se exploró lo mismo en jabalíes urbanos de Barcelona (España).

La prevalencia de *Salmonella* fue mayor en jabalíes que co-habitaban con ganado vacuno (35.67%, CI 95% 28.19 - 43.70) que en aquellos que no (17.54 %, CI 95% 8.74 - 29.91), de manera similar, la riqueza de serotipos es mayor en el primer grupo. Además, la probabilidad de que un jabalí sea portador de *Salmonella* aumenta cuanto mayor es el rebaño de vacas con el que comparte el hábitat. El hallazgo una cepa de *S*. Enteritidis resistente a ciprofloxacina y ácido nalidíxico en jabalí es una causa de preocupación para la salud pública. Las diferentes prevalencias de *Salmonella* en ganado vacuno (21.92%, 95% CI 13.10-33.14) y cabra montés (0.96%, 95% CI 0.2-2.8) y la ausencia de serotipos compartidos sugieren la ausencia de nexo (más información en el Estudio I y II).

La prevalencia de *E. coli* O157:H7 fue baja: 3.41% en jabalí (IC 95% 0.94 - 8.52) y 1.88% en cabra montés (IC 95% 0.39 - 5.38), mientras que este patógeno no fue aislado del ganado doméstico (Estudio III). Respecto a *Campylobacter*, las muestras de cabra montés fueron negativas. *C. jejuni* se aisló de un jabalí (0.67%, IC 95% 0.02 – 3.66), sin embargo, *C. jejuni* fue la especie predominante en el ganado vacuno: 9.09% (IC 95% 3.02 – 19.95). *C. lanienae* fue la especie más frecuente en jabalí (12%, IC 95% 7.27 – 18.3) y se encontró simultáneamente en una vaca. Este estudio (IV) indica que, de nuevo, la cabra montés parece no desempeñar un papel importante en la epidemiología de *Campylobacter*. Además, a pesar de que el jabalí y el ganado vacuno tienen su propia especie predominante de *Campylobacter*, existe una potencial transmisión de *C. lanienae* y *C. jejuni* entre ambos hospedadores.

Además, tanto los animales salvajes como domésticos parecen poco importantes como reservorio de resistencia antimicrobiana (Estudio III). La frecuencia de resistencia en *E. coli* indicador fue reducida (0 a 7.9%). Sin embargo, se detectó resistencia a una cefalosporina de tercera generación en jabalí, y a fluoroquinolonas tanto en éste como en ganado vacuno. Esto sugiere el potencial del jabalí como centinela de resistencia antimicrobiana en una gran variedad de ambientes.

En jabalíes urbanos, *Salmonella* se encontró en un 5% (IC 5% 0.61-16.91) y *Campylobacter coli* en un 4.88% (IC 95% 0.6-16.53). Otros *Campylobacter* termófilos fueron moderadamente prevalentes (19.51%, 95% CI 8.82-34.87), mientras que *C. jejuni* y *E. coli* O157:H7 no fueron aislados. La frecuencia de resistencia antimicrobiana fue mayor en *Enterococcus faecium* (95% de los aislados era resistente a uno o más agentes), seguido por *Enterococcus faecalis* (50%) y *Escherichia coli* (10%). Además, describimos por primera vez resistencia a linezolid en un aislado procedente de fauna salvaje. Ya que estos resultados tienen implicaciones en la salud pública, es necesario llevar a cabo un seguimiento sanitario de la fauna en entornos urbanos.

Introduction

The relevance of wildlife research

About Zoonoses and Emerging Infectious Diseases

Zoonoses are infections acquired from animals and pose a risk to public health. Particularly, zoonoses from wildlife represent the most significant threat to global health of all Emerging Infectious Diseases (Jones et al, 2008). This term (EID) refers to "infections that have newly appeared in a population or have existed but are rapidly increasing in incidence or geographic range" (Morse, 1995) and includes animal diseases that have recently crossed the species barrier (Bengis et al, 2002). EID can affect humans, livestock or wildlife, or any combination thereof. It has long been known that the "zoonotic pool" (introductions of infections from other animal species) is an important and potentially rich source of emerging diseases for the human population (Morse, 1995). On the other hand, humans have been implicated in wildlife EIDs that have had or still have severe consequences in wild populations (Daszak et al, 2000).

According to Cutler et al (2010), the emergence of infectious diseases is caused by a complex multifactorial set of changing circumstances including intensive agricultural practices, globalization, encroachment of humans into natural habitats, climate change, and others. In addition, Gummow (2010) mentions the poor governance resulting in the breakdown in veterinary and medical services as a cause of re-emergence of diseases.

Generally, emerging infectious diseases in humans are associated with a range of underlying causal factors that include interactions with zoonotic pathogens within a host-parasite continuum between wildlife, domestic animals and humans (Daszak et al, 2000), which I have schematically represented in Figure 1. It is estimated that 60% of emerging human pathogens are zoonotic, and of these, more than 71% have wildlife origins (Cutler et al, 2010). Unfortunately, most research efforts have traditionally been focused on humans or economically relevant species such as livestock and only in recent years has this begun to change. This mentality shift seemed inevitable in light of new discoveries and knowledge. For example, Jones et al (2008) showed the urgency for identification of potentially new zoonotic pathogens in wildlife populations as a forecast measure for human EIDs. This study also suggests that efforts to conserve areas rich in wildlife diversity by reducing anthropogenic activity may have reduced the likelihood of future zoonotic disease emergence, a fact that strengthens the arguments for a global conservation effort in direct benefit for the human population.

Humans are exposed to wildlife zoonoses mainly via the spread of the infection to livestock and, in cases where wildlife represents a human food source, through hunting and the consumption of contaminated meat; additionally, contact with or ingestion of water

contaminated with faeces and urine of infected animals poses a route for transmission between wildlife and humans (Van Campen and Rhyan, 2010). The use of recreational waters has also been related to pathogen transmission (Craun et al, 2005). Although it is assumed that many emerging diseases of humans, domestic animals and wildlife are maintained in reservoir hosts, these reservoirs are rarely identified (Haydon et al, 2002), especially when they are wild reservoirs.

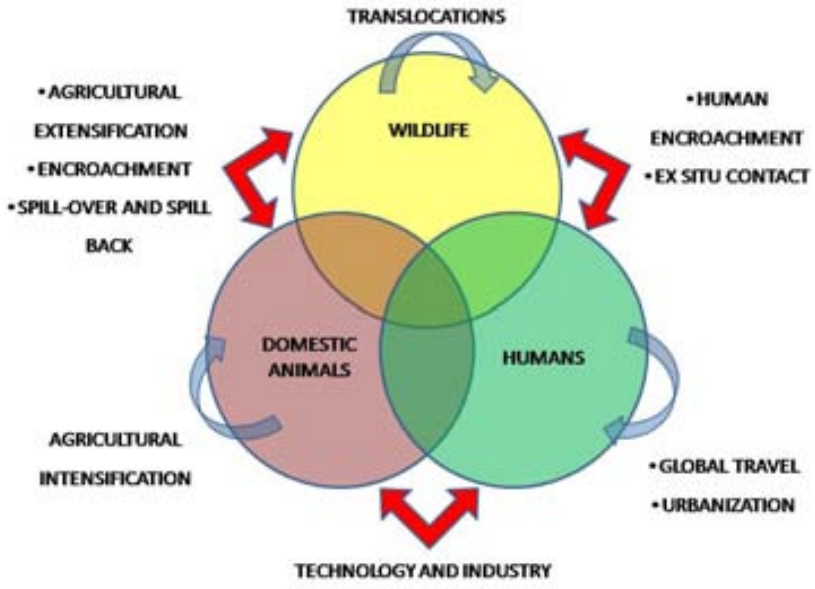

Figure 1. Host-parasite continuum between wildlife, domestic animals and humans. Arrows indicate factors driving disease emergence within (blue arrow) or among groups (red arrow). Adapted from Daszak et al, 2000.

The wildlife-livestock interface

Anywhere the wildlife-livestock interface occurs; it should be regarded as a two-way street with the potential for the transmission of pathogens in either direction (Bengis et al, 2002). From a conservationist point of view, it is also important to remember that one of the deleterious effects of pathogen transmission is a loss in biodiversity due to the possibility of depopulation and local extinction (Gummow, 2010).

Interactions between wild and domestic animals in Europe are believed to be increasing due to different factors such as (1) the concentration of large populations of wild animals in small, delimited natural areas due to the high human distribution and density (Gummow, 2010); (2) European wildlife politics and consumer preferences moving the animal breeding industry from more intensive to more extensive farming systems (Gortázar et al, 2007); (3) wildlife populations becoming more abundant since management through feeding , translocations and fencing (Gortázar et al, 2007) and (4) the increase in recent decades of forest areas at the

expense of agricultural areas (Martin et al, 2011), providing additional refuge and sometimes also food resources to wildlife, among others.

In developing countries, on the other hand, the growth of industrial livestock production to meet the global demand on protein and the wildlife trade will inevitably be linked to emerging disease outbreaks in the near future (Karesh et al, 2005).

The occurrence of transmission of infection between wildlife and domestic livestock is highly dependent on both spatial and temporal elements, which vary in function of the infectious agent involved, the presence or necessity of a vector, and others (Bengis et al, 2002). Disease transmission between wildlife and livestock can be of particular concern in relation to wild ungulates, since these often share habitat and resources with domestic livestock (Boehm et al, 2007). The main opportunity for transmission of infectious agents between wildlife and livestock is thought to occur on pasture, especially in grazing systems where wildlife and livestock share access to agricultural pastures: ingestion or investigation of forage contaminated with faeces during grazing may lead to transmission opportunities (Van Campen and Rhyan, 2010). Setting up barriers to prevent wildlife contact with domestic livestock may be useful in some situations, as well as hygienic measures such as the proper disposal of the hunting carcasses (Gortázar et al, 2007). However, control of diseases may be complex in most situations, since elements that influence transmission are also related to seasonal and climatic cycles or fluctuations that affect animal numbers and distribution or vector abundance (Bengis et al, 2002).

Disease can be transmitted by direct or indirect contacts between infected and susceptible individuals. Direct contacts are more likely to be important in intra-specific transmission and both faecal-oral and urinary-oral routes are considered major transmission routes for infectious diseases of wildlife and livestock (Van Campen and Rhyan, 2010).

“Spill-over” is the term used for the transmission of infectious agents from reservoir animal populations, which are often domesticated species, to sympatric wildlife; but there can also be a reverse spill-over, or “spill-back”, from wildlife to susceptible domestic animals (Daszak et al, 2000). The story of brucellosis and tuberculosis in North America exemplifies this situation: after near eradication in cattle, wildlife (mainly deer) have maintained and transmitted the infection back to adjacent cattle populations (Van Campen and Rhyan, 2010). Consequently, when studying pathogens in wild populations, the nature and spatiotemporal availability of natural resources (e.g. pastures, watering holes) shared by livestock and wildlife should be systematically considered. Such parameters can strongly determine research findings, for

example Avagnina et al (2012) found a low frequency of certain pathogens in game meat from Alpine wild ungulates and attributed this to the infrequent pasture-sharing of these populations with domestic animals. In that study area, livestock are only grazed in the mountainous areas during the summer, therefore limiting the transmission of pathogens from farmed animals to wild fauna.

Similarly, particularities of the pathogen under study should be considered, e.g. pathogens that infect more than one host species are likely to be encountered in several host populations, some of which may constitute infection reservoirs (Haydon et al, 2002), and should be targeted in the study of the wildlife-livestock interface. According to these authors, accumulating epidemiologic evidence is often the best first step in identifying a reservoir. A host may be a "dead-end host", unable to maintain the infection without continuing transmission from other species, or it may be a "maintenance host", able to maintain the infection without continual introduction from other species, as defined by Van Campen and Rhyan (2010).

It should be emphasized that each population interface (wildlife/wildlife or wildlife/livestock) should be regarded as unique when considering risks of interspecies disease transmission. The same disease at the interface of the same two species may behave differently in different areas or situations. This can be for a variety of reasons, including different climatic environments, different management of one or both species, shared food, water and mineral sources, population densities, predation, concurrent diseases, and so forth. A solid scientific basis is strongly needed for the suggestion of disease control measures, since these can create or increase conflicts between veterinary authorities, hunters, conservationists, livestock breeders and the general public (Gortázar et al, 2007).

One step beyond: the "Wildlife – livestock – human continuum"

Humans, wildlife and domestic animals exist within a continuum of habitat, pathogens and environment (Daszak et al, 2001), which has been called the "Wildlife – livestock – human continuum" (Daszak et al, 2006), and understanding how diseases are transmitted and move within this interface will allow better planning of control measures (Gummow, 2010). However, most previous instances are not encouraging since the success rate of controlling zoonoses in wildlife is poor (Mathews, 2009). Changing circumstances are also posing new challenges; for example, since a pathogen can appear in new places if its reservoir host or vector becomes more widely disseminated (Morse, 1995), risk of zoonoses transmission is

likely to increase in the following years if the positive trends in abundance and distribution of certain wild populations are maintained. Due to the complexity of the diseases that humanity faces at the wildlife-livestock-human continuum, a multidisciplinary approach is required (Cook and Karesh, 2008).

Keeping in mind the concept of the "wildlife-livestock-human continuum", the relevance of conducting studies such as this Thesis becomes clear. In the era of the "One health" initiative, integrative approaches are encouraged to expand scientific knowledge, which will ultimately enhance animal, environmental and public health. In our case, we hope that the results of this Thesis will trigger collaboration between veterinary, medical and environmental authorities, and with the different social sectors involved such as hunters, game managers or farmers.

The importance of game meat

Throughout Europe, wild ungulates are becoming increasingly abundant, and in some instances, since the natural predators are extinct or their populations reduced, hunting is the only method implemented for the control of wild ungulate abundance. At the same time, hunting is a popular leisure activity in many European countries and also an important economic sector in many regions. These facts lead to a huge harvesting of game carcasses, which are a valuable protein source, and the use of this natural resource as a food intended for human consumption is an interesting option that needs to be evaluated. Traditional dishes of rural areas often include game meat; in fact, meat from wild animals is considered the first source of animal protein in the history of human nutrition. However, game meat today makes up only to a small percent of the overall food supply in most regions (Paulsen et al, 2012). Currently, there is a great interest in the general public in maintaining local particularities and traditions, as well as an interest in finding alternative and more sustainable meat production systems and protein sources, which make game meat an attractive and appreciated food. Moreover, the consumer's perception on game meat from free-ranging wild populations is that this is "happy meat", i.e. that these animals live freely and contently, according to their nature and natural behaviour. They are not transported to slaughter and are generally die instantaneously. Maybe for these reasons, game meat is much valued and its consumption in Europe is increasing.

However, concerns arise with regards to food safety and microbiological quality. Anyone attending a hunt can observe the visible contamination of the carcasses with soil or gut content and large openings of the body cavities. As an example, *Salmonella* can be isolated at

every step of the game meat chain (Paulsen et al, 2012), or the same PFGE profile has been found in STEC from faeces and carcasses of wild boar and red deer, meaning that cross-contamination occurs during processing (Díaz-Sánchez et al, 2013). Several studies emphasize the need for implementing sound hygienic procedures, particularly during the evisceration, transport, skinning and cutting operations (Atanassova et al, 2008; Avagnina et al, 2012; Gill, 2007) and proper handling in all phases of game production, in order to guarantee food that meets the EU quality and safety standards. Avagnina et al (2012) call for a rigorous application of good harvesting practices by hunters, as well as for putting into effect traceability requirements, as required by the European Regulation.

One study on the microbiological quality of game meat of wild ungulates in the Italian Alps (Avagnina et al, 2012) showed that the quality of meat is highly dependent on the first phases of hunting and anatomical location of the wound, with a shot to the abdominal cavity associated with carcass contamination. Furthermore, these authors found higher bacterial loads in wild boar than in ruminant species, which may be related to differences between species and/or shooting/evisceration times, and the presence of *Yersinia* spp. was also more frequent in wild boar meat. Differences in the hunting methods that can explain this are, for example, that shots frequently produce wounds in wild boar that are not fatal allowing animals to continue running and thus increasing contamination levels, or that wild boars are more frequently dragged after death (Atanassova et al, 2008). Also, ethological differences can be responsible for a more severely contaminated skin in wild boar than in ruminants and therefore a higher contamination load in wild boar carcasses (Atanassova et al, 2008).

Membré et al (2011) suggest that improving hunting practices across European countries and encouraging good hygiene practices would maintain the microbiological quality of large wild game meat. This is a major challenge considering the differences in game species diversity in the European countries and regions and the different pathogen prevalence in these wild populations, as well as the differences in hunting methods and processing practices (Avagnina et al, 2012).

The proper training of hunters may be a key solution for improving the hygienic quality of game meat. More studies are needed on the proper handling and management of carcasses, in light of the influence of different environments and hunting techniques upon meat hygienic quality.

The wild hosts

Author's note: In this section, only wild hosts are described. Please find information on free-ranging livestock in Studies I to IV.

Wild boar

The wild boar (*Sus scrofa*) or Eurasian wild pig has one of the widest geographic distributions of all terrestrial mammals (Oliver and Leus, 2008), which includes Europe, Asia and North Africa. In addition this range has been greatly expanded by human introduction to the American continent, Australia, New Zealand and several islands in the Pacific Ocean (Rossell and Herrero, 2007); as a result, this species currently occurrs on all continents except Antarctica. Owing to this huge range and to differentiation into subspecies, certain variability in the biology of wild boar between populations can be observed, e. g. in relation to reproduction rate, gregariousness, and other characters. In this chapter I focus on the Western Europe populations since their situation corresponds to a wider extent with the reality of the wild boar populations studied in this Thesis. In the Iberian Peninsula, two subspecies -*S. s. castilianus* and *S. s. baeticus*- have been reported, though this differentiation should be confirmed with molecular biology techniques (Rossell and Herrero, 2007).

Until four or five months of age, wild boars are called piglets and have a light brown coat with longitudinal darker lines. Subsequently, they develop a uniform brown-reddish colouration until they are between 10 and 12 months of age, when they acquire the adult coat, which is brown-greyish. They show a marked sexual dimorphism, with males being more corpulent with larger tusks.

The wild boar is said to have been an important resource of subsistence hunters since the earliest times, and it is nowadays one of the most important game species for recreational hunting. Although over-hunting and changes in land use have resulted in the fragmentation of its range and its regional extinction in determined areas, the species remains widely distributed and is often locally abundant (Oliver and Leus, 2008). In Europe it is present in most continental areas, including regions where it had disappeared, such as Sweden or Britain.

Worldwide, the wild boar occupies a wide variety of temperate and tropical habitats, from semi-desert to tropical rain forests, temperate woodlands, grasslands and reed jungles; it even ventures onto agricultural land to forage. In fact, in many countries it is considered a pest due to its predation on crops. In Europe, it prefers broadleaved forests and especially evergreen oak forests, but may also be found in more open habitats such as steppe, Mediterranean shrubland, marsh, farmland and peri-urban areas and is found from sea level to subalpine pastures higher than 2400 m (Rossell and Herrero, 2007).

The species is omnivorous, though vegetable matter is predominant, including a variety of mast, seeds, roots, green plant matter, fungi and agricultural crops (Schley and Roper, 2003). The animal matter consumed by wild boars belongs to both invertebrates (mainly to insects, earthworms and snails) and vertebrates (mainly small mammals like rodents and shrews, but also birds) (Schley and Roper, 2003). Wild pigs are generally crepuscular (most active in the early morning and late afternoon) or nocturnal, and spend a total of 4 to 8 hours foraging or traveling to feeding areas (Oliver and Leus, 2008). The home range estimates for adult females and adult males vary greatly, and larger home ranges of male subadults are related to their expulsion from their original groups and the subsequent dispersion phase.

Wild boars are gregarious, forming herds of usually between 6-20 individuals but herd size varies depending on locality and season. The basic nucleus of the herd is one or more females and their last offspring, while subadult males from previous litters may form other nuclei. Adult males are usually solitary animals, but they can remain near a family group of females and their offspring, and are part of the herd during the mating season. The reproductive activity in *S. scrofa* tends to be seasonal (in most populations the main rut period takes place in autumn, between September and December) and positively correlated with the availability of the principal food resources and related climatic factors. Females can get pregnant during their first or second year, and give birth to 1 -8 piglets (Fernández-Llario, 2006) after 120 days of pregnancy (Rossell and Herrero, 2007). Juvenile mortality averages 15% in the first three months (Oliver and Leus, 2008), their only relevant predator being the wolf (*Canis lupus*) and occasionally large raptors hunting on piglets (Fernández-Llario, 2006; Rossell and Herrero, 2007).

In Spain, a high interannual variability is observed, reported densities ranging from one or less to fifteen individuals /km^2 (Rossell and Herrero, 2007). The trends of the Iberian populations show a large increase in recent decades, as in other European countries (Sáez-Royuela and Tellería, 1986). This has been attributed to socio-economic changes that have led to a progressive abandonment of the rural areas, intensive agriculture, lack of predators, and other effects.

At the global level, there are no major threats to the species, which is categorized as Least Concern by the IUCN Red List. However, Oliver and Leus (2008) consider that at a more local level threats exist, mainly habitat destruction and hunting pressures (for food, sport or in reprisal for crop damage), in particular in areas near human habitation. In fact, in many countries or local communities wild pigs constitute the single or most important game species

for subsistence and/or recreational hunters. Wild boars are also susceptible to a variety of infectious diseases which can decimate their populations and are also threatened by genetic contamination through contact with free-ranging domesticated pigs or released non-native or hybrid wild boars (Fernández-Llario, 2006). The most relevant diseases are tuberculosis, salmonellosis and metastrongylosis. Other diseases that should be taken into account are Aujezsky's disease, brucellosis, classical swine fever and toxoplasmosis, just to name a few. It is mandatory to examine wild boar meat for the presence of the nematode *Trichinella* spp. if it is intended for human consumption.

Since wild boar is the ancestor of most domestic pig breeds, many diseases are shared and can be transmitted. This can have negative impacts on the farming industry but also on public health, especially considering that wild boar meat is consumed in large amounts throughout Europe and worldwide. However, in contrast to the abundance of literature available on the prevalence of food-borne pathogens in domestic pigs, little is known about the occurrence of food-borne pathogens in wild boars. These can be important as a source of infection for domestic animals and humans: Wacheck et al (2010) found 61% of the animals carrying at least one (out of 6) food-borne pathogen.

Wild boars in urban environments

Currently more than 50% of the global human population lives in urban areas and urbanization is expected to increase in the upcoming decades (UN DESA, 2009). As urbanization intensifies, habitat modifications and fragmentation will continue, resulting in alterations of the local flora and fauna. However, studies have revealed huge interspecific variations in responses to urbanization (Davis et al, 2012). The process of wild animal populations becoming adjusted to specific conditions of urban environments is called "synurbization" (Luniak, 2004), and is occurring in several species and locations, such as in the red fox in England, gray squirrels in the USA (Parker and Nilon, 2012), and many others. Synurbic populations of wild boar in particular are found in Barcelona (Figure 2), Berlin and several other cities in Poland, Japan and the USA (Cahill et al, 2010). Urban-adapted species occur at higher densities in urban and suburban environments, mainly supported by a non-fluctuating and non-seasonal abundance of resources, either accidental or intentional (Bradley and Altizer, 2007).

In addition, urbanization likely affects the host-parasite interaction because it potentially alters the distribution and composition of suitable wildlife habitat, increases host contact rates, or promotes a shift in the exploitation of food resources, among others. Davis et al (2012), for

example, showed that snakes infected with faecal parasites were closer to the edge of an urban forest than uninfected ones. The dynamics of infectious disease in wildlife can also be affected by urbanization: the prevalence of chronic wasting disease in mule deer (*Odocoileus virginianus*) is higher in populations from developed areas than those from natural areas in Colorado (Farnsworth et al, 2005). Remarkably, the prevalence of wildlife-related zoonoses can also be influenced by urbanization, with implications not only for wildlife management but also for public health. Some studies that illustrate this are Bradley et al (2008) - West Nile Virus antibody prevalence in songbirds-, Kellner et al (2012) - *Baylisascaris procyonis* in urban raccoons, *Procyon lotor* - or Liccioli et al (2012) - gastrointestinal parasites in urban coyotes, *Canis latrans*.

Determining how urban landscapes influence wildlife infectious disease will become increasingly important for predicting human disease risks as well, since the majority of emerging human infectious diseases are zoonotic (Bradley et al, 2008).

Figure 2. A. Wild boar feeding from a bin in the peri-urban area in Barcelona. B. One citizen feeding wild boars directly in Collserola Park (Barcelona, Spain).

The Iberian ibex

Capra pyrenaica, whose common name in English is under debate (Sarasa et al, 2012), is endemic to the Iberian Peninsula. In this Thesis I will use the term Iberian ibex though other names are Iberian wild goat, Pyrenean Ibex or Spanish Ibex (Herrero and Pérez, 2008). This species formerly occurred throughout Spain, southwest France, Andorra, and Portugal, but today is extinct in France and Andorra and no longer occurs in the Pyrenees. As is the case with other European wild caprids, knowledge on the ecology, behaviour, conservation status and management of the Iberian ibex is scarce (Acevedo and Cassinello, 2009). It is relevant to mention its importance as a trophy-hunting species, with some trophy prices exceeding several thousand Euros; this can be an important source of revenue to local communities in rural areas (Herrero and Pérez, 2008). Of the four subspecies described - *C. p. lusitanica*, *C. p. pyrenaica*, *C. p. victoriae, C. p. hispanica* - only the latter two are currently present.

C. p. victoriae occurs in the central Spanish mountains and has been re-introduced to certain sites in Spain (Granados et al, 2001) and northern Portugal (Moço et al, 2006), while *C. p. hispanica*, the subspecies studied in this Thesis, occupies the mountainous chain along the Mediterranean coast. The present distribution of this species is the result of both natural and artificial expansion processes (Acevedo and Cassinello, 2009). The species occurs in rocky habitats from sea level to 3,400 m (Granados et al, 2007). The typical habitats are cliffs and screes interspersed with scrub or pine trees, but even small rocky patches in arable farmland and on the coast may be occupied by this species (Herrero and Pérez, 2008). It can live in close proximity to humans, and is a familiar and popular species of intrinsic curiosity. When appropriate habitat is available, it disperses rapidly and can easily colonise new areas.

The Iberian ibex is a medium-sized mountain ungulate. Both sexes are endowed with persistent horns that approximately indicate the animal's age. In the male, these horns are larger and S-shaped. Males are additionally greater in size and weight, have a beard and larger black areas in the coat. The color of the coat varies seasonally: it is light brownish in summer and gets darker in winter (Granados et al, 2007). Thanks to their large and flexible hooves they are able to move and run on steep slopes and rocky terrain (Acevedo and Cassinello, 2009).

This is a gregarious species, forming herds that vary in size and composition throughout the year. Most of the year males and females remain separated, but this sexual segregation disappears during the rut. Then, mixed groups of all ages and both sexes are composed (Granados et al, 2007). It is a polygamous species that mates at the end of autumn and beginning of winter (Alados and Escós, 2012). Females start to breed at the age of 30 months,

approximately, while successfully breeding males are primarily older than eight years. Pregnancy lasts around 155 days; therefore births occur from April to June, and in exceptional occasions, there may be a twin birth (Granados et al, 2007).

The feeding habits of the Iberian ibex are considered highly adaptive, depending on plant availability; they are "mixed feeders" (browser and grazer), and these behaviours vary with location (Acevedo and Cassinello, 2009). Altitudinal movements are frequent in the Iberian ibex populations, and partly related to food availability (Alados and Escós, 2012). During summer, the herds move to the summits where they find green pastures. During winter the Iberian ibexes from certain mountainous regions are forced to descend to the valleys due to the abundance of snow and food scarcity. Predators are thought to have little impact on the Iberian ibex populations, since its distribution is not coincident with that of wolves, only red foxes (*Vulpes vulpes*) and golden eagles (*Aquila chrysaetos*) are able to hunt juveniles (Alados and Escós, 2012).

Iberian ibex numbers have increased steadily since the early 1990s (Herrero and Pérez, 2008). In 2002, the total Iberian ibex population was estimated to be around 16 000 individuals, divided into 53 nuclei (Pérez et al, 2002). Its current distribution is a result of both natural and artificial expansion, since there have been translocations (Acevedo and Cassinello, 2009) and escapes from game enclosures (Pérez et al, 2002). Despite this positive trend, in some populations there have been strong oscillations in the abundance (Granados et al, 2001). The alteration and fragmentation of habitats (through fires or infrastructure development) may impact certain populations (Herrero and Pérez, 2008). Morevoer, distribution overlap with the introduced aoudad (*Ammotragus lervia*) and a possible competition for resources might pose a conservation threat to the Iberian ibex, although this is yet unclear (Acevedo et al, 2007). Outbreaks of sarcoptic mange (*Sarcoptes scabiei*) occur sporadically (Herrero and Pérez, 2008), and can even lead to local extinction (León-Vizcaíno et al, 1999). According to Granados et al (2007) other diseases affecting Iberian ibex are oestrosis (*Oestrus* sp.), brucelosis (*Brucella* sp.), brochopneumonia (*Pasteurella multocida*) and queratoconjunctivitis (*Chlamydophila psittaci* and *Mycoplasma* sp.). An outbreak of Contagious Agalactic Syndrome, caused by *Mycoplasma agaliactae*, has been described by Verbisck et al (2010) coinciding with a hard draught, high ibex density and concentration of both ibex and domestic ruminants in summer pastures and around mountain lakes. Gonzalez-Candela et al (2006) found several potential pathogens in Iberian ibex that are frequent in sympatric domestic small ruminant herds, suggesting that these herds could act as reservoirs; similarly Cubero-Pablo et al (2000) reported a considerable

seroprevalence of *Chlamydophila* sp. However, further research is needed on the Iberian ibex health status to determine the impact of potential pathogens.

It is thought that in previous decades the Iberian ibex population was prevented from growing and expanding by competition with domestic livestock, which restricted ibexes to marginal habitats (Herrero and Pérez, 2008). In fact, this is indicated by Acevedo et al (2007) in relation to the population from Castilla-la-Mancha. Nevertheless, this species is now abundant and its range and population are currently expanding. For this reason, it is assessed as "Least Concern" by the IUCN. Hunting reservations and protected areas are thought to have played a crucial role in species recovery, though most of the range occupied by wild goats is outside protected areas (Herrero and Pérez, 2008). However, Acevedo et al (2011) forecasted a reduction in the Andalusian Iberian ibex populations attributable to competition with sympatric species and land use-changes. There is thus uncertainty regarding the conservation status of this species, with the identification of other threats such as overabundance or human disturbance (Acevedo and Cassinello, 2009).

The zoonotic pathogens

Salmonella enterica

Salmonella enterica is a Gram-negative bacillus from the Enterobacteriaceae family (Prescott et al, 1999) whose main taxonomic descriptor is the serotype (Foley and Lynne, 2008). Serotyping is a technique that uses differences in the polysaccharide portion of the lipopolysaccharide layer (O antigen) and the filamentous portion of the flagella (H antigen) present on the surface of *Salmonella* to separate strains into distinct serotypes (serovars). There are over 2600 identified *Salmonella* serotypes (EFSA, 2013).

The primary route of *Salmonella* infections in humans and other animal species is faecal-oral transmission; in addition, in cattle and swine the respiratory tract and tonsils are potential sites of invasion (De Jong and Eckdahl, 1965; Fedorka-Cray et al, 1995). *Salmonella* entering via the faecal-oral route are able to colonize the small intestine, colon and cecum, by adhering to the mucosa with the fimbriae and pili present on the bacterial cell surface. As reviewed by Foley and Lynne (2008), *Salmonella* have evolved intricate measures to invade host cells following epithelial attachment.

Infections by *Salmonella enterica* are a significant public health concern around the world, and a number of different *Salmonella* are capable of causing human disease (Foley and Lynne, 2008). In the European Union, serotypes Enteritidis and Typhimurium have been the most frequently reported in human cases in recent years (EFSA, 2012a; 2013). However, in general, every serotype of *S. enterica* ssp. *enterica* should be considered as potentially harmful to humans (EFSA, 2012a). In general terms, *Salmonella* serotypes can be classified as host-restricted (almost exclusively associated with a single species, e.g. Typhi in humans), host-adapted (frequent in one species but can also cause disease in others) or un-restricted (ubiquitious) serotypes, which usually induce a self-limiting gastroenteritis in a broad range of unrelated host species (Uzzau et al, 2000). Serotypes Enteritidis and Typhimurium belong to the latter classification, and are additionally capable of causing systemic disease in a wide range of host animals.

In humans, typhoid and paratyphoid fever are caused by serotypes Typhi and Paratyphi (A, B or C), respectively. The rest of the serotypes cause non-typhoid salmonellosis, whose most common manifestation is mild to moderate gastroenteritis consisting of diarrhoea, abdominal cramps, vomiting and fever (Foley and Lynne, 2008). The symptoms are usually self-limiting and typically resolve within 2 to 7 days; in a small number of cases, septicemia and invasive infections of the organs can occur. Fluoroquinolones and ceftriaxone are the recommended

drugs for *Salmonella* septicemia. Salmonellosis is also associated with long-term or chronic sequelae, like reactive arthritis (EFSA, 2013).

S. Enteritidis is more frequently associated in cases of human illness to the consumption of eggs or poultry meat, while *S*. Typhimurium is associated with the consumption of pig, bovine and poultry meat (EFSA, 2013). Animals can go unnoticed as intermittent or persistent carriers, but they may also show fever, diarrhea, abortion or death.

Reptiles and birds are typically colonized by *Salmonella*, but generally by serotypes that are host-specific and are not very pathogenic to humans except for children or adults with some immunodeficiency (Hilbert et al, 2012). *Salmonella* can be found in a variety of wild animals living in the wild, and considerable differences in *Salmonella* prevalence have been reported between species and regions (Paulsen et al, 2012). In some instances, e. g. in Antarctic fauna, *Salmonella* serotypes are indicators of exposure to human activities (Iveson et al, 2009).

Salmonellosis is defined by Gortázar et al (2007) as a disease in which wildlife is suspected to be important for disease control. In addition, it is of economic and zoonotic relevance and may affect wildlife management and conservation. According to Hilbert et al (2012), the role of wildlife is of increasing interest owing to (1) its potential as a transmission or accumulation vector in contact with domestic animals, (2) outbreaks reports from direct contact between wildlife and humans, (3) the possible contribution of meat of wild animals that are carriers of *Salmonella* to human salmonellosis and (4) the potential of wildlife vectors as a source of contamination of non-animal based food or food facilities.

Escherichia coli O157:H7

Escherichia coli (*E. coli*) is a Gram-negative bacterium from the Enterobacteriaceae family (Prescott et al, 1999). However, some strains can be pathogenic to both humans and animals. Pathogenic *E. coli* strains use a multi-step scheme of pathogenesis, which consists of colonization of a mucosal site, evasion of host defenses, multiplication and host damage (Kaper et al, 2004). *E. coli* diarrhoeagenic strains have been classified into six categories: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic E. coli (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Nataro and Kaper, 1998).

Additionally, *E. coli* can be defined by its serogroup (shared O (somatic) antigen) and serotype (specific combination of O and H (flagellar) antigen) (Kaper et al, 2004). Hence, *E. coli* O157:H7 is a serotype, and furthermore, it is typically classified as EHEC (Bardiau et al, 2010; García et al, 2010). EHEC are in turn a subset of VTEC (Verocytotoxin-producing *E.coli*) (Nataro and Kaper, 1998). This is a parallel nomenclature used to refer to *E. coli* strains that produce one or more toxins of the Stx family, also called STEC (Shiga-toxin producing *E.coli*) (Nataro and Kaper, 1998). Most of the VTEC serotypes are not associated with human disease; therefore, EHEC is used only to denote a subset of these that contain the locus of enterocyte effacement pathogenicity island (Kaper et al, 2004) and includes a clinical connotation (Nataro and Kaper, 1998). Henceforth, I will refer to VTEC since this is the nomenclature used by the European Food Safety Authority. Please note that Verocytotoxin-producing *E.coli* can be also called verotoxigenic *E. coli* or verocytotoxigenic *E. coli* (EFSA, 2013).

VTEC are a group of *E. coli* capable of producing potent cytotoxins (verocytotoxins) that inhibit protein synthesis within eukaryotic cells (EFSA, 2009). VTEC infections are important zoonotic diseases able to cause severe disease in humans. The serogroups *E. coli* O157:H7 and *E. coli* O157:H- (VTEC O157) are currently those most frequently reported in the European Union to be associated with human disease (EFSA, 2013). Moreover, VTEC O157 is also commonly associated with the most severe cases of disease, i.e. haemorrhagic colitis and the potentially life-threatening haemolytic uremic syndrome (EFSA, 2009). Transmission occurs via the faecal-oral route and frequently through ingestion of contaminated food or water; direct contact with infected animals or humans; and rarely, inhalation (reviewed by García et al, 2010).

The most recent EFSA report available (EFSA, 2013) includes the large outbreak caused by VTEC O104:H4 that occurred in Germany in summer 2011; therefore the number of cases

reported that year increased more than 2.5 times in relation to 2010. The identified food vehicle was fenugreek sprouts imported from outside the EU (EFSA, 2013). Even without these data, the EU trend for VTEC infections continued to significantly increase for the fourth consecutive year.

It is known that young cattle (especially between 3-24 months of age) are an important reservoir of VTEC, but cattle outside this age range are less likely to excrete the pathogen (EFSA, 2009). Likewise, sheep, goats and wild ruminants seem to be important reservoirs for VTEC (EFSA, 2013). Cattle particularly can shed periodically or seasonally ubiquitously VTEC O157 at a highly variable prevalence without suffering apparent illness (García et al, 2010). VTEC is also reported in non-ruminant populations: many of these animal species are believed to be transient hosts, who only excrete the organism for a short period after infection (EFSA, 2009), but there may be other important reservoirs. This and other human pathogenic serogroups may be found in a range of different animal species and food categories; however, the frequency varies strongly among the EU states (EFSA, 2012a).

In light of the virulence of certain VTEC O157, García et al (2010) underscore the need to be vigilant for this pathogen and to apply "One health" approaches to minimize the potential for zoonotic transmission and disease outbreaks. Considering the information currently available, it can be suggested that wildlife, or at least certain species, should be included in *E. coli* O157:H7 monitoring, e.g., red deer are known to be a natural reservoir of O157:H7 in Spain (Díaz-Sánchez et al, 2013); furthermore, this pathogen has also been detected in wild boar (Sánchez et al, 2010) in this country. Similarly, in Belgium wild cervids are considered a potential source of VTEC (Bardiau et al, 2010b). Therefore, further studies are needed in order to determine the potential role of wild boar in the epidemiology of *E. coli* O157:H7.

Thermophilic *Campylobacter*

Campylobacter is a Gram-negative bacterium with a sole polar flagel on one or both sides (Prescott et al, 1999). It is a microaerophile (i.e., it does not tolerate the levels of atmospheric oxygen (20%), and thus must be grown at an oxygen concentration of 2 -10%) and nutritionally fastidious (shows complex nutritional requirements) (Man, 2011). The genus *Campylobacter* is morphologically diverse – curved, spiral or rod-shaped species are included. They can be found in the intestinal tract, the reproductive organs or the oral cavity.

The genus *Campylobacter* includes non-pathogenic and pathogenic species for animals and humans. Campylobacteriosis in humans is caused by thermotolerant (also called thermophilic) *Campylobacter* species, with the infective dose being usually very low (EFSA, 2013). *Campylobacter* can be transmitted through direct contact with animals or their products (Prescott et al, 1999), with broiler meat the main source for food-borne campylobacteriosis in humans in the EU (EFSA, 2013). Other diseases associated with *Campylobacter* infections are Inflammatory Bowel Disease, oral diseases, meningitis and urinary tract infection (Man, 2011). *C. jejuni* infection is also related to severe sequelae such as Guillain-Barré Syndrome (EFSA, 2013).

Campylobacter naturally occurs in aquatic environments and members of this genus often colonize humans, other mammals, birds, reptiles and shellfish, but birds are considered its main reservoir. With regards to food-producing animals, *Campylobacter* spp are very prevalent in poultry, cattle and sheep as well as in pets such as dogs or cats; however, in animals *Campylobacter* is rarely associated with mortality (EFSA, 2013).

C. jejuni is the most well-known species since it is an important cause of human gastroenteritis worldwide (Man, 2011). *C. jejuni* can be isolated in large amounts from surface waters, and is very common in the gastrointestinal tract of animals (Prescott et al, 1999). Many cattle herds and poultry flocks shed this microorganism. An emerging highly virulent clone causes outbreaks of ovine abortions in the USA and its zoonotic nature has been recently suggested (EFSA, 2013). In humans, *C. jejuni* causes enteritis after multiplying in the small intestine and producing toxins. Principal foods implicated in its transmission are milk, pork and poultry products, usually undercooked. The incubation period lasts between 2 and 10 days and after this period fever and profuse diarrhoea with fresh blood in the faeces occurs. Campylobacteriosis is normally self-limiting and most cases recuperate within 5 to 8 days. In severe cases with invasion, erythromycin (a macrolide) is used. *C. coli* and *C. fetus* are also a

recognized pathogen causing human gastroenteritis. The latter also causes reproductive disease and abortion in cattle and sheep.

In the EU, *Campylobacter* is the most commonly reported gastrointestinal bacterial pathogen since 2005, and the species most frequently associated are *C. jejuni* followed by *C. coli* and *C. lari* (EFSA, 2013). The reasons for this continued increase are not completely known. It is difficult to understand all aspects of its epidemiology since it is a multi-host pathogen highly prevalent in the environment; in addition, it is suspected that climate factors play an important role.

Some *Campylobacter* species have been labeled as emerging, but others are not considered emerging pathogens since they are newly identified or little is known about their pathogenic potential (Man, 2011). Due to the difficulty in the isolation of the more fastidious *Campylobacter* and to the methodology generally used, which is not always suitable for the recovery of the emerging *C.* species, the contribution of these species to the etiology of human gastroenteritis may be underestimated. The most frequent emerging species isolated from patients with gastroenteritis are *C. concisus* and *C. upsaliensis*.

Studies of *Campylobacter* in wildlife are scarce, but remarkably a variety of wild mammals carry potentially pathogenic emerging *Campylobacter* spp. In fact, the EFSA (2013) considers as principal *Campylobacter* reservoirs “wild and domesticated birds and mammals”. Perhaps due to this concern, an increase in studies on zoonotic *Campylobacter* carried by wildlife has recently occurred, investigating wild reptiles (Wang et al, 2013), mammals (Díaz-Sánchez et al, 2013) or birds (Griekspoor et al, 2013; Sanad et al, 2013).

Antimicrobial resistance

A new global threat

Host and bacteria have coevolved over millions of years; on the other hand, the evolution of antimicrobial resistance (AMR) is relatively recent (Beceiro et al, 2013). Some bacteria are considered to have been resistant since ancient times, referred to as "naturally" or "intrinsically" resistant, but others have acquired resistance (WHO, 2011). It is assumed that whenever antimicrobial agents are used, bacteria inevitably develop resistance mechanisms either through spontaneous mutation or by acquiring genes from other bacteria (da Costa et al, 2013), and hence the appearance of AMR has been driven by both appropriate and inappropriate use of antimicrobial agents for human and animal health and food production, although there are probably other selective pressures (Allen et al, 2011).

It is concerning that AMR can compromise the effectiveness of the antibiotic treatment in severe infections (EFSA, 2012b), and furthermore, can increase the virulence or fitness of certain bacterial species in some environments, often by helping these species to colonize new niches (Beceiro et al, 2013). AMR is found in both pathogens and commensal bacteria. For example, *Salmonella* is a public health concern not only because of its frequency in zoonotic events, but also because many strains are resistant to a number of antimicrobial agents (Foley and Lynne, 2008). On the other hand, commensal *E. coli* strains rarely cause disease except in immunocompromised hosts or where the normal gastrointestinal barriers are breached (Kaper et al, 2004). Commensal species are also called indicator bacteria, since they form a reservoir of resistance genes, which may transfer between bacterial species (EFSA, 2008). *E. coli* for instance is known to be adept at horizontal gene transfer (CoNDitSoAT, 2006). In this Thesis, the indicator bacteria tested for AMR are *E. coli*, *Enterococcus faecium* and *Enterococcus faecalis.* These species naturally occur in the intestine of the majority of animals and are suitable indicators (EFSA, 2012b).

AMR in bacterial isolates from food and food-producing animals is variably prevalent depending on the agent, the bacterial species and the animal species. Nevertheless, resistant bacteria occurring in animals can spread to humans via food-borne routes and environmental routes such as water and direct animal contact (EFSA, 2012b). Wildlife can also contribute to the spread of AMR, even though wild animals are not treated with antimicrobial agents, resistant bacteria occur in them (Radimersky et al, 2010).As in the case of pathogens, the wildlife-human interface may be an important pathway of AMR transmission that is being studied in different species and circumstances, e.g. Palomo et al (2013) describe the spread of quinolone-resistant *Salmonella* among humans, livestock and white stork (*Ciconia ciconia*) and

Sayah et al (2005) evaluate resistance patterns in *E. coli* from human septage, domestic and wild animals (cervids and birds) and surface water.

As a global threat and a food safety problem, the fight against AMR requires a holistic and multifaceted approach with coordination and exchange of information among the agricultural, food, veterinary and health sectors (WHO, 2011). To unify efforts, the most relevant world agencies of human and animal health, namely the World Health Organization (WHO) and the World Organization for Animal Health (OIE), have categorized antimicrobial agents as a function of their importance. This allows the identification of those agents that should be used prudently and for which risk management strategies should be prioritized. Table 1 is a list of the antimicrobial agents included in the susceptibility test implemented for this Thesis. Please note that most antimicrobial agents selected are categorized as Critically Important and/or Veterinary Critically Important and represent the high variability of antimicrobial classes. Specific information on each antimicrobial class with regards to the classification criteria is given in Table 2.

In this Thesis, to report antimicrobial resistance we use as much as possible epidemiological cut-off values (not clinical breakpoints). This is because our purpose is not to determine the probability of treatment failure in wildlife, since they are likely to never receive antibiotic treatment, but to detect "non-wild type" microorganisms (i.e., not susceptible). The epidemiological cut-off value detects any deviation in susceptibility from the wild-type population, and therefore, enables early detection of developing resistance (EFSA, 2012b). Hence, what we report in this Thesis is "microbiological resistance" and not "clinical resistance".

WHO and OIE classification criteria

Currently, the Third Revision of Critically Important for Human Medicine (WHO, 2012) is the reference document with definitions and categories. The criteria on which the definitions are based are:

-Criterion 1: An antimicrobial agent which is the sole, or one of limited available therapy, to treat serious human disease.

-Criterion 2: An antimicrobial agent that is used to treat diseases caused by either: (1) organisms that may be transmitted to humans from non-human sources or, (2) organisms that may acquire resistance genes from non-human sources.

Interpretation of the categories is the following:

-Critically Important Antimicrobial (CIA): Those antimicrobials which meet both Criterion 1 and Criterion 2.

-Highly Important Antimicrobial (HIA): Those antimicrobials which meet either Criterion 1 or Criterion 2.

-Important Antimicrobial (IA): Those antimicrobials those which meet neither Criterion 1 nor Criterion 2.

Similarly, in the List of Antimicrobials of Veterinary Importance (OIE, 2007) criteria and categories for veterinary antimicrobials are defined:

-Criterion 1: It is met when more than 50% of the respondents of the questionnaire for the preparation of the draft list identified the importance of the antimicrobial class.

-Criterion 2: It is met when compounds within the class are identified as essential against specific infections and there is a lack of sufficient therapeutic alternatives.

The categories established are:

-Veterinary critically important antimicrobials (VCIA): meet both criteria 1 and 2.

-Veterinary highly important antimicrobials (VHIA): meet criterion 1 or criterion 2.

-Veterinary important antimicrobials (VIA): meet neither criteria 1 or 2.

Antimicrobial agents tested in this Thesis

Table 1. Selection of antimicrobial agents and its classification.

Antimicrobial	WHO category	OIE category	Vet. species	Observations
Aminoglycosides				
Streptomycin			AP, AV, B, CP, E, L, O, P, S	
Kanamycin			AV, B, E, P, S	
Apramycin	CIA	VCIA	AV, B, L, O ,S	Apramycin: Veterinary use only
Gentamicin			AV, B, CM, CP, E, L, O, S	
Amikacin			E	
Lincosamides				
Lyncomycin	HIA	VHIA	AP, B, AV, CP, O, P, S	
Macrolides				
Erythromycin	CIA	VCIA	AP, AV, B, CP, E, L, O, P, S	
Aminopenicillins				
Amoxicillin Ampicillin	CIA	VCIA	AV, B, CP, E, O, P, S	
Natural penicillins				
Penicillin G	CIA	VCIA	AV, B, CM, CP, E, L, O, S	Also called Benzylpenicillin
Aminopenicillin plus betalactamase inhibitor				
Amoxicillin-clavulanic acid	-	VCIA	AV, B, CP, E, O, S	Combination not listed by the WHO
Phenicols				
Florphenicol	HIA	VCIA	AV, B, CP, E, L, O, P, S	Florphenicol: Veterinary use only
Chloramphenicol			Not listed	
Cyclic polypeptides				
Colistin	CIA	VHIA	AV, B, CP, E, L, O, S	
Quinolones				
Nalidixic acid	CIA	VCIA	B	1st G
Ciprofloxacin			AV, B, S	2nd G (fluoroquinolones)
Diaminopyrimidines				
Trimethoprim	HIA	VCIA	AV, B, CP, E, L, O, S	
Tetracyclines				
Tetracycline	HIA	VCIA	AP, AV, B, CM, CP, E, L, O , P , S	
Sulfamides				
Sulfamethoxazole	HIA		Not listed	Sulfonamides and combinations are VCIA
Streptogramins				
Quinupristin/dalfopristin	HIA		Not listed	
Oxazolidinones				
Linezolid	CIA		Not listed	Only used in human medicine
Monobactams				
Aztreonam	CIA		Not listed	
Glycopeptides				
Vancomycin	CIA		Not listed	Last resort in vet. med.
Cephalosporins				
Cefotaxime	CIA		Not listed	3rd G
Ceftazidime				
Cefoxitin	HIA			2nd G
Carbapenem and other penems				
Imipenem	CIA		Not listed	

AV=Avian, AP=Bee, B=Bovine, CM=Camel, CP=Caprine, E=Equine, L=Rabbit, O=Ovine, P=Fish, S=Swine. G= Generation

Table 2. Specific information on each antimicrobial class. Sources: WHO (2012) and OIE (2007).

Antimicrobial class	In humans, sole or limited therapy for:	May result from transmission from non-human sources of:	In veterinary medicine, used in:
Aminoglycosides	As part of treatment of enterococcal endocarditis and MDR tuberculosis.	*Enterococcus* spp., Enterobacteriaceae and *Mycobacterium* spp.	Septicaemia, digestive, respiratory and urinary diseases.
Lincosamides	-	*Enterococcus* spp. and *Staphylococcus aureus* including MRSA	Mycoplasmal pneumonia, infectious arthritis and hemorrhagic enteritis of pigs.
Macrolides	*Legionella*, *Campylobacter* and MDR *Salmonella* and *Shigella* infections.	*Campylobacter* spp. and *Salmonella*	*Mycoplasma* infection in pig and poultry, hemorrhagic digestive disease in pigs and liver abscesses (*Fusobacterium necrophorum*) in cattle (few alternatives), respiratory infections in cattle.
Oxazolidinones	MDR MRSA and MDR *Enterococcus* spp.	*Enterococcus* spp. and MRSA	-
Carbapenem and other penems	MDR Enterobacteriaceae.	Enterobacteriaceae	-
Glycopeptides	MDR MRSA and MDR *Enterococcus* spp	*Enterococcus* spp. and MRSA	-
Monobactams	MDR Gram negatives, especially with limited other options including for ESBLs.	Enterobacteriaceae	-
Penicillins	Limited therapy for *Listeria, Enterococcus* spp.	*Enterococcus* spp., Enterobacteriaceae	Septicaemias, respiratory and urinary tract infections. Few economical alternatives available.
Phenicols	In certain geographic settings: bacterial meningitis, typhoid and non-typhoid fever and respiratory infections	Enterobacteriaceae	Importance in treating some fish diseases, useful alternative in respiratory infections of cattle, swine and poultry. Florphenicol: pasteurellosis in cattle and pigs.
Cyclic polypeptides	MDR Enterobacteriaceae	Enterobacteriaceae	Septicaemias, colibacillosis, salmonellosis and urinary infections.
Tetracyclines	Infections due to *Brucella, Chlamydia* spp. and *Rickettsia* spp.	*Brucella* spp.	Treatment of many bacterial and chlamydial diseases. Few economical alternatives available.
Sulfamides + dihydrofolate reductase inhibitors (diaminopyrimidines)	In certain geographic settings, for acute bacterial meningitis, systemic non-typhoidal salmonella infections and other infections	Enterobacteriaceae	Essential because of diseases covered (bacterial, coccidial and protozoal infections), and use in multiple animal. Few economical alternatives available.
Quinolones	*Campylobacter* spp., invasive disease due to *Salmonella* spp. and MDR *Shigella* spp. infections.	*Campylobacter* spp. and Enterobacteriaceae	Septicaemias and infections such as colibacillosis. Fluoroquinolones have no equally efficacious alternative in the treatment of chronic respiratory disease in poultry (*E. coli*).
Cephalosporins (3^{rd} G)	Acute bacterial meningitis and disease due to *Salmonella* in children. MDR Enterobacteriaceae	Enterobacteriaceae	-
Cephalosporins (2^{nd} G)	In certain geographic settings, one of limited therapies for sepsis in children	Enterobacteriaceae	-
Streptogramins	-	*Enterococcus* spp. and MRSA	Virginiamycin is an important antimicrobial in the prevention of necrotic enteritis (*Clostridium perfringens*)

MDR= Multi-drug resistant. MRSA= Methicillin-resistant *Staphylococcus aureus*. ESBL= Extended-Spectrum Beta-Lactamase. G=Generation

Hypothesis and objectives

Hypothesis and objectives

In a natural environment where livestock and wild ungulates co-habit ("Els Ports de Tortosa i Beseit"), previous works have detected a spill-over of *Mycobacterium bovis* and *Salmonella enterica* (Mentaberre et al, in press) from cattle to wild boar. Further questions arise in regards to these and other zoonotic pathogens, since this area is a National Game Reserve where both wild boar and Iberian ibex are hunted and consumed. For this reason, food-borne pathogens were selected for this Thesis, as well as antimicrobial resistance as an indicator of "how wild are wild animals" (Osterblad et al, 2001), and surveyed in both game species and livestock.

The specific objectives were:

To further investigate whether livestock presence affects the prevalence, diversity and antimicrobial resistance of *Salmonella enterica* in sympatric wild ungulates (Study I and II).

To investigate whether *Escherichia coli* O157:H7 (Study III) and thermophilic *Campylobacter* (Study IV) are shared between wild ungulates and sympatric livestock in extensive farming conditions.

To determine the presence of antimicrobial resistance in *E. coli* and *Salmonella* in co-habiting wild and domestic species in a natural environment (Study I to III).

To investigate which thermophilic *Campylobacter* species are carried by wild ungulates (Study IV).

To explore the prevalence of the above-mentioned pathogens and the presence of AMR in indicator bacteria in an urban wild boar population (Study V).

Study I:

Effect of cattle on *Salmonella* carriage, diversity and antimicrobial resistance in free-ranging wild boar (*Sus scrofa*) in notheastern Spain.

Plos One, 7(12):e51614.

Abstract

Salmonella is distributed worldwide and is a pathogen of economic and public health importance. As a multi-host pathogen with a long environmental persistence, it is a suitable model for the study of wildlife-livestock interactions. In this work, we aim to explore the spill-over of *Salmonella* between free-ranging wild boar and livestock in a protected natural area in NE Spain and the presence of antimicrobial resistance. *Salmonella* prevalence, serotypes and diversity were compared between wild boars, sympatric cattle and wild boars from cattle-free areas. The effect of age, sex, cattle presence and cattle herd size on *Salmonella* probability of infection in wild boars was explored by means of Generalized Linear Models and a model selection based on the Akaike's Information Criterion. Prevalence was higher in wild boars co-habiting with cattle (35.67%, CI 95% 28.19 - 43.70) than in wild boar from cattle-free areas (17.54 %, CI 95% 8.74 - 29.91). Probability of a wild boar being a *Salmonella* carrier increased with cattle herd size but decreased with the host age. Serotypes Meleagridis, Anatum and Othmarschen were isolated concurrently from cattle and sympatric wild boars. Apart from serotypes shared with cattle, wild boars appear to have their own serotypes, which are also found in wild boars from cattle-free areas (Enteritidis, Mikawasima, 4:b:- and 35:r:z35). Serotype richness (diversity) was higher in wild boars co-habiting with cattle, but evenness was not altered by the introduction of serotypes from cattle. The finding of a *S*. Mbandaka strain resistant to sulfamethoxazole, streptomycin and chloramphenicol and a *S*. Enteritidis strain resistant to ciprofloxacin and nalidixic acid in wild boars is cause for public health concern.

Introduction

Interactions in the wildlife-livestock interface are currently increasing in the EU since animal husbandry is moving from more intensive to more extensive farming systems (Gortázar et al, 2007). This fact often enhances disease transmission between wildlife and livestock and may be of particular concern in relation to wild ungulates, which frequently share habitat resources with domestic livestock (Boehm et al, 2007). The wild boar is especially considered a carrier and reservoir of several zoonotic pathogens (Meng et al, 2009; Wacheck et al, 2010).

Among the pathogens shared between wildlife and domestic animals, little is known about *Salmonella* spp. (one of the most common genera of zoonotic bacteria of worldwide economic and health importance (Uzzau et al, 2010)) and the role of the wildlife-livestock interface in its transmission. This microorganism is considered a true multi-host pathogen with a long environmental persistence (Murray, 1991). These characteristics (broad host range of some serotypes and long environmental persistence) make it a suitable model for studying interactions between wildlife and livestock in natural environments.

Most of the studies on *Salmonella* in wildlife focus on vectors such as insects, rodents and birds in the farm environment (some examples are Davies and Breslin (2003), Garber et al (2003) and Liebana et al (2003)), and to our knowledge only one study relates the *Salmonella* prevalence and serotypes in wild large mammals to those found in co-habiting livestock: Glawischnig and colleagues (2000) report an outbreak of salmonellosis (caused by serotype Dublin) in chamois (*Rupicapra rupicapra*) which had its origin in sick cattle grazing in the same pasture.

From a public health perspective, wildlife can play an important role in the complex *Salmonella*-wildlife-human cycle (Hilbert et al, 2012) since wildlife has been shown to be a common reservoir of this pathogen, in addition, *Salmonella* can be isolated at virtually every step of the game meat chain (Paulsen et al, 2012) and healthy animals can shed *Salmonella* over long periods of time. *Salmonella* is also of public health concern because many strains are resistant to a number of antimicrobial agents: data show that 44% of the *Salmonella* samples isolated from animal slaughter and veterinary diagnostic sources were resistant to at least one antimicrobial agent (Foley and Lynne, 2008).

In wildlife, investigations of antimicrobial resistance are highly variable in their results, mainly depending on the host species, the bacterial species and the geographic location, but it is assumed that livestock and humans may be sources of antimicrobial resistance in wildlife

(some examples, though not of *Salmonella*, are Dolejska et al, 2007; Literak et al, 2010; and Skurnik et al, 2006).

It has been stated that an ecological approach may help to understand pathogen dynamics and host-pathogen interactions, but few studies have actually applied tools from population ecology to environmental microbiology and veterinary research (some examples on *Salmonella* are Lankau et al, 2012; Patton et al, 2009; and Santos et al, 2007). This may be essential from a public health perspective, e.g. Perron et al (2007) found that asymptomatic *Salmonella* Typhimurium DT104 isolates showed a greater phenotypic and genotypic diversity within pig herds than disease-associated ones. Especially in the case of research in wildlife diseases, this multidisciplinary approach must be adopted (Daszak et al, 2007; Hudson et al, 2002).

In this work, we aim to assess the effect of livestock presence on the prevalence and the components of diversity (richness and evenness) of *Salmonella* in the free-ranging wild boar population in the Ports de Tortosa i Beseit National Game Reserve, northeast Spain. In this game reserve, cattle occupy specific areas during the greater part of the year. Also, we assessed antimicrobial resistance in both wild boar and cattle in this natural area, where human activities are minimal.

Materials and Methods

Study area

The study area is located within the National Game Reserve Els Ports de Tortosa i Beseit in northeastern Spain, which is also part of the Natural Park of the same name. It is a calcareous mountain region with high orographic complexity that results in a rugged and abrupt terrain with numerous ravines and steep slopes. About 28% of the surface is above 1000 m.o.s.l., with the highest peak being Mont Caro (1442 m). The predominant habitat is pine grove (39%) followed by oak grove (15%), and, due to the dry Mediterranean climate, rivers account for only 0.2%. The most abundant wild ungulates are the Iberian ibex (*Capra pyrenaica*) and the wild boar (*Sus scrofa*), which are exploited for hunting purposes. Wildlife and cattle share pastures in some valleys of the study area, therefore, we chose three hunting areas (HA, hereafter) that are free of cattle presence and five HA are grazed by cattle either year-round or during the hunting season (see Figure 3: areas have been called A to H). Areas were categorised as grazed or cattle-free, and the animals were grouped according to this category for some analyses. Despite all HA belong to the same ecosystem and the ubiquitous nature of

Salmonella and its high survival rate in soil (Winfield and Groisman, 2003); we took into account that differences between areas in the landscape composition could have confounding effects on the results. Therefore, we checked for differences among HA by Geographic Information System (GIS) analysis. In brief, we have used CORINE land cover data CLC2006 (European Environment Agency, 2007) with a working scale of 1:100.000, a minimum mapping unit 25 hectares and a minimum width of linear elements 100 metres. Later we estimated the surface of the main landscape classes using the ArcGIS 10 (Esri® ArcMap 10) by area and a MANOVA analysis for comparing landscape features (e.g., mean slope, mean altitude, percentage of sclerophyllous vegetation, percentage of mixed forest, percentage of coniferous forest and percentage of transitional woodland-shrub, all of them as response variables) between two categories (grazed vs ungrazed, as explanatory factor). No difference in terms of land use and landscape composition was observed among our sampling areas (Pillai statistic = 0.93, df = 1 p = 0.47), hence we assume that landscape characteristics will have minimum effect on the observed patterns of *Salmonella* occurrence.

Animal sampling

Wild boar

Altogether, 214 individual faecal samples were obtained from hunter-harvested wild boars during the regular hunting season (October to January) from 2007-2008 to 2010-2011. Fifty-seven samples were obtained from the cattle-free HA and 157 from the grazed ones. The difference in sample size is due to a different hunting effort (mean hunting days/year in HA with cattle is 23, while it is 8 in cattle-free HA, $F = 117.48$ df = 1, $p < 0.001$, ANOVA) owing to easier access and orography in grazed areas, which results in a higher amount of wild boars hunted in those areas each year (mean number of wild boars hunted/year in cattle free HA is 50, while it is 110 in HA with cattle, $F = 7.41$, df = 1, $p = 0.01$, , ANOVA).

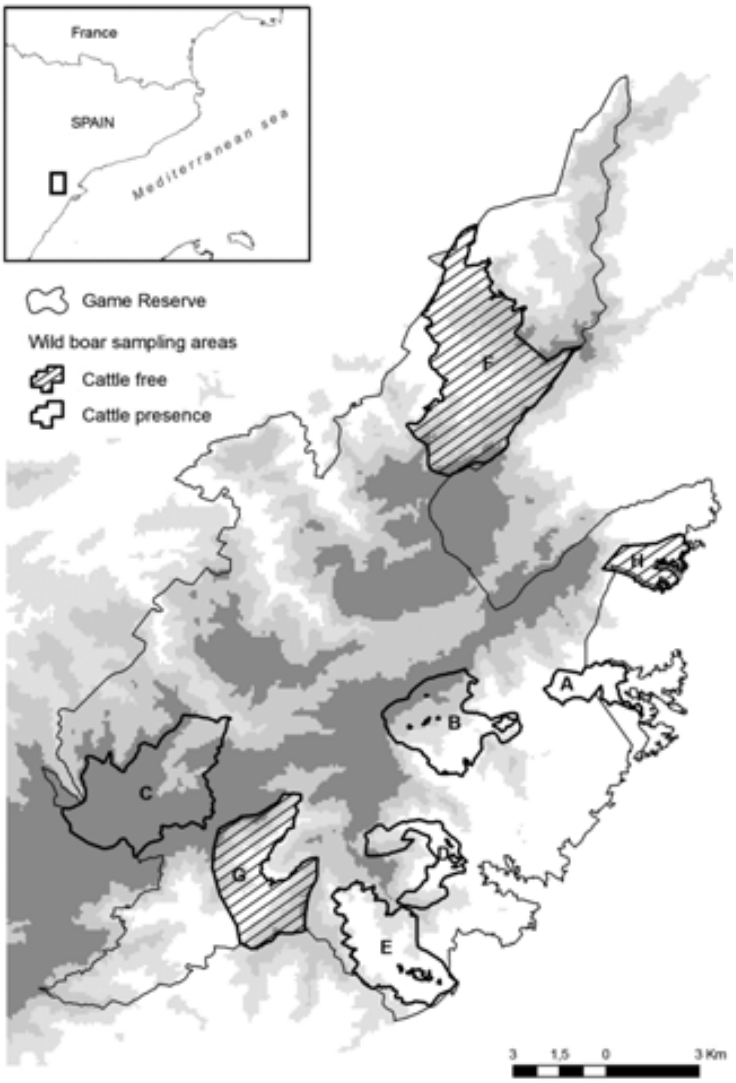

Figure 3. Sampling areas with cattle presence and absence.

On the other hand, to collect further information on the animals, sex was visually determined by direct observation of genitalia and age was estimated based on tooth eruption pattern and replacement as well as dental attrition (Boitani and Mattei, 1992). So as to minimize error in age determination, four age classes were used: piglets (up to 5 months), juveniles (6 to 12 months), yearlings (between 13 and 24 months) and adults (over 24 months). Finally, Juveniles and Piglets were grouped together due to the small sample size of these age classes. Faeces were collected directly from the rectum and stored in sterile containers. They were refrigerated and sent to the laboratory within the following 24 hours.

Cattle

Seventy-three cattle samples were collected from 2008 to 2010. Previously, information was obtained from the Reserve's managers on herd location (five herds, size 30, 50, 60, 70 and 170 individuals). The farming conditions in the National Game Reserve are free-ranging with

supplemental feeding in the dry season (summer) and herds are small, which sometimes made it difficult to locate the animals (e.g., 60 animals in a 1823 ha area). The herd in area A (see Fig. 3) belongs to a bullfighting breed while the rest (B – E) are herds aimed at meat production. Cattle sampling was preferably performed on days that wild boars were also sampled. When cattle were located, animals were counted to assess aggregation and observed until defecation. Then, faeces were stored in a sterile container and refrigerated and sent to the laboratory within the following 24 hours.

Ethics statement

No permit or approval was needed for this work, since it does not imply extraordinary activities in the National Game Reserve. Faecal samples from the animals were collected specifically for this study. All animals were legally hunted and sampled with the permission of the National Game Reserve. Wild boars are hunted by groups of local hunters by the traditional method of this region (drive hunting) as allowed by the National Game Reserve. Hunters allowed the sampling of the harvested animals and the use of these samples for scientific purposes. Cattle were also sampled on public land and since samples were environmental there was no need for animal management.

Microbiological analyses

Cultures of *Salmonella* were performed according to ISO 6579:2002 Annex D (International Organization for Standardization, 2007), which is the method recommended by the European Union Reference Laboratory for *Salmonella* in faecal and environmental samples. For all samples a 1/10 dilution in buffered peptone water (BPW) was made, then incubated at 37ºC ± 1°C for 18 h ± 2 h. Next, Modified semi-solid Rappaport-Vassiliadis (MSRV) (Difco) agar plates were inoculated with three drops (a total volume of 0.1 ml) of BPW culture. Plates were incubated at 41.5 ºC ± 1°C for 24h ± 3 h and if negative, they were incubated for an additional 24h ± 3 h. Suspected growth of *Salmonella* was confirmed by plating on both Xylose Lysine Desoxycholate agar (XLD) (bioMérieux) and on chrom ID^{TM} *Salmonella* agar (SM ID2) (bioMérieux). The plates were incubated for 24h ± 3 h at 37ºC ± 1°C.

Colonies of presumptive *Salmonella* were subcultured on Columbia 5% sheep blood agar (bioMérieux) and incubated for 24h ± 3 h at 37ºC ± 1°C. Identity of isolates as *Salmonella* spp. was confirmed by a commercially available biochemical method Enterotube ^{TM} II (BD BBL ^{TM}). Serological typing of one isolate per sample was performed based on the Kauffmann-White scheme (Grimont and Weill, 2007). Phage Typing was performed at the National Center of

Microbiology, Institute of Health Carlos III (Madrid, Spain) by using Anderson's scheme (Anderson et al, 1977).

Antimicrobial susceptibility testing

A set of isolates was selected in order to characterize the frequency of antimicrobial resistance within each host group (cattle, wild boars from cattle-free areas, wild boars from grazed areas). At least one isolate from each serotype within each host group was tested for antimicrobial resistance. Most serotypes were isolated only once within each host group. When serotypes were more frequent, additional isolates were tested only when they belonged to different places or times with respect to the isolate already tested.

Antimicrobial resistance was tested by the agar diffusion method to obtain the Inhibition Zone Diameter (IZD) against amoxicillin-clavulanate, cefoxitin, amikacin, apramicin, imipenem and aztreonam while the broth microdilution method was performed to determine the minimum inhibitory concentrations (MIC) of sulfamethoxazole, gentamicin, ampicillin, ciprofloxacin, cefotaxime, ceftazidime, tetracycline, streptomycin, trimethoprim, chloramphenicol, florfenicol, kanamycin and nalidixic acid. Epidemiological cut-off/breakpoint values are shown in Table 3.

Table 3. Antimicrobial agents used and cut-off values.

Method	Antimicrobial agent	Disk content / concentration range	Cut-off value / break-point	Reference
Disk diffusion	Amoxicillin-clavulanate	30 µg	14 mm	VAV 2005
	Cefoxitin	30 µg	15 mm	CLSI
	Amikacin	30 µg	15 mm	CLSI
	Apramicin	20 µg	20 mm	Rosco diagnostica
	Imipenem	10 µg	20 mm	CLSI
	Aztreonam	30 µg	18 mm	CLSI
Broth microdilution	Sulfamethoxazole	8-1024 µg/ml	256 µg/ml	EFSA
	Gentamicin	0.25-32 µg/ml	2 µg/ml	EFSA
	Ampicillin	0.5-32 µg/ml	8 µg /ml	EFSA
	Ciprofloxacin	0.008-8 µg/ml	0.064 µg /ml	EFSA
	Cefotaxime	0.06-4 µg/ml	0.5 µg /ml	EFSA
	Ceftazidime	0.25-16 µg/ml	2 µg /ml	EUCAST
	Tetracycline	1-64 µg/ml	8 µg /ml	EFSA
	Streptomycin	2-128 µg/ml	16 µg /ml	EFSA
	Trimethoprim	0.5-32 µg/ml	2 µg /ml	EFSA
	Chloramphenicol	2-64 µg/ml	16 µg /ml	EFSA
	Florfenicol	2-64 µg/ml	16 µg /ml	EUCAST
	Kanamycin	4-128 µg/ml	32 µg /ml	CLSI
	Nalidixic acid	4-64 µg/ml	16 µg /ml	EFSA

VAV: http://www.vigilanciasanitaria.es/vav/. CLSI: CLSI M100-S21, EFSA: EFSA Journal 2012; 10:2742. EUCAST: www.srga.org/eucastwt/WT_EUCAST.htm

Statistical analysis

To assess the effect of cattle on *Salmonella* infection probability in wild boar, we fitted a set of independent generalized linear models (GLM) using a binomial distribution and the logit link function (Zuur et al, 2009) in which the response variable was explained by the single and the additive effects of age, sex, cattle presence and cattle herd size, and their two-way interactions. Herd size was included as an explicative variable, since it has been previously shown to be related to *Salmonella* prevalence and shedding (Foley and Lynne, 2008). Cattle density was not considered because it is too low in our study area and shows little variation (range 0.02- 0.42 adult cow/ha). Wild boar abundance, which would be a likely factor affecting *Salmonella* infection in wild boar, was not different between areas with and without cattle presence ($F = 0.04$, $df = 1$, $p = 0.84$, ANOVA) and was therefore not included in the models. Complete information allowing for statistical analyses was known for 204 animals. Animals

sampled from regular hunting activity are assumed to be representative of the healthy population (Thulke et al, 2009).

For all statistical models, we performed a model selection procedure based on the information-theoretic approach and the Akaike's Information Criterion corrected for small sample sizes (AICc) (Burnham and Anderson, 2002; Johnson, 2002). In short, competing models are ranked in relation to the difference between their Akaike scores with the score of the best model (Δi), which has the lowest AICc. Models with $\Delta i < 2$ units have substantial support for explaining the observed variability in the variable of interest. Subsequently, we estimated the Akaike weight (w_i), defined as the relative probability that a given model is the best model among those being compared. Once the best model was selected, the explained deviance (ED) was calculated as a measure of explained variability of each response variable (Zuur et al, 2007). Additional recommended readings for guidance are Anderson et al (2000) and Anderson et al (2001).

Diversity was compared between host populations by means of its components: richness and evenness. Richness is in this case the number of serotypes found in each host group, while evenness is the relative distribution of isolates among serotypes. Evenness was assessed by the probability of interspecific encounter (PIE), which is defined by Hurlbert (1971) as the probability that two randomly sampled individuals from the assemblage represent different species (i.e., *Salmonella* isolates are "individuals" and serotypes are "species").

We faced the problem that richness strongly depends on sample size (Hurlbert, 1971); therefore, it could not be directly compared. Richness was corrected for sample size with the use of bootstrapping. The statistical analyses were performed using R software version 2.15.1 (R Development Core Team, 2011), including prevalence estimates, which were estimated by package "epiR" 0.9-43 version (Stevenson et al, 2012), and EcoSim 7.72 (Gotelli and Entsminger, 2012).

Results

Wild boar

The prevalence of *Salmonella* among wild boars from cattle-free areas (n = 57) was 17.54 % (CI 95% 8.74 - 29.91) (see serotypes and antimicrobial resistance in Table 4). Their counterparts with contact with cattle showed prevalence two times higher (35.67% CI 95% 28.19 - 43.70, n =

157, see Table 5), with this difference being statistically significant ($\chi^2 = 5.62$, df = 1, p = 0.02). However, all animals from both groups were apparently healthy.

Cattle

The *Salmonella* prevalence among cattle was 21.92% (CI 95% 13.10 - 33.14, n = 73). See Table 6 for information about serotypes and antimicrobial susceptibility.

Antimicrobial resistance

Two strains (2.98%) showed antimicrobial resistance; one *Salmonella* Enteritidis and one *Salmonella* Mbandaka strain (see Tables 4 - 6). The resistant *Salmonella* Enteritidis was carried by a wild boar from a cattle-free area (H), and showed resistance against ciprofloxacin and nalidixic acid. To the contrary, the *Salmonella* Mbandaka strain was carried by a wild boar from the cattle-grazed area A and was resistant to sulfamethoxazole, streptomycin and chloramphenicol.

Table 4. Serotypes and antimicrobial resistance of *Salmonella* isolates from wild boar from cattle-free areas in a Natural Park in northeastern Spain

Serotype	**Number of isolates**	**Area**	**Number of antibiograms**	**Antimicrobial resistance**
4:b:-	3	F,G	2	Susceptibility
Enteritidis	2	H	2	CIPR,NAL Susceptibility
Ohio	1	G	1	Susceptibility
42:l,v:z	1	H	1	Susceptibility
Shangai	1	F	1	Susceptibility
Mikawasima	1	H	1	Susceptibility
35:r:z35	1	F	1	Susceptibility

CIPR= Ciprofloxacin, NAL= Nalidixic acid.

Table 5. Serotypes and antimicrobial resistance of *Salmonella* isolates from hunted wild boars in areas with cattle presence in a Natural Park in northeastern Spain.

Serotype	Number of isolates	Area	Number of antibiograms	Antimicrobial resistance
Meleagridis	13	A	13	Susceptibility
4:b:-	8	A,B,D	4	Susceptibility
Muenster	6	A,B	3	Susceptibility
42:b:e,n,x,z15	4	D,C	2	Susceptibility
Newport	3	A	2	Susceptibility
Anatum	2	A	1	Susceptibility
Othmarschen	2	A	1	Susceptibility
Carnac	2	A, B	2	Susceptibility
16:l,v:1,5,7	2	A,D	1	Susceptibility
Stoneferry	1	A	1	Susceptibility
Stanley	1	A	1	Susceptibility
Spartel	1	A	1	Susceptibility
Offa	1	A	1	Susceptibility
Mikawasima	1	A	1	Susceptibility
Mbandaka	1	A	1	SMX, STR, CHL
Kottbus	1	A	1	Susceptibility
Enteritidis	1	A	1	Susceptibility
58:K:-	1	A	1	Susceptibility
48:	1	D	1	Susceptibility
38:l,v:z54	1	A	1	Susceptibility
38:l,v:z53	1	E	1	Susceptibility
Tomegbe	1	A	1	Susceptibility
Paratyphi B	1	A	1	Susceptibility

SMX=Sulfamethoxazole, CHL=Chloramphenicol, STR= Streptomycin.

Table 6. Serotypes and antimicrobial resistance of *Salmonella* from cattle from a Natural Park in northeastern Spain.

Serotype	Number of isolates	Herd size	Number of antibiograms	Antimicrobial resistance
Anatum	10	170	3	Susceptibility
Meleagridis	4	170	4	Susceptibility
Kedougou	1	170	1	Susceptibility
Othmarschen	1	50	1	Susceptibility

Inter-specific overlap of Salmonella serotypes

A wide variety of *Salmonella* serotypes are shown in Tables 4 to 6. Some serotypes have been found in both wild boar from cattle-free and cattle-grazed areas (Enteritidis, 4:b:-, Mikawasima and 35:r:z35). Serotypes 4:b:- and Enteritidis were the most frequent found in wild boars from cattle-free areas (30% and 20% of the total isolates, respectively), while they were present in a lower frequency in wild boars from cattle-grazed areas (1.79% and 14.29%, respectively). Cattle serotypes were only shared with wild boars from cattle-grazed areas (e.g., Meleagridis, the most frequent serotype in this group was the second most frequent in cattle, or Anatum and Othmarschen also appeared in sympatric wild boars). This overlap suggests some degree of spill-over between cattle and wild boar. The fact that these serotypes were simultaneously isolated from both host species in the same place (A) and its PFGE pattern (see Mentaberre et al, in press) indicate a direct association between *Salmonella* isolates from cattle and wild boar.

Phage-typing of *Salmonella* serotype Enteritidis strains revealed no association between these isolates: the strain from area A showed an unrecognizable lytic pattern, while the strains from the cattle-free area H were PT1 and 14C.

Salmonella serotype richness and evenness

In the smallest group, i.e. wild boars from cattle-free areas, serotype richness was 7 (See Table 4). This value had to be compared with serotype richness in cattle and in wild boars from cattle-grazed areas at a sample size equal to 57. Serotype richness was clearly lower in cattle: 4 serotypes was the maximal value (this means that the probability of being smaller than 7 is 100%) and also the most probable (610 out of 1000 repetitions, i.e. probability $_{(Richness=4)}$ = 0.61). The group of wild boars from cattle-grazed areas had 12 as a most probable value (p $_{(Richness=12)}$ = 0.22), and probability that its richness was greater than 7 was 0.99.

These results indicate higher *Salmonella* serotype richness in wild boars from cattle-grazed areas than their cattle-free counterparts: when sampling an equal number of wild boars from both areas we would have a greater number of different serotypes in wild boars from grazed areas. However, richness in cattle was lower than in both groups of wild boars.

The index for evenness was lower in cattle ($PIE_{Cattle} = 0.57$) than in both groups of wild boars ($PIE_1 = 0.91$, $PIE_2 = 0.92$), indicating that two *Salmonella* isolates taken randomly from wild boars will be different serotypes with a probability higher than 0.9, while two *Salmonella* isolates randomly detected in cattle will differ with a probability of 0.57. Hence a certain serotype seems to be predominant in cattle, but this is not the case in wild boars despite their co-habiting with cattle.

Salmonella infection probability in wild boar

The best model for explaining *Salmonella* probability of infection (see Table 7) in wild boars was the additive effects of cattle herd size and age class ($\beta_{Herd\ size} = 0.011 \pm 0.002$, n = 204, $w_i = 0.51$, explained deviance = 11.56%). As can be seen in Figure 4, *Salmonella* infection probability increases with herd size, but decreases with age, especially after the first year of life (piglets and juveniles). The pure effect of the variables was 7.53 and 2.85% for herd size and age class, respectively, and there was no shared deviance. The relative importance of the variables also supports our results ($R_{i\ Herd\ size}=0.99$, $R_{i\ Age\ class}=0.96$, $R_{i\ Sex}=0.01$, $R_{i\ Cattle\ presence}= 0$). The second model with substantial support (see Table 5, $\Delta_i < 2$, $w_i = 0.51$) was not selected due to the principle of Parsimony. The same trend was observed in cattle: the probability of a cow being a *Salmonella* carrier increased as herd size increased ($z = 2.78$, $\beta_{Herd\ size} = 0.07$, SE = 0.02, $p < 0.01$, explained deviance = 23.68%).

Table 7. Model selection for the probability of *Salmonella* carriage in wild boars.

Biological Models	***K***	**AICc**	**Δi**	***wi***
Herd size + age class	**4**	**234.02**	**0.00**	**0.51**
Herd size * age class	**6**	**234.30**	**0.28**	**0.45**
Herd size	2	240.17	6.15	0.02
Herd size + sex	3	241.64	7.63	0.01
Herd size * sex	4	243.70	9.68	0.00
Cattle presence + age class	4	249.40	15.38	0.00
Cattle presence * age class	6	250.09	16.07	0.00
Cattle presence	2	252.45	18.43	0.00
Age class	3	254.16	20.14	0.00
Cattle presence + sex	3	254.40	20.38	0.00
Sex + age class	4	256.20	22.18	0.00
Cattle presence * sex	4	256.21	22.19	0.00
Null	1	257.36	23.34	0.00
Sex * age class	6	258.22	24.20	0.00
Sex	2	259.18	25.16	0.00

K=number of parameters, AICc=Akaike's Information Criterion corrected for small sample sizes, Δi = difference of AICc with respect to the best model, *w*i = Akaike weight. In bold, models with substantial support.

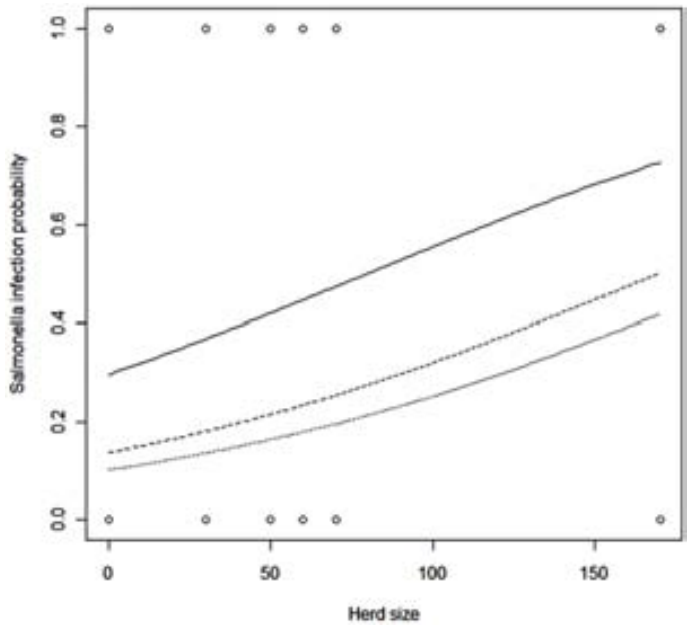

Figure 4. Relationship between *Salmonella* carriage probability in wild boar age classes and size of the cattle herd cohabiting in the area. Legend: solid line=piglets and juveniles (intercept=0.3), dashed line= yearlings (intercept= 0.14), dotted line= adults (intercept= 0.1). Slope=0.01.

Discussion

The prevalence found in the wild boars with contact with cattle in our study (35.67%) is the highest described to date in this species in the literature. On the other hand our results confirm the presence of *Salmonella enterica* serotypes of medical importance in wildlife; e.g., Enteritidis, Newport and Mbandaka were among the 10 most frequent serotypes causing salmonellosis in humans in the EU, and specifically the *S*. Enteritidis phage-type 1 is among the most frequent (EFSA, 2012a). Other serotypes found in the study area have also been related to human outbreaks (e.g., Anatum, Paratyphi B and Kottbus, among others); and salmonellosis caused by *Salmonella enterica* serotype Enteritidis has been diagnosed in sympatric Iberian ibex (Navarro-Gonzalez et al, in press), with unknown consequences at the population level. The high prevalence of *Salmonella* in wild boars and the fact that wild boars shed this pathogen to a higher extent than wild ruminants (Hilbert et al, 2012) make it possible that wild boar plays an important role in the transmission and maintenance of *Salmonella* in the study area and likewise in similar multi-host systems.

The prevalence observed in the wild boars from cattle-free areas (17.54%) is similar to that found in wild boars from Portugal: 22.1%; serotypes Typhimurium and Rissen (Vieira-Pinto et al, 2011). Lower prevalences have been described either from tonsils of wild boar in Switzerland (12%; serotypes Enteritidis, Veneziana and Stourbridge: (Wacheck et al, 2010) or from tissues of wild boars in northern Spain (7.5%; serotypes Worthington and 38:l,v:z35 (Millán et al, 2004)).

Since *Salmonella* prevalence in wild boar from cattle-grazed areas was higher than prevalence in cattle itself, *Salmonella* sources other than cattle, but linked to its presence, may exist. For example, other wild hosts (wild birds and rodents) attracted by free access to cattle feed or other domestic animals (dogs or cats) could play a role in the epidemiology of *Salmonella* in the study area. Wild boar may be exposed to numerous *Salmonella* sources more directly than cattle due to its omnivorous and opportunistic feeding habits (Schley and Roper, 2003) that include potential *Salmonella* carriers, such as mice and birds (Liebana et al, 2003), and especially its rooting behaviour may favour the transmission through inhalation, a potential route of *Salmonella* infection in pigs (Fedorka-Cray et al, 1995).

Cattle seem to contribute to the *Salmonella* prevalence in wild boar by introducing their own serotypes to the environment, which can be regarded as an additive effect. We also found that *Salmonella* in cattle and in sympatric wild boars increased when the cattle herd size increased.

Nevertheless, it should be considered that herd-related factors other than herd size, such as food, breed or health status may be related to the *Salmonella* prevalence. Also, although not having considered cattle density as an explanatory variable due to its little variation among herds in the study area (see Materials and Methods section), the bullfighting herd in area A is not only the largest, but also that with the highest density. Another difference is that fighting bulls, although under extensive management conditions, have to be kept inside enclosures given that they are potentially dangerous. In this case, these enclosures are not an effective biosecurity barrier, as evidenced by the results. Indeed, shared serotypes and simultaneous isolation in cattle and wild boar occurred only in this hunting area A. Similarly, Skov et al (2008) found *Salmonella* in wildlife surrounding farms where and when *Salmonella* was also detected in livestock. This is important because even at a low prevalence, wildlife can be a source of *Salmonella* for livestock (Carlson et al, 2011).

As shown, serotypes found in previous studies about *Salmonella* in wild boar differ widely from our isolates. The wide number of serotypes found in our study area and the fact that most serotypes were isolated only once during the study period or in one animal only may reflect 1) a high diversity of *Salmonella* sources within the Reserve; 2) a high heterogeneity in the exposure of the wild boars to these sources; 3) a low intra-specific transmission of these serotypes; or 4) the separation of wild boars and their *Salmonella* strains by natural barriers (e.g., steep terrain). Indeed, Methner et al (2010) detected different epidemiological groups of *Salmonella* serotype Cholerasuis in wild boars from certain regions of Thuringia (Germany) that were separated by natural (mountains) or artificial barriers (arterial roads).

This may explain why richness was higher in wild boars (both from cattle-grazed and cattle-free areas) than in cattle. Cattle are supposed to live in more homogenous conditions that determine exposure within herd (e.g., food and water), which is supported by the lower serotype richness found in cattle. The highest richness was found in wild boars from cattle-grazed areas, and this confirms an additive effect of cattle on the *Salmonella* of sympatric wild boars, as explained above with *Salmonella* prevalence. However, PIE was similar in wild boars from cattle-grazed and cattle-free areas, suggesting that wild boar may be a spill-over host: serotype evenness has not been altered by the acquisition of serotypes from cattle, which dominate in cattle but not in the *Salmonella* population from wild boars.

It should be noted that serotyping only one isolate per sample may have underestimated richness. However, for our aim (comparing richness between host groups) this methodology should suffice since the underestimation would be the same in each group. Additionally, the

protocol, included enrichment to favour the detection of *Salmonella,* and enrichment media can have an effect on the strain/serotype detected as shown by other studies (Gorski et al, 2012; Singer et al, 2009). Thus, serotyping more than one isolate would not be more representative.

On the other hand, antimicrobial resistance is an anecdotal finding in wild boar. Interestingly, two very different patterns of resistance have been found and they are directly related to the origin of the animals. The *Salmonella* serotype Mbandaka strain resistant to chloramphenicol, sulfamethoxazole and streptomycin was carried by a wild boar from a cattle-grazed area. Streptomycin and sulfonamides are, along with others, antimicrobial agents for which veterinary-associated *Salmonella* isolates show the greatest percentage of resistance (Foley and Lynne, 2008). In fact, serotype Mbandaka is the most frequent (20%) serotype isolated from cattle in Spain (EFSA, 2012a); therefore, although this serotype was not isolated from cattle in our study area, its origin is possibly linked to cattle or associated factors.

The resistance profile displayed by a *S*. Enteritidis strain found in a wild boar from a cattle-free area (ciprofloxacin and nalidixic acid) is of concern since ciprofloxacin belongs to the second generation of fluoroquinolones and is today the antimicrobial of choice for treatment of severe or invasive *Salmonella* infections in humans (EFSA, 2010). This resistance profile is not usually found in Spanish cattle (EFSA, 2010) suggesting the existence of a different source of antimicrobial resistance in the National Game Reserve. This resistance profile is frequent in *S*. spp from fowl and pigs, both particularly in Spain (EFSA, 2010; VAV, 2005); but to our knowledge this type of farming does not occur in the study area nor within a short distance; on the other hand, the highest levels of resistance among *S*. Enteritidis isolates from humans in 2010 were observed for nalidixic acid, 18.7 %, and ciprofloxacin, 9.3 % (EFSA, 2010).

Caleja et al (2011) reported a high frequency of antimicrobial resistance among *S*. Typhimurium and *S*. Rissen isolates from wild boar in Portugal against ampicillin, amoxicillin-clavulanic acid, streptomycin, chloramphenicol, tetracycline, sulfonamides and trimethoprim-sulfamethoxazole. Behaviour of wild boar, especially their feeding habits, makes this species prone to pathogen exposure and may also be linked to antimicrobial resistance carriage. Therefore, it may be a suitable sentinel for *Salmonella* presence, prevalence and antimicrobial resistance in a natural environment.

Conclusions

This study provides evidence of the association of *Salmonella* infection in free-ranging wild boar and herd size of sympatric cattle in extensive farming conditions. It also suggests that sources of *Salmonella* other than livestock exist in the National Game Reserve. The spill-over of *Salmonella* between cattle and wild boar, although increasing serotype richness in the latter, did not alter the evenness of the *Salmonella* population in wild boar and thus may be sporadic or a dead-end. The presence of antimicrobial resistance in isolates from wildlife of a protected area is a matter of concern.

Study II:

Lack of evidence of spill-over of *Salmonella enterica* between cattle and sympatric Iberian ibex (*Capra pyrenaica*) from a protected area in Catalonia, NE Spain.

Transboundary and Emerging Diseases, doi:10.1111/tbed.12037

Abstract

Salmonella enterica is a zoonotic agent of worldwide importance found in a wide range of wild hosts. However, its prevalence in many popular game species has never been assessed. Iberian ibex (*Capra pyrenaica*) is the main game caprinae of the Iberian Peninsula and around two thousand individuals are hunted every year for trophy or for home consumption. In this work, 313 Iberian ibexes from the Ports de Tortosa i Beseit National Game Reserve (NE Spain) were tested for *Salmonella enterica* in faeces and antimicrobial susceptibility was determined. The exact location of shooting or capture was recorded with a GPS device in order to study the links of *Salmonella* infection with cattle presence and human proximity. Additionally, samples were taken from cattle grazing inside this reserve (n=73). Only three Iberian ibexes (0.96%, 95% CI 0.2-2.8) were positive to *Salmonella* (serotype Enteritidis, Bardo and 35:r:z35), while prevalence was moderate in cattle: 21.92% (95% CI 13.10-33.14, serotype Meleagridis, Anatum, Kedougou and Othmarschen). All isolates were susceptible to the antimicrobial agents tested. Moreover, a case of fatal septicaemic salmonellosis in an 11-year-old male Iberian ibex is described where *Salmonella enterica* serotype Enteritidis was isolated from the lung, liver and spleen samples. The low prevalence of *Salmonella* in Iberian ibex and the lack of shared serotypes suggest no association to cattle. Despite this, game meat aimed for human consumption should be examined and it is strongly recommended that hunters and game keepers manipulate animals and carcasses under maximal hygienic conditions in order to avoid environmental contamination and human contagion.

Introduction

Salmonellosis continues to be the second most frequent zoonose in the European Union (EFSA, 2012a). However, many gaps exist in our knowledge about its presence and distribution in wildlife, which has been stated to be a common reservoir. Particularly, game species may play a more important role in its epidemiology, displaying an increased risk for public health owing to (1) its meat being consumed by humans and (2) being abundant species potentially involved in environmental contamination (e.g. water sources, recreational waters or pastures). In fact, disease transmission between sympatric wildlife and livestock can be of particular concern in relation to wild ungulates, which frequently share habitat resources with domestic livestock (Boehm et al, 2007).

The Iberian ibex is among the species about which there is a lack of information. It is the main game caprinae of the Iberian Peninsula (around two thousands of ibexes are hunted each year in Spain, according to the official data (Ministerio de Agricultura, Alimentación y Medio Ambiente, 2012) and is rapidly increasing its abundance and distribution (Acevedo and Cassinello, 2009). Furthermore, Iberian ibex has been re-introduced to areas of Spain and Portugal (Moço et al, 2006; Herrero and Pérez, 2008) and there is a proposal for its re-introduction in the French Pyrenees (Crampe, 1991). Thus, information about the health status of this species and its role in the epidemiology of shared pathogens is of increased interest. Males are mainly exploited for trophy-hunting while harvesting of females and juveniles is aimed for home consumption by hunters and their families. Carcass processing is done under inadequate hygiene conditions at the place of shooting (evisceration, head cut off and offal abandoned in situ). This poses a risk of transmission to risk groups (i.e. hunters, game keepers, ramblers) and to other host species such as wild boars, foxes or scavengers in general, which may be attracted to offals, and also cause environmental contamination.

To our knowledge, only one work has assessed the presence of *Salmonella* in Iberian ibex, but this was carried out by sampling nasal, ocular and vaginal swabs of the Southern Spain populations (see Gonzalez-Candela et al, 2006). In these populations, presence of livestock in poor health conditions has triggered disease outbreaks of sarcoptic mange in the past (Leon-Vizcaino et al, 2001) and contact to livestock has also been related to infectious keratoconjunctivitis outbreaks (Verbisck et al, 2010). In our research we studied the presence of *Salmonella enterica* in Iberian ibexes and co-habiting extensive-grazing cattle in a protected area to explore if transmission between both species occurs, as well as possible links with *Salmonella* sources of human origin. We also screened for antimicrobial resistance, but we did

not expect to find resistant isolates, due to the farming system (extensive) and the low human activity inside the park.

Materials and Methods

Study area

The study area was the National Game Reserve "Els Ports de Tortosa i Beseit" (28 587.17 ha), which is a protected area located within the Natural Park "Els Ports" in northeastern Spain (40º 48' 28'' N, 0º 19' 7'' E). It is a calcareous mountainous region with a high orographic complexity, which results in a ragged and abrupt terrain formed by numerous canyons and steep slopes. About 28% of the surface is above 1000m over sea level (m.a.s.l.), being the highest peak Mont Caro (1442 m). All municipalities (9) are excluded from the park limits and situated at the periphery at less than 550 m.a.s.l. The predominant habitat is pine grove (39.1% of the total surface) followed by oak grove (14.6%). Rivers account only for 0.2%, as typical of its dry Mediterranean climate. The Iberian ibex (*Capra pyrenaica*) and the wild boar (*Sus scrofa*) are the most abundant species, and the only ones allowed for big game exploitation. The Iberian ibex population is estimated to be 3458 individuals in 2011. Farming activity is extensive and herds occupy well-defined areas, being cattle the most abundant livestock.

Iberian ibex sampling

Three hundred and thirteen Iberian ibexes (*Capra pyrenaica* subspecies *hispanica*) were either hunter-harvested (n = 283), box-trapped (n = 27) or found ill (n = 3) from 2007 to 2011, and faecal samples were obtained directly from the rectum. Due to characteristics of the hunting method, faecal samples had to be stored at -18 ºC until sending them to the laboratory. However, samples collected from captured and sick animals were stored in refrigeration (4ºC) and sent to the laboratory within 24 hours after collection. Despite this different management of the samples, *Salmonella* spp. are known for their tolerance of freezing even for over one year (Archer, 2004). Thus, we can assume that the isolation of this microorganism from faeces is not being highly affected by storage at this temperature.

In addition, the location of shooting or capture from each animal was recorded with a GPS device.

Cattle sampling

Seventy three faecal samples were taken from cattle between 2008 and 2010. Previously, information regarding herds' size, location and grazing periods was obtained from the Reserve's managers. Herds are small and kept in extensive farming, which sometimes made it difficult to locate the animals within a valley (e.g. 62 head of cattle in a 1823 ha surface). When animals were found they were counted and observed until defecation. Then, fresh faeces were collected in a sterile container and stored at refrigeration (4 ºC) until they were sent to the laboratory within the next 24 hours.

Microbiological analyses

For *Salmonella* culturing we applied the ISO 6579:2002 Annex D (International Organization for Standardization, 2007), method recommended by the EU's CRL (European Union Community Reference Laboratory) for *Salmonella* in faecal and environmental samples. Briefly, samples were cultured in buffered peptone water (BPW, 1/10 dilution) and incubated at 37ºC ± 1°C for 18 h ± 2 h. Next, Modified semi-solid Rappaport-Vassiliadis (MSRV) (Difco) agar plates were inoculated with three drops (a total volume of 0.1 ml) of BPW culture. Plates were incubated at 41.5 ºC ± 1°C for 24h ± 3 h and if negative, they were incubated for an additional 24h ± 3 h.

Suspected growth of *Salmonella* was confirmed by plating out to both Xylose Lysine Desoxycholate agar (XLD) (bioMérieux) and on chrom ID^{TM} *Salmonella* agar (SM ID2) (bioMérieux). The plates were incubated for 24h ± 3 h at 37ºC ± 1°C.

Colonies of presumptive *Salmonella* were subcultured on Columbia 5% sheep blood agar (bioMérieux) and incubated for 24h ± 3 h at 37ºC ± 1°C. Identity of isolates as *Salmonella* spp. was confirmed by a commercially available biochemical method Enterotube ^{TM} II (BD BBL ^{TM}). Serological typing was performed based on the Kauffmann-White scheme (Grimont and Weill, 2007). Phage Typing was performed in the Centro Nacional de Microbiología, Instituto de Salud Carlos III (Madrid, Spain) by using Anderson's scheme (Anderson et al, 1977).

Antimicrobial susceptibility

Isolates belonging to different serotypes and to different days or places were selected for antimicrobial susceptibility testing. Thus, twelve isolates were tested for antimicrobial resistance by agar diffusion method to obtain data of the Inhibition Zone Diameter (IZD) against amoxicillin-clavulanate, cefoxitin, amikacin, apramicin, imipenem and aztreonam while broth microdilution method was performed to determine the minimum inhibitory

concentrations (MIC) of sulfamethoxazole, gentamicin, ampicillin, ciprofloxacin, cefotaxime, ceftazidime, tetracycline, streptomycin, trimethoprim, chloramphenicol, florfenicol, kanamycin and nalidixic acid. Cut-off/breakpoint values are shown in Table 3 (Study I).

Human activity

The spatial location of possible human-wildlife interaction points inside the Natural Park (houses, picnic areas, etc) was also obtained from the Reserve's managers and spatial data were visualized with ArcGIS 9.2.

Results

Salmonella prevalence and antimicrobial resistance

Only three Iberian ibexes were positive to *Salmonella enterica* (0.96%, CI 95% 0.20-2.78, see Figure 5 to know the location of the positive animals). Due to this low prevalence, further statistical analyses were not performed with respect to distance to human-wildlife interaction points or cattle facilities. *Salmonella* prevalence among cattle was definitely higher: 21.92% (CI 95% 13.10-33.14, n=73). Table 8 shows serotypes and frequency of *Salmonella* isolates from both host species; it is remarkable that no serotype was found to be shared between cattle and ibexes.

None of the isolates showed resistance to the antimicrobial agents tested. *Salmonella enterica* serotype Enteritidis was isolated from a clinical case (see below for a detailed description) and showed by phago-typing an unrecognizable lytic pattern.

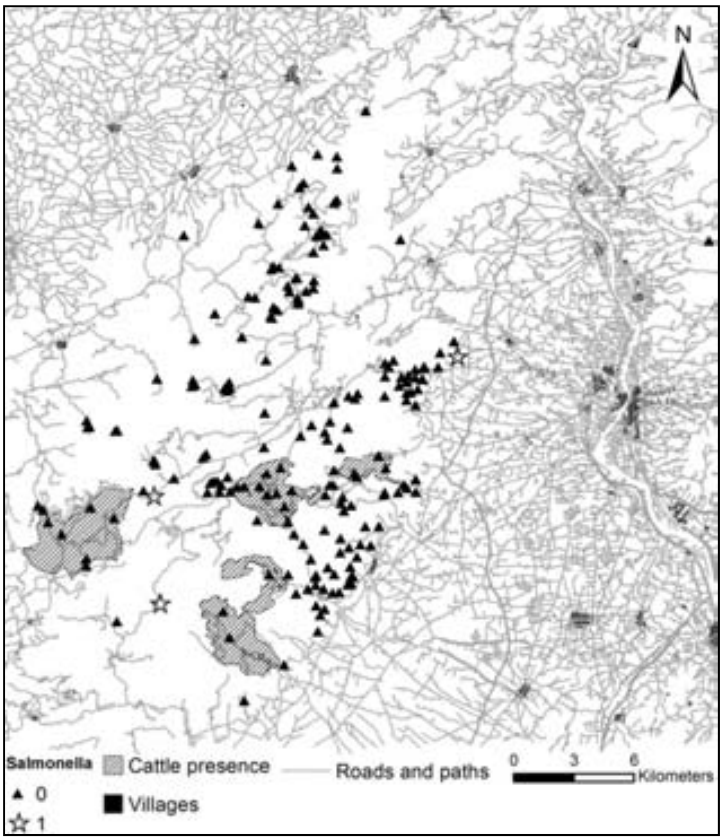

Figure 5. Map of the study area showing places with cattle presence and localization of Iberian ibex sampling.

Table 8. *Salmonella* serotypes from Iberian ibex and cattle.

Host	Serotype	Number of isolates
Iberian ibex	35:r:z35	1
	Bardo	1
	Enteritidis	1
Cattle	Anatum	10
	Meleagridis	4
	Kedougou	1
	Othmarschen	1

Clinical case

As part of a regional passive health surveillance programme of game species, any dead animal found within this Game Reserve is necropsied at the Veterinary Faculty (Universitat Autònoma de Barcelona). In February, 2008, a free-ranging 11-year-old male Iberian ibex was found extremely weak by the Park Rangers. The animal was easily caught, euthanized owing to welfare concerns, and frozen until it was carried to the Veterinary Faculty where a postmortem examination was performed.

The ibex weighed 40 Kg (average body weight for healthy males 80-100 Kg), had a poor body condition and absence of fat stores. External examination also revealed a high number of ticks

mainly at the base of the ears. The main internal findings included moderate splenomegaly and ileo-cecal lymphadenopathy, and few multifocal white-yellow 1- 5 mm foci of necrosis in the kidney (Figure 6), liver and lung. Incidental findings were two vesicles of *Cysticercus tenuicollis*, one under the epicardium and the second one under the visceral pleura in the lung, and multifocal mild nodular verminous pneumonia. Tissue specimens were fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin wax. Two-micrometer sections were mounted on glass slides and stained with haematoxylin and eosin (H/E). Selected sections were stained with Gram method. Samples from lung, kidney, and spleen were sent for routine microbiological analysis.

On histopathological examination, microscopic lesions were consistent with gram-negative bacterial sepsis, with embolic necrotic foci and fibrin thrombi in the lung, liver, spleen and kidney. Despite macroscopically there were only a few lesions in the liver, microscopically random suppurative foci were seen scattered throughout the entire section .Those foci had mainly degenerated neutrophils and fibrin in the centre, and were surrounded by lesser numbers of lymphocytes, plasma cells and macrophages. Intracellular and extracellular gram-negative coccobacilli were occasionally seen in those foci, mainly in the kidney. Intestinal samples were too autolyzed for evaluation. Because of the isolation in pure culture of *Salmonella* sp. from all tested organs and the histopathological lesions a septicaemic salmonellosis was diagnosed. The phagotyping of this isolate showed a non-recognizable pattern.

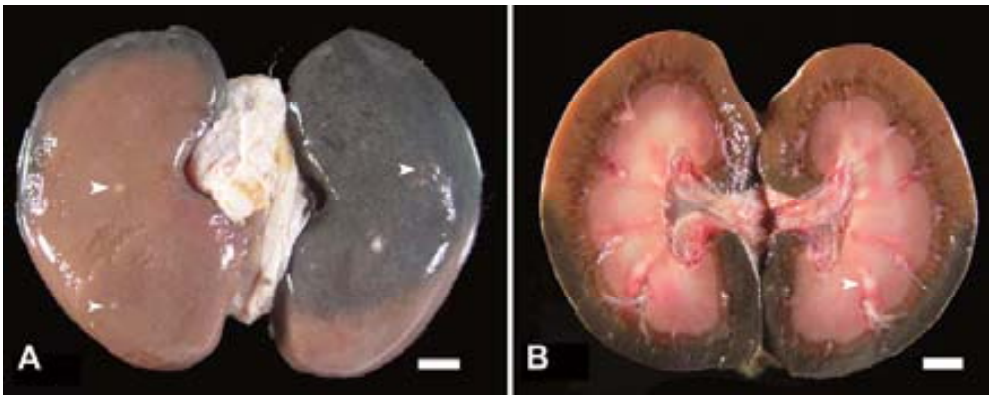

Figure 6. A. Multifocal embolic nephritis in an Iberian ibex (*Capra pyrenaica*) with septicaemic salmonellosis. B. Cut surface of the kidney in (A). Black discoloration of the kidney is due to autolytic changes (pseudomelanosis).

Discussion

Salmonella infection in wild animals may be the result of the transmission of this microorganism from domestic animals or humans and consequently reflect the local serotypes present in the environment (Murray, 1991; Skov et al, 2008). However, *Salmonella* prevalence in Iberian ibex in this study is very low and the serotypes found in this species were not found in cattle, and vice versa. Although contact between the positive ibexes and cattle cannot be ruled out, both the much lower prevalence in the Iberian ibex and the difference in the serotypes isolated suggest that there is not a link between *Salmonella* infection in these species. In fact, *Salmonella* infection in Iberian ibex seems occasional and not related to cattle, despite apparently sharing common geographical areas and natural resources. Fresh faeces were all negative to *Salmonella*, which supports that freezing of samples did not considerably affect the probability of *Salmonella* isolation.

Our results are in line with previous studies done in the same and related species. In fact, although there is little knowledge about the epidemiological situation of *Salmonella* in wild, free-living mammals, especially wild European ungulates, in those few studies carried out the prevalence of *Salmonella* in faeces has been always low or inexistent (e.g. in Wahlstrom et al, 2003; Lillehaug et al, 2005). In a faecal and serologic survey on abortive agents performed in Alpine ibex (*Capra ibex*) *Salmonella* was not found among more than 600 samples from 14 colonies in the Swiss Alps (Marreros et al, 2011).

Our study area can be considered a high-risk area due to the high *Salmonella* prevalence detected in wild boar (up to 35%: Navarro-Gonzalez et al, 2012) and moderate prevalence in cattle. The low presence of *Salmonella* in Iberian ibex could be due to its trophic behaviour and to a different use of space compared to cattle and wild boar by selecting higher altitudinal ranges, which would imply low habitat overlap and low contact rate with these species.

Similarly to the results of this work, a survey conducted in the same study area found a complete absence of tuberculosis in Iberian ibex while prevalence in wild boar and cattle was high. The authors suggested the existence of avoidance behaviour of contaminated pastures or disease resistance (Mentaberre et al, 2010).

Although *Salmonella* is present in a very low prevalence in the studied population, it had fatal consequences for this clinical case and its real impact in the Iberian ibex population may be under-estimated. Since no active surveillance is performed in many places, sick and dead animals may go unnoticed in places where they are difficult to find, especially if clinically

affected animals die of acute disease. In animals, sub-clinical infections are more common than clinical cases. In fact, a distinction should be made between a healthy *Salmonella* carrier, and an animal with salmonellosis (Mörner, 2001). Carrier animals are considered an important source of infection because they shed bacteria intermittently in faeces. The clinical and pathological syndromes of salmonellosis typically vary from localized enterocolitis to septicaemia; abortion may also occur (Brown et al., 2007). Stressors that compromise immune competence or disrupt the enteric bacterial ecosystem are often implicated in salmonellosis (Brown et al., 2007). The lesions observed at necropsy in the studied Iberian ibex were suggestive of a septicaemia, with histological lesions and the isolation of *S*. Enteritidis confirming this. Clinical salmonellosis in wild ungulates is rare and most reports refer to cases of animals in captive situations (Foreyt et al, 2001; Hattel et al, 2007) and very few to free-ranging ungulates (Nettles et al, 2002).

In another work about *Salmonella* in wild boar in this study area (Mentaberre et al, in press), a *Salmonella* Enteritidis strain found in a wild boar hunted some months after this clinical case showed the same non-recognizable pattern of phage lysis. The Pulse Field Gel Electrophoresis pattern and resistance profile were also the same. The wild boar was found in a valley 8 km away, however, the link between these animals is unknown. This distance can certainly be covered by an Iberian ibex but home ranges from male Iberian ibexes are generally small during winter (Such-Sanz et al, 2007), when the sick Iberian ibex was found. Further research is needed to clarify the existence of a common *Salmonella* source and contact between Iberian ibex and wild boar. On the other hand, wild boars carrying *Salmonella* 35:r:z35 have also been found in the study area but the PFGE showed different percentage of similarity between them (76.3%).

A human source can be considered for the *Salmonella* Enteritidis found in the clinical case since the location of this case coincides with the most outer limits of the Reserve, i.e. in closer proximity to a city or village. Nonetheless, even if there may be a relation to a human source or not, the impact of human activity on *Salmonella* infection in Iberian ibex seems to be anecdotic, at least under the terms of our study.

Although our results suggest that Iberian ibexes do not constitute a significant reservoir for human salmonellosis, any *Salmonella* serotype should be regarded as potentially zoonotic (EFSA, 2012a). As nearly two thousands of Iberian ibex are hunted annually for human consumption, a putative risk of transmission of *Salmonella* from this species to humans must be assumed. In general, after being hunted, game animals are skinned and eviscerated in the

field under insufficient hygienic conditions or in private barns, garages, basements or kitchens. Such practices may greatly increase the risk of faecal contamination from the animal's own intestines as well as reducing product quality. In the case of cervids, it has been stated that when the environment for deer is relatively heavily contaminated with *Salmonella*, from other animals or perhaps occasionally from an infected deer, then the organism may be present in substantial numbers on the hides and in the rumens of deer, and some may be transferred to meat during carcass dressing (Gill, 2007). Then, the possibility of sporadic occurrence of these cases and the absence of mandatory veterinary inspection of game pieces makes at risk the population of the area (hunters mainly) which will be sensitive to contract unexpected zoonosis.

In light of these results, Iberian ibex appears to be an accidental or dead-end host for many of the studied pathogens until now. This highlights the importance of increasing our knowledge on the wild species in particular and of avoiding referring to “wildlife” in general in epidemiologic issues; instead of this, particularities of the species and the study area have to be considered.

In conclusion, we recommend hunters and game keepers to manipulate animals and carcasses under maximal hygienic conditions in order to avoid environmental contamination and human contagion.

Study III:

Antimicrobial resistance in indicator *E. coli* and *E. coli* O157:H7 from wild boar, Iberian ibex and free-ranging sympatric livestock in a natural environment (NE Spain).

Under review.

Abstract

In the last half a century, wild ungulates have greatly increased in abundance and range throughout Europe. This new situation presents a concern for public health because these animals are known carriers of resistant bacteria and zoonotic pathogens of special relevance. In this work, we tested for antimicrobial resistance to a set of antimicrobial agents in indicator *E. coli* and for the presence of the zoonotic pathogen *E. coli* O157:H7 in free-ranging livestock and sympatric wild boars (*Sus scrofa*) and Iberian ibex (*Capra pyrenaica*) in a National Game Reserve in NE Spain. Statistical significance was assessed with the Fisher's exact test. The frequency of resistance in indicator *E. coli* from all three species was low, ranging from 0 to 7.9%. The resistance score (total number of resistances per total number of possible resistances) was significantly lower in Iberian ibex. Remarkably, one isolate carried by wild boar showed resistance to a third-generation cephalosporin and fluoroquinolone-resistance was detected in isolates from cattle and sympatric wild boar. Four wild boars (3.41%, 95% CI 0.94 - 8.52) and three Iberian ibexes (1.88%, 95% CI 0.39 - 5.38) carried *E. coli* O157:H7, which was not found in livestock faeces. All *E. coli* O157:H7 isolates were susceptible to all antimicrobial agents tested. Despite that free-ranging livestock and sympatric game ungulates appeared to be low-significance reservoirs of antimicrobial resistance, we highlight the potential role of wild boar as a sentinel for antimicrobial resistance in a broad range of environmental conditions.

Introduction

Antimicrobial resistance (AMR) may compromise the treatment of severe human diseases (EFSA, 2012b), and thus monitoring and reporting its occurrence is a priority for health surveillance agencies worldwide. This phenomenon has been partly associated with the misuse of antimicrobial agents in intensive animal food production (Allen et al, 2010); in fact, a lower occurrence of resistant bacteria has been repeatedly observed in extensive or organic farming systems when compared to intensive ones (Alvarez-Fernandez et al, 2012; Blake et al, 2003; Berge et al, 2010; Heuer et al, 2002). In recent decades, the likelihood of wild ungulates coming into contact with human waste or livestock has undergone a huge increase throughout Europe, mainly due to intensive game management (Gortázar et al, 2006) and large population increases in most European ungulate species (Apollonio et al, 2010). In addition, wild ungulates have been declared reservoirs of food- and vector-borne pathogens (Gortázar et al, 2007).

Moreover, it has been shown that resistance profiles of the gut bacteria of wild mammals are influenced by their proximity to human activities (Allen et al, 2010), and many studies have found similarities in the patterns of resistance in samples from livestock and small fauna, e.g. rodents (Kozak et al, 2009; Literak et al, 2009), insects (Literak et al, 2009; Rybarikova et al, 2010) or birds (Rybarikova et al, 2010) sampled in the farm environment. Thus, a question that now remains is whether free-ranging ungulates in close contact with free-ranging livestock carry indicator bacteria with similar resistance profiles. Indicator (commensal) *E. coli* is suitable for such a study, since it is common in animal faeces and provides information on resistance in a population (EFSA, 2012b).

On the other hand, some *E. coli* are known to be etiologic agents of food-borne diseases, for example Shiga-toxin producing *E. coli* (STEC) (Garcia et al, 2010). Miko et al. (2009) underline that wild animals and their meat are underestimated as reservoirs for STEC and as possible sources for human infections. The number of cases of human STEC infection in the European Union has increased for three consecutive years, with O157:H7 being the most frequently reported serogroup associated with human disease (EFSA, 2012a). This serotype is part of the gut microbiota of many animal species, and ruminants have been identified as a major reservoir, particularly cattle (Hussein, 2007). Wildlife has also been connected to food-borne outbreaks of *E. coli* O157:H7, such as the case of feral swine grazing near a spinach field (Jay et al, 2007), or the black-tailed deer jerky (Keene et al, 1997).

In this work, we carried out an intensive two-year sampling of both free-ranging game ungulates (wild boar - *Sus scrofa* - and Iberian ibex - *Capra pyrenaica*) and livestock herds sharing habitat in a game reserve in NE Spain in order to explore the host-specific patterns of antimicrobial resistance in indicator *E. coli*. A previous study demonstrated the spill-over of *Salmonella enterica* (Mentaberre et al, in press) between livestock and wild boars in the same area. For this reason and due to its zoonotic relevance, *E. coli* O157:H7 was also screened and tested for AMR. Wild boar is a very abundant and popular game species in Europe and its meat is much appreciated, while the Iberian ibex is an endemic species of the Iberian Peninsula and is also largely hunted and consumed. In fact, according to the official statistics, more than 160 000 wild boars and 3000 Iberian ibexes were hunted in Spain in 2010 (Ministerio de Agricultura, Alimentación y Medio Ambiente, 2013).

Wild boars have been previously described as carriers of *E. coli* O157:H7 (Mora et al, 2012; Sanchez et al, 2010; Wahlstrom et al, 2003) and other STEC strains that are potential human pathogens (Miko et al, 2009; Wacheck et al, 2010). Sanchez et al. (2010) found indistinguishable PFGE types in *E. coli* O157:H7 isolates from a wild boar and a human patient and Mora et al. (2012) report similarities between STEC from wildlife and humans, data that strongly suggest that wild boar and other wild animals play a role in human infection. Furthermore, STEC has also been isolated from wild boar meat and meat products from Spain (Diaz-Sanchez et al, 2012). Although transmission must occur between wildlife and livestock (Jay et al, 2007; Mora et al, 2012), to our knowledge no previous work has tested this pathogen in the faeces of large wild mammals and sympatric free-ranging livestock. Specifically in Iberian ibex, the carriage of *E. coli* O157:H7 has never been assessed, but a study on its close relative the alpine Ibex (*Capra ibex*) found a considerable prevalence of STEC (6 out of 27; Hofer et al, 2012).

Concisely, in this work we test the following predictions: although no environment can be considered pristine of antimicrobial resistance (Allen et al, 2010), we expect wildlife to be almost free of antimicrobial resistance since the use of antimicrobials in the study area can be ruled out and human activities, and thus, selective pressure, are reduced. Also, we expect to find *E. coli* O157:H7 at a low prevalence in both large wild mammals and sympatric livestock.

Materials and Methods

Study area

The study area is located within the National Game Reserve and Natural Park "Ports de Tortosa i Beseit" (NGR hereafter), in northeastern Spain. It is a calcareous mountain region with high orographic complexity that results in a rugged and abrupt terrain with numerous ravines and steep slopes. About 28% of the surface is above 1000 m.o.s.l., with the highest peak being Mont Caro (1442 m). The predominant habitat is pine grove (39%) followed by oak grove (15%), and, due to the dry Mediterranean climate, rivers account for only 0.2%. Wildlife and livestock share pastures in some canyons in the study area.

Animal sampling

Wild boar

In total, 143 individual faecal samples were obtained from hunter-harvested wild boars during the regular hunting season (October to January) from 2009 to 2011. The location of each hunting session was recorded, sex was visually determined by direct observation of genitalia and age was estimated based on tooth eruption pattern and replacement as well as dental attrition (Boitani and Mattei, 1992). Faeces were collected directly from the rectum and stored in sterile containers. They were refrigerated and sent to the laboratory within the subsequent 24 hours.

Iberian ibex

A total of 184 Iberian ibexes were either hunter-harvested (n = 154), box-trapped (n = 28) or found sick (n = 2) from 2009 to 2011, and faecal samples were obtained directly from the rectum. Due to the characteristics of the hunting method, faecal samples had to be stored at -18 ºC until being sent to the laboratory. However, samples collected from captured and sick animals were stored in refrigeration (4º C) and sent to the laboratory within 24 hours after collection. *E. coli* are known for their cold-shock response (Phadtare, 2004) and thus, we can assume that the isolation of this microorganism from faeces is not highly affected by storage at this temperature.

Free-ranging livestock

Fifty-two samples from livestock were collected from 2010 to 2011. Previously, information was obtained from the Reserve's managers on herd location. The farming conditions in the

NGR are free-ranging (extensive) with supplemental feeding in the dry season (summer) and herds are small, which sometimes made it difficult to locate the animals (e.g., 60 animals in a 1823 ha area). Forty-six samples were obtained from cattle (5 herds, 380 head in total) and 4 from the only horse herd in the NGR (32 head). Furthermore, two herds made up of both sheep and goats grazing in the periphery of the Reserve were sampled. For each herd, a pool was made with several droppings and processed as one sample.

Livestock sampling was preferably performed on days that wild boars were also sampled. When livestock were located, animals were counted and observed until defecation. Then, faeces were collected and stored in a sterile container and refrigerated until being sent to the laboratory within the subsequent 24 hours.

Microbiological analyses

In total 25g of faeces were diluted in buffered peptone water (225ml). Once diluted, one loop was cultured in agar MacConkey (direct plating) in order to obtain one colony of indicator Escherichia coli to be used for resistance testing per animal/pool (EFSA, 2008; EFSA, 2012c).

Indicator E. coli

Faeces were cultured on MacConkey agar plates (bioMérieux, Marcy l´Etoille, France) at 37ºC for 18-20h. One compatible colony was selected and confirmed by PCR (Heininger et al, 1999).

E. coli O157:H7

Faecal samples were processed according to the ISO 16.654:2001 protocol to obtain *E. coli* O157. This protocol applies immunomagnetic separation to select O157 positive *E. coli*. One suspected colony per sample was confirmed by PCR as *E. coli* O157:H7 as described previously (Desmarcherlier et al, 1998; Gannon et al, 1997; Heininger et al, 1999).

In some cases the faecal sample was insufficient to perform both procedures. Therefore, the population size for the estimation of the prevalence of *E. coli* O157:H7 was slightly smaller (160 Iberian ibexes and 117 wild boars).

Antimicrobial susceptibility testing

All *E. coli* O157:H7 isolates were tested for antimicrobial resistance. With regards to indicator *E. coli*, a set of isolates was selected in order to characterize the frequency of antimicrobial resistance within each host group. In the case of wildlife, a selection was made according to its origin within the NGR, in order to representatively sample the study area (altogether, 63

isolates were selected from wild boar and 89 from Iberian ibex). All isolates from livestock were tested for antimicrobial resistance (n = 44, from which: 38 from cattle, 4 from horse and 2 from small ruminants).

Antimicrobial resistance was tested by the agar diffusion method to obtain the Inhibition Zone Diameter (IZD) against amoxicillin-clavulanate, cefoxitin, amikacin, apramycin, imipenem and aztreonam while the broth microdilution method was performed to determine the Minimum Inhibitory Concentrations (MIC) of sulfamethoxazole, gentamicin, ampicillin, ciprofloxacin, cefotaxime, ceftazidime, tetracycline, streptomycin, trimethoprim, chloramphenicol, florfenicol, kanamycin and nalidixic acid. The epidemiological cut-off values are shown in Table 9.

Table 9. Antimicrobial agents and epidemiological cut-off values.

Method	Antimicrobial agent	Disk content/ concentration range	Cut-off value	Reference
Disk diffusion	Amoxicillin-clavulanate	30 µg	17 mm	EUCAST
	Cefoxitin	30 µg	19 mm	EUCAST
	Amikacin	30 µg	18 mm	EUCAST
	Apramicin	20 µg	20 mm	Rosco diagnostica
	Imipenem	10 µg	24 mm	EUCAST
	Aztreonam	30 µg	27 mm	EUCAST
Broth microdilution	Sulfamethoxazole	8-1024 µg/ml	64 µg/ml	EFSA
	Gentamicin	0.25-32 µg/ml	2 µg/ml	EFSA
	Ampicillin	0.5-32 µg/ml	8 µg /ml	EFSA
	Ciprofloxacin	0.008-8 µg/ml	0.064 µg /ml	EFSA
	Cefotaxime	0.06-4 µg/ml	0.25 µg /ml	EFSA
	Ceftazidime	0.25-16 µg/ml	0.5 µg /ml	EFSA
	Tetracycline	1-64 µg/ml	8 µg /ml	EFSA
	Streptomycin	2-128 µg/ml	16 µg /ml	EFSA
	Trimethoprim	0.5-32 µg/ml	2 µg /ml	EFSA
	Chloramphenicol	2-64 µg/ml	16 µg /ml	EFSA
	Florfenicol	2-64 µg/ml	16 µg /ml	EFSA
	Kanamycin	4-128 µg/ml	8 µg /ml	EUCAST
	Nalidixic acid	4-64 µg/ml	16 µg /ml	EFSA
	Colistin	2-4 µg /ml	2 µg /ml	EFSA

EFSA: EFSA Journal 2012; 10:2742. EUCAST: www.srga.org/eucastwt/WT_EUCAST.htm

For the comparison of our data with that from intensively-reared livestock, Table 10 shows the frequencies of resistance in cattle and pigs from the European Union (EFSA 2012b) and Spain (VAV Network data, personal communication).

Statistical analysis

Percentages of resistance in *E. coli* were compared between host species with the Fisher's exact test and the significance level set at $p < 0.05$. Furthermore, the resistance scores between host species were compared, as explained by Murray et al. (1990). This score is the sum of times any resistance occurred per the total number of isolates and antimicrobial agents tested x 100. For the prevalence of *E. coli* O157:H7 its 95% CI was calculated. The statistical analyses were performed with R Software (R Development Core Team 2.14.0, 2011), specifically with package epiR for obtaining the 95% CI.

Results

Indicator E. coli

Eight wild boars (12.7%), 4 cows (9.09%) and 3 Iberian ibexes (3.37%) were carriers of resistant *E. coli* (7.65% of the total tested samples). These frequencies were not statistically different, however the difference between wild boar and Iberian ibex was almost significant ($p = 0.052$). Due to the small number of Ibexes carrying resistant bacteria, its relation to the proximity to human settlements could not be assessed. The resistance-carrying isolates from cattle came from 3 different herds.

Table 10 shows the percentage of isolates from each host species showing resistance to each antimicrobial agent tested. Frequencies of resistance ranged from 0 to 7.9%. Resistance to sulfamethoxazole, tetracycline, ampicillin and streptomycin was found in both wild species as well as in livestock. Additionally, resistance to trimethoprim was found in isolates from wild boar and Iberian ibex, and resistance to kanamycin and cefotaxime only in the former. Finally, resistance to ciprofloxacin and nalidixic acid were found in bacteria from cattle and wild boar. No statistically significant differences were found for any antimicrobial agent tested in any host species. It is remarkable that those agents whose resistance is widespread and frequent in domestic species in the European Union and Spain (see Table 10) are detected in our study area both in bacteria from livestock and wildlife: e.g. resistance against tetracycline, streptomycin, sulfamides, ampicillin and trimethoprim. In other cases, such as cefotaxime,

resistance is not so frequent in these domestic species, but we did find this resistance in wild boar.

Table 10. Frequencies of resistance in each host species of the National Game Reserve, and in pigs and cattle from Spain and the European Union.

	National Game Reserve			Spain		EU	
Antimicrobial agent	**Wild boar**	**Iberian ibex**	**Livestock**	**Pigs[a]**	**Cattle[a]**	**Pigs[b]**	**Cattle[b]**
Ciprofloxacin	3.2	0	6.8	29.1	3.1	2	15
Sulfamethoxazole	6.3	1.1	2.2	70.1	35.2	37	34[2]
Gentamicin	0	0	0	7.2	3.1	2	9
Ampicillin	4.8	1.1	2.2	69.1	15.6	21	28
Cefotaxime	1.6	0	0	1.1	0	1	3
Ceftazidime	0	0	0	1.4	0	-	-
Tetracycline	7.9	3.3	2.2	90.3	48.8	48	38
Streptomycin	4.8	1.1	2.2	75.9	35.5	44	33
Trimethoprim	3.2	1.1	0	71.2	17.6	-	-
Chloramphenicol	0	0	0	27.3	9.8	7	17
Florfenicol	0	0	0	1.8	5.9	-	-
Kanamycin	6.3	0	0	16.2	2.7	-	-
Nalidixic acid	1.6	0	4.5	18.3	3.1	2	13
Colistin	0	0	0	0	0	-	-
Amoxicillin - clavulanate	0	0	0	-	-	-	-
Cefoxitin	0	0	0	-	-	-	-
Amikacin	0	0	0	-	-	-	-
Apramicin	0	0	0	-	-	-	-
Imipenem	0	0	0	-	-	-	-
Aztreonam	0	0	0	-	-	-	-

[a] VAV Network data, personal communication. [b] EFSA Journal 2012, 10:2598.
- Not determined.

When considering resistance to more than one agent, one strain showed resistance to six agents, three strains to five agents, four strains to three agents and one strain to two agents. The same resistance profile was rarely detected more than once (see Table 11 for the resistance profile of the resistant *E. coli* strains detected in our study area and the animal species from which they originated). Resistance scores were: 0.56% for Iberian ibex, 2.83% for wild boar and 1.46% for livestock. Significant differences ($p < 0.001$) were found only between Iberian ibex and the other species, indicating that antimicrobial resistance in Ibex is more rare.

Table 11. Phenotypic profile of the resistant *E. coli* strains isolated from the three host species.

Host species (number of isolates)	Resistance profile
Wild boar	CIPR,SMX,AMP,CEFOT,TET,NAL
Wild boar	SMX,AMP,TET,STR,KAN
Iberian ibex	SMX,AMP,TET,STR,TMP
Wild boar	SMX,TET,STR,TMP,KAN
Wild boar	CIPR,AMP,STR
Wild boar	SMX,TET,TMP
Cattle	SMX, TET, STR
Cattle	CIPR, AMP, NAL
Cattle	CIPR,NAL
Cattle	CIPR
Wild boar (2)	KAN
Iberian ibex (2), Wild boar	TET

CIPR: Ciprofloxacin, SMX: Sulfamethoxazole, AMP: Ampicillin, CEFOT: Cefotaxime, TET: Tetracycline, NAL: Nalidixic acid, STR: Streptomycin, KAN: Kanamycin, TMP: Trimethoprim.

E.coli O157:H7

Four wild boars were positive for *E. coli* O157:H7 (3.41%, 95% CI 0.94-8.52). A similar prevalence was found in Iberian ibex: 3 animals were positive (1.88%, 95% CI 0.39-5.38), while this microorganism was not isolated from any livestock sample. We detected no statistically significant difference among these host groups. All isolates were susceptible to the antimicrobial agents tested. The PCR revealed differences between hosts: all three isolates from Iberian ibex, as well as three isolates from wild boar, were Stx1-negative and Stx2-positive; however, one isolate from wild boar was both Stx1 and Stx2-postitive.

Discussion

Indicator E. coli

Overall, the resistance frequency in indicator *E. coli* in our study is 7.65%; from 196 *E. coli* isolates tested, only 15 were resistant to at least one antimicrobial agent, and when considering antimicrobial agents individually, frequencies were between 1% and 10%, which is defined by the EFSA (2012b) as low resistance level. This demonstrates that the finding of AMR in our study area could be considered occasional, possibly accidentally introduced by abiotic or biotic factors that are still undetermined.

In contradiction to our findings, Schierack et al. (2009) found susceptibility to all antimicrobial agents tested in 42 *E. coli* isolates from wild boars in Germany (however, those isolates came from only 21 individuals). However, it is difficult to compare our results to other studies due to differences in the procedures used. For example, the frequency of resistance to at least one antimicrobial agent strongly depends on the number of agents tested and the agents themselves, and they greatly differ between studies. Different testing methods, such as broth microdilution or agar disk diffusion, yield results in different units (MIC and IZD, respectively), which also hampers comparison. Finally, interpreting resistance using either epidemiological cut-off values or clinical breakpoints displays different results in the frequency of resistance.

In addition to these considerations, great variations are observed depending on the species, the ecosystem and the geographic location. For example, Gilliver et al (1999) found a very high prevalence of AMR in wild rodents from England, while Osterblad et al (2001) found almost complete absence of AMR in wildlife from Finland). In some cases, this variation has been connected to the presence of farms (Allen et al, 2011) or interactions with farm waste (Cole et al, 2005), livestock rates (Guenther et al, 2010), human proximity (Wheeler et al, 2012) or human density (Skurnik et al, 2006). Skurnik et al (2006) also reported a higher resistance score in extensively-reared farm animals from the French Pyrenees compared to wildlife from the same area. We also tested this resistance score in our host groups, and it was lower in Iberian ibex than in wild boar or livestock; however, there was no statistically significant difference between wild boar and livestock. This suggests that resource sharing of livestock with wild boar may be higher than with Iberian ibex, as well as exposure to AMR sources, and hence, AMR is distributed more evenly between livestock and wild boar. The fact that Skurnik et al (2006) found a higher resistance score in extensively-reared livestock than in wildlife could be due to the great variety of wild species tested by these authors (e.g squirrel or bat), which may not be sympatric to livestock, may not share nutritional resources or may avoid interaction.

A Belgian survey of wild boar found generally low levels (0-12%) of AMR in *E. coli* (Martin et al, 2007). These authors suggest the possibility of the introduction of resistant strains from livestock to wild boars and cervids, and the results of Kozak et al. (2009) suggest direct or indirect transmission from farm animals to wild small mammals. However, free-ranging livestock appears not to be a main source of AMR in our study area. These free-ranging livestock from the NGR show resistance levels much lower than those reported by the EFSA and the VAV Network for intensively-reared livestock, but similar to our results on sympatric wildlife. Resistance to widely used antimicrobial agents (such as resistance to tetracycline,

sulfamides or streptomycine) is also very frequent in intensively-reared pigs or cattle and could be simply wide-spread in the environment. However, other potential sources of AMR in the study area may include other wildlife such as birds, rodents or insects, which can act as vectors of resistant bacteria or resistance genes from farms or human settlements, human interaction or even physical forces like wind and watersheds (Allen et al, 2010). Indeed, multidrug-resistant bacteria have been isolated from a range of wild birds and mammals with no known previous exposure to antimicrobial agents; a fact that suggests that resistance is not confined to the ecological niche where it emerged (da Costa et al, 2013). Although we do not know if our findings are related to the presence of antimicrobial residues in the environment, this is very unlikely due to the farming conditions, the low human density and the remoteness of the study area.

Resistance to fluoroquinolones and cephalosporins

Despite the low prevalence of AMR, we found resistance to more agents than we expected, particularly because one study with comparable cut-off values (Guenther et al, 2010) did not find resistance to cephalosporins or fluoroquinolones in small wild mammals from German rural areas. This may indicate that the impact of human activities in our study area is higher than expected and suggests that protected natural environments are not exempt from the introduction of anthropogenic AMR. Similarly, Mokracka et al. (2012), found multi-resistance in *E. coli* from a wild boar shot in the buffer zone of a National Park in Poland. Other plausible explanations are direct or indirect contact of wildlife with resistant microorganisms from other sources or even with antimicrobials.

In any case, some of our findings raise concerns: one wild boar was carrying resistance to a third generation cephalosporin (cefotaxime), and resistance to fluoroquinolones (ciprofloxacin) was detected in both wild boar and cattle. These antimicrobial agents are listed as "critically important antimicrobials for human medicine" by the WHO (World Health Organization, 2007) and the carriage of resistant bacteria by wildlife should be regarded as a risk for the spread of AMR in natural environments. In fact, wild boars have been found to be carriers of cefotaxime-resistant *E. coli* in countries as diverse as Poland (Mokracka et al, 2012), the Czech Republic (Literak et al, 2010) and Portugal (Poeta et al, 2009), and the authors of these studies define the wild boar as a reservoir of extended-spectrum beta-lactamase (ESBL) producing *E. coli*.

Taking into account all the issues discussed here, we consider wild boar as a suitable sentinel for AMR in a natural environment. Among wildlife, wild boar may be a better sentinel than other species due to the fact that they are omnivorous, root and feed from soil, can be found

in many different habitats and can travel long distances, factors that may expose them to a greater extent to AMR sources. All of these traits make wild boars a good indicator of the diversity of AMR circulating in a natural environment. Iberian ibex, on the contrary, is a browser that feeds mainly on leaves from trees and bushes, not on the ground, and may prefer higher altitudinal ranges to avoid sharing pastures with livestock. Poeta et al. (2009) already proposed the wild boar specifically for the monitoring of ESBL producing *E. coli*, and, in light of our results, we think that this could be extended to AMR in general.

E. coli O157:H7

On the other hand, it was encouraging that the *E. coli* O157:H7 isolates were susceptible to all antimicrobial agents tested. Again, we find AMR to be lower in wildlife than in intensively-reared livestock: the VAV Network reports high frequency of resistance in *E. coli* O157:H7 isolated in 2009 (VAV Network data, personal communication). This suggests that *E. coli* O157:H7 sources in wild animals might be not common to those in livestock.

With regards to the prevalence of *E. coli* O157:H7 in wild boars, our results were within the range of what has been previously reported in this species in other parts of Spain: 3.3% (Sánchez et al, 2010) and 0.38% (Mora et al, 2012). Three out of four *E. coli* O157:H7 positive wild boars were piglets. Since in our sample only 10 animals were piglets, this represents a high percentage of positivity among piglets (30%). Further research is needed to elucidate if the carriage of this microorganism in wild boar is age-dependent. The fact that these positive piglets came from the same canyon and were sampled within one month of each other suggests that there may have been a common source of contamination, though momentary in time and space.

Out of the three *E. coli* O157:H7 positive Iberian ibexes, two animals were hunted and apparently healthy, but one was found sick and died within a few hours. This animal showed hemorrhagic enteritis (data not shown). We were not able to determine the cause of death due to the advanced autolysis of the organs, and so further research is needed to determine if this microorganism can cause disease in Iberian ibex. Even if the prevalence detected was low, the impact of this potential disease on the local population could be important, as has been suggested with regards to *Salmonella* in this population (Navarro-Gonzalez et al, in press).

Conclusions

For monitoring AMR in wildlife and the environment, there is urgency for the standardization of testing methods and the interpretation of criteria to allow comparisons between species and locations. Free-ranging livestock act like wild ungulates as low-significance reservoirs of antimicrobial resistance in this natural environment. Furthermore, antimicrobial resistance seems evenly distributed between wild boar and livestock, but is less frequent in Iberian ibex. This finding may be related to the behavior and habits of these species. For several reasons, for the purposes of choosing a wild species as an AMR sentinel, wild boar may be a very good indicator of the AMR circulating in a natural environment. Finally, prevalence of *E. coli* O157:H7 in wild ungulates was low, and may be unrelated to livestock.

Study IV:

Thermophilic *Campylobacter* spp. shared between free-ranging cattle and sympatric wild ungulates from a natural environment (NE Spain).

Abstract

Campylobacter infections are a public health concern and an increasing cause of food-borne zoonoses in the European Union. However, little is known about the spill-over from free-ranging livestock to sympatric wild ungulates, especially regarding uncommon *Campylobacter* species. In this study, we aim to determine the prevalence of *Campylobacter coli, C. jejuni* and other thermophilic *C.* spp. in game ungulates (wild boar -*Sus scrofa*- and Iberian ibex – *Capra pyrenaica*) and free-ranging sympatric livestock in a National Game Reserve in NE Spain. Furthermore, we explore the extent to which *Campylobacter* species are shared among these co-habiting hosts. Faecal samples from Iberian ibex (n=181), small ruminants (n=2) and horses (n=4) were negative for *C.* spp. Three wild boars out of 150 were positive for *C. coli* (2%, 95% CI 0.41 - 5.73) and one was positive for *C. jejuni* (0.67%, 95% CI 0.02 – 3.66). The latter was predominant in cattle: 9.09% (n= 55, 95% CI 3.02 – 19.95). While *C. coli* was not isolated from this host, in one sample *C. fetus* was detected. *C. lanienae* was the most frequent species in wild boar at 12% (95% CI 7.27 – 18.3) and one cow from the same canyon as some of these wild boars also carried *C. lanienae.* These results suggest that wild boar and cattle have their own predominant *Campylobacter* species, while Iberian ibex do not seem to play an important role in the epidemiology of *Campylobacter*. However, there is a potential spill-over of *C. lanienae* and *C. jejuni*, and thus, further research is needed to elucidate the factor determining inter-specific transmission.

Introduction

Campylobacter infections pose a serious public health problem as they are the principal bacterial cause of bacterial gastroenteritis throughout the world (EFSA Panel on Biological Hazards, 2012; FAO/WHO, 2009; Humphrey et al, 2007). Moreover, a growing number of Campylobacter species other than *Campylobacter jejuni* and Ca*mpylobacter coli* have been recognized as emerging human and animal pathogens. In fact, the true prevalence is probably underestimated because the methods used for routine isolation favour the growth of some species over others (Man, 2011). The symptoms of campylobacteriosis range from self-limited, mild diarrhoea to severe inflammatory bloody diarrhoea. Occasionally, infection has also been associated with postinfectious sequelae including septicemia and neuropathies, such as Guillain-Barré and Miller Fisher Syndrome (Allos, 2001; Godschalk et al, 2004; Nachamkin et al, 1998). The predominant route of transmission is consumption and handling of contaminated poultry meat. Other foodstuffs, untreated drinking water and milk have also been associated with the illness, but poultry products are considered the major source of infection (Olson et al, 2008; Pires et al, 2010). Additionally, it has been suggested that environmental *C. jejuni* are a negligible source of infection. Several studies have identified wildlife and water-borne sources as being implicated in *Campylobacter* infection (Ethelberg et al, 2005; Karenlampi et al, 2007). Information on the ecology of *Campylobacter* in wildlife is scarce; it is not believed to cause disease in wildlife and wildlife's role as a reservoir remains undetermined (Sippy et al, 2012). In particular, there is a lack of information about the carriage of uncommon *Campylobacter* species in wild mammals (Lee et al, 2011). In some instances, *Campylobacter* prevalence in wildlife has been found to be low, but this strongly depends on the species sampled and the geographic location. In the Nordic countries for example, Kemper et al (2006) found one reindeer positive for *C. hyointestinalis* out of 2500, and Lillehaug et al (2005) found only one wild cervid positive for *C. jejuni* out of 324. A broader survey of Swedish wildlife (Wahlstrom et al, 2003) found a great variability in the prevalence of thermophilic *Campylobacter*: it was low in moose, hare and roe deer, but considerable (>10%) in gulls, wild boar and Canada geese, and absent in red deer and fallow deer samples. It has been suggested that such results in the northern regions of Europe are due to the climate conditions, which may be limiting for enteropathogen survival (Kemper et al, 2006), but the status as a *Campylobacter* carrier is not known for many wild species and habitats.

Research on the link of wildlife to *Campylobacter* in domestic animals has been assessed mainly in agricultural settings, and especially focused on wild birds (Colles et al, 2008; Colles et al, 2011; Craven et al, 2000; Sippy et al, 2012), small mammals (Jones et al, 2007; Meerburg et

al, 2006; Sippy et al, 2012) or insects (Hald et al, 2004). However, there is a gap of knowledge with regards to the potential spill-over from free-ranging or extensive livestock to co-habiting wild ungulates, and vice versa. For example, Díaz-Sánchez et al (2013) found no relation between *Campylobacter* spp. in large game animals and the presence of livestock in hunting estates. However, further determination at the species level of both isolates from wildlife and livestock could shed light on the epidemiology of *Campylobacter* in the livestock-wildlife interface.

In this study, we aim to determine (1) the prevalence of thermophilic *Campylobacter* in large wild mammals and free-ranging sympatric livestock and (2) the extent to which *Campylobacter* species are common to wild and domestic species that share resources (mainly pastures), indicating a potential inter-specific transmission. In fact, *Campylobacter* transmission between wildlife and farm animals is considered to possibly be bidirectional (Sippy et al, 2012). For this purpose, we studied the presence of thermophilic *Campylobacter* in a National Game Reserve (Els Ports de Tortosa i Beseit, NE Spain) where wild boar (*Sus scrofa*) and Iberian ibex (*Capra pyrenaica*) share resources (pastures, water ponds) with free-ranging livestock. Both wild ungulates are important as popular game species and meat sources: around 3000 Iberian ibexes and 160 000 wild boars are hunted each year in Spain (Ministerio de Agricultura, Alimentación y Medio Ambiente, 2013). Wild boar in particular has been described as a carrier of *C. coli* (Wahlstrom et al, 2003) in Sweden, and as a carrier of *C. jejuni* in the same country (Wahlstrom et al, 2003) and in Spain (Díaz-Sánchez et al, 2013). On the other hand, Wacheck et al (2010) did not isolate *Campylobacter* from any of 153 wild boars in Switzerland. Nonetheless, *Campylobacter* has been isolated from wild boar meat (2.1%, Atanassova et al, 2008), indicating that it could enter the food chain. Moreover, Jay-Russell et al (2012) described *Campylobacter* in gastrointestinal and oral cavity specimens in 12 of 30 feral swine (40%), including six species: *C. coli*, *C. fetus*, *C. hyointestinalis*, *C. jejuni, C. lanienae* and *C. sputorum*.

However, to our knowledge no previous study has assessed the presence of *Campylobacter* in either the Iberian ibex or in its closest relative, the Alpine ibex (*Capra ibex*). Like the domestic small ruminants (Schilling et al, 2012), the ibex may serve as silent *Campylobacter* carriers.

Materials and Methods

Study area

The study area is located within the National Game Reserve “Els Ports de Tortosa i Beseit” in northeastern Spain, part of the Natural Park of the same name. It is a calcareous mountain region with high orographic complexity that results in a rugged and abrupt terrain with numerous ravines and steep slopes. About 28% of the surface is above 1000 m.o.s.l., with the highest peak being Mont Caro (1442 m). The predominant habitat is pine grove (39%) followed by oak grove (15%), and, due to the dry Mediterranean climate, rivers account for only 0.2%. The most abundant wild ungulates are the Iberian ibex and the wild boar, which are exploited for hunting purposes. Wildlife and cattle share pastures in some of the canyons in the study area.

Animal sampling

Wild boar

In total, 150 individual faecal samples were obtained from hunter-harvested wild boars during the regular hunting seasons (October to January) of 2009-2010 and 2010-2011. The location of each hunting session was recorded, sex was visually determined by direct observation of genitalia and age was estimated based on tooth eruption pattern and replacement as well as dental attrition (Boitani and Mattei, 1992). So as to minimize error in age determination, four age classes were used: piglets (up to 5 months), juveniles (6 to 12 months), yearlings (between 13 and 24 months) and adults (over 24 months). Faeces were collected directly from the rectum and stored in sterile containers. They were refrigerated and sent to the laboratory within 24 hours. The culture was performed immediately after reception.

Iberian ibex

181 Iberian ibexes were either hunter-harvested (n=153) or captured (n=30) from 2009 to 2011, and faecal samples were obtained directly from the rectum. Due to characteristics of the hunting method, faecal samples were stored at -18°C until being sent to the laboratory. However, samples collected from captured animals were stored in refrigeration (4°C) and sent to the laboratory within 24 hours after collection. The culture was performed immediately after reception. In addition, the sex and age and the location of shooting or capture from each animal was recorded with a GPS device.

Free-ranging livestock

61 samples from livestock were collected from 2010 to 2011. Previously, information was obtained from the Reserve's managers on the location of the herds. The farming conditions in the National Game Reserve are free-ranging with supplemental feeding in the dry season (summer). The herds are small (in total, 5 herds and 380 head), which sometimes made it difficult to locate the animals (e.g., 60 animals in a 1823 ha area). 55 samples were obtained from cattle and 4 from the only horse herd in the Reserve (32 head). Furthermore, two herds of sheep and goats grazing in the periphery of the Reserve were sampled. For each herd, a pool of several droppings was collected and processed as one sample.

Livestock sampling was preferably performed on days that wild boars were also sampled. When livestock were located, animals were counted and observed until defecation. Then, faeces were stored in a sterile container and refrigerated and sent to the laboratory within the following 24 hours. The culture was performed immediately after reception.

Microbiological analyses

Isolation of Campylobacter from the samples

For isolation and detection of thermophilic *Campylobacter* from the samples under investigation, different protocols were used (Table 12). These included direct plating on selective agar plates for which modified Charcoal Cefoperazone Desoxycholate Agar (mCCDA,CM739, Oxoid, Basingstoke, UK) was used. In some cases, an enrichment step was included using Bolton [CM0983, Oxoid supplemented with antibiotics (SR0183) and 5% lysed horse blood (SR0048) (both from Oxoid)] or Preston broth [Nutrient broth Nº 2, CM0067, Oxoid that was prepared according to the manufacturer's instructions and supplemented with 5% lysed horse blood (SR0048) and antibiotics (SR0204 and SR0232E; Oxoid)]. Both enrichment broths were combined with either mCCDA or Campyfood agar (CFA Ref 43471, bioMérieux, Marcy l'Etoile, France).

For direct plating of stool samples, a swab was dipped into the sample and streaked onto selective plates and incubated 48 h at 42ºC under microaerobic conditions (Genbag microaerobic atmosphere generator, Ref 45532, bioMérieux,). In 96 cases, charcoal swabs were taken from the faeces at the time of sampling and also processed for the isolation of *Camyplobacter*.

For enrichment protocols, 1 g of fresh faeces was aseptically transferred to a 10 ml sterile screw-cap bottle and 9 ml of broth was added. Enrichment was performed for 4-6 h at 37ºC followed by 48 h at 42ºC for Bolton broth and for 48 h at 42ºC for Preston broth under microaerobic conditions. After this incubation period, 200 µl were cultured for 48 h on the two selective agar plates (mCCDA and CFA) as described above.

Identification of suspected Campylobacter colonies

Following incubation the plates were examined and up to five colonies with *Campylobacter*-typical morphology (according to the manufacturer's instructions) were cultured onto blood agar plates (bioMérieux) at 37ºC for 48 hours in a microaerobic atmosphere for further identification using conventional PCR previously described in Ugarte-Ruiz et al (2012). DNA was liberated by boiling a colony, suspended in 600 µl of sterile double-distilled water, for 10 minutes.

Table 12. Methods used for the isolation of *Campylobacter* from faeces.

Method	**Enrichment step**	**Selective agar plate***
A1	none	mCCDA
B1	Bolton, 37°C, 4-6 hr + 42°C, 48h	mCCDA
B2	Bolton, 37°C, 4-6 hr + 42°C, 48h	CFA
P1	Preston, 42°C, 48 hr	mCCDA
P2	Preston, 42°C, 48 hr	CFA

*All selective agar plates were incubated at 42°C for 48 h.

Species-specific identification of many Campylobacters is problematic because of their fastidious growth characteristics, along with the absence of suitable biochemical assays and the existence of atypical strains. Genotypic identification methods such as PCR targeting ribosomal genes are commonly used to identify several fastidious bacteria including *Campylobacter* spp. Moreover, Gorkiewicz et al (2003) recommend 16S rDNA sequence analysis as an effective, rapid procedure for the unambiguous identification of the majority of Campylobacters. Nevertheless, the lower levels of 16S rDNA variations found between some species make it difficult to identify samples to the species level (Oporto et al, 2011). Several studies have shown that an analysis based on the DNA gyrase B subunit gene (*gyr*B) sequence has a great degree of resolution and is considered a rapid and effective method for identifying bacterial species and for examining phylogenetic relationships (Kawasaki et al, 2008; Suzuki et al, 2001; Yamamoto et al, 1996).

The gyrB gene encodes the subunit B protein of DNA gyrase, a type II topoisomerase that catalyzes the negative supercoiling of bacterial DNA. The partial nucleotide sequences of *gyr*B from the *C*. spp strains were determined as follows. As the similarity of small-subunit rDNA (16S rDNA) sequences is considered a powerful tool for bacterial classification (Olsen et al, 1993), we used it to complete *gyr*B results.

GyrB analysis

Cell lysates were centrifuged (600 G for 10 min) and supernatants were used for conventional PCR for amplification of the gene *Gyr*B using the primer pair GyrB1 (AARCGYCCNGGHATGTATAT) and GyrB2 (CCDARNGCNGTDATCATATT). The primers were designed by Oligo 6.0 software (Molecular Biology Insights, Cascade, CO, USA). Their specificity was verified using 28 strains previously identified as *Campylobacter* spp. using the protocol described above. PCR amplification of the 1352 bp product was performed in 40 µl containing 2.1 µl of lysed cell supernatant, 20 µl of a PCR master mix (kit QIAGEN Multiplex PCR, Hilden, Germany), and 0.384 µM of each primer (Invitrogen, Life Technologies). The amplification was performed in a Thermal Cycler (C1000, Biorad Laboratories, Hercules, CA, USA) with denaturation for 15 min at 95ºC, 40 cycles of 30 s at 94ºC, 90 s at 50ºC and 1 min at 72ºC, and a final 10 min extension at 72ºC. Amplicons were detected by gel electrophoresis using 2% agarose gels containing 10 mg ml^{-1} SYBR green Stain (Invitrogen, Life Technologies) for 30 min at 400 mA. A DNA molecular weight marker (100 bp low ladder, Biotools, B&M Labs, Spain) was included for reference. Bands were visualized under UV light and gel images were taken with a UV Biorad Molecular Imager (Biorad Laboratories). All *Gyr*B-derived amplicons were purified using the QIAquick PCR Purification kit (Qiagen, Hilden, Germany) and the purified amplicons were sequenced by Stabvida (Lisbon, Portugal).

16s rDNA analysis

The 16s rDNA gene sequences of some of our isolates were determined by PCR amplification and direct sequencing. The genomic DNA used was extracted and purified as previously described by Lawson et al (1989). A continuous segment of the 16S rDNA gene of the isolates was determined from PCR-amplified products, derived from universal primers pA (5'-AGAGTTTGATCCTGGCTCAG; positions 8-27, *Escherichia coli* numbering) and pH* (5'-AAGGAGGTGATCCAGCCGCA; positions 1541–1522). PCR product detection and sequencing was carried out as described for *Gyr*B.

All 16S rDNA sequences were compared with the NCBI GenBank nucleotide database using BLASTN and aligned along the sequences of the most closely related species and other representative species within the genus *Campylobacter* from GenBank using ClustalW. In addition, the identification of the phylogenetic neighbors and calculations of pair-wise 16S rDNA gene sequence similarities were achieved, using the EzTaxon server (http://www.eztaxon.org/).

Statistical analysis

Prevalence was compared with the Fisher's exact test and the significance level set at $\alpha = 0.05$. All statistical analyses were performed with R Software (R Development Core Team 2.14.0. 2011), including the 95% CI with package epiR.

Results

Campylobacter other than C. coli and C. jejuni

Twenty strains (eighteen from wild boar and two from cattle) were identified by the PCR as *Campylobacter* sp. For nineteen strains, the partial nucleotide sequences of *gyr*B did not show enough similarity with the previously described sequences to elucidate which species they belonged to. However, they showed a high similarity among them (96-99%). Therefore, as described in the Materials and Methods section, the 16S rDNA gene was used to identify to the species level.

The 16S rDNA gene sequences of six representative strains (4 wild boar, 2 cow), based on *gyr*B sequencing results, were determined by PCR amplification and direct sequencing. Comparative 16S rDNA gene sequence analysis revealed 99.9% similarity between three of the wild boar isolates and the *C. lanienae* strain S-K FAVW (Gorkiewicz et al, 2003) while one wild boar strain shared a similarity of 98.9%. Curiously, one of the cow strains shared a 98.8% homology with the *C. lanienae* strain S-K FAVW. Furthermore, comparative 16S rDNA gene sequence analysis revealed 100% similarity between the second strain found in cows and *C. fetus subsp. venerealis* NCTC 10354^T (Table 13).

Table 13 also shows that the 16S rDNA gene sequence of the three mentioned strains tested also exhibited a high level of similarity with *C. lanienae* NCTC 13004^T (99.5% sequence similarity), while the last wild boar *Campylobacter* exhibited a similarity of 99.2%. In addition, a similarity of 98.4% was found between one of the cow strains and *C. lanienae* NCTC 13004^T.

Table 13. Results of 16S rDNA sequencing and homology searches.

Isolate	Nucleotides sequenced (pb)	Nearest blast hit	% Homology
28-WB	1315	*C. lanienae* strain S-K FAVW	99.9%
28-WB	1315	*C. lanienae* NCTC 13004^T	99.5%
210-WB	1306	*C. lanienae* strain S-K FAVW	99.9%
210-WB	1306	*C. lanienae* NCTC 13004^T	99.5%
1061-WB	1310	*C. lanienae* strain S-K FAVW	99.9%
1061-WB	1310	*C. lanienae* NCTC 13004^T	99.5%
1104-WB	1316	*C. lanienae* strain S-K FAVW	98.9%
1104-WB	1316	*C. lanienae* NCTC 13004^T	99.2%
2078-C	1337	*C. lanienae* strain S-K FAVW	98.8%
2078-C	1337	*C. lanienae* NCTC 13004^T	98.4%
145-C	1325	*C. fetus subsp. venerealis* NCTC 10354^T	100%

WB= wild boar strain, C=cattle strain

Analyses of the 16S rDNA gene sequences of the four wild boar strains confirmed their assignment to the genus *Campylobacter* as *C. lanienae*. Since the other 14 wild boar isolates shared 96 - 99% *gyr*B gene sequence similarity with the four 16S rDNA sequenced strains, these were also considered as *C. lanienae.*

Prevalence of Campylobacter spp.

Prevalence and 95% CI are shown in Table 14. No Iberian ibex, horse or small ruminant was positive for thermophilic *Campylobacter*. The overall prevalence of thermophilic *Campylobacter* was 14.67% in wild boar and 11.48% in cattle.

Specifically, 3 wild boars were positive for *C. coli* (2%, 95% CI 0.41 - 5.73) and one for *C. jejuni* (0.67%, 95% CI 0.02 – 3.66). Eighteen wild boars, or 12% (95% CI 7.27 – 18.3), carried *Campylobacter lanienae*. No age or sex differences were detected in the prevalence of *C.* spp. This was not significantly different in relation to either cattle presence or absence in the area of sampling of wild boars.

C. coli was not isolated from cattle faeces, but 5 cows were positive for *C. jejuni* (9.09%, 95% CI 3.02 – 19.95). Additionally, one isolate (1.82%, 95% CI 0.05 – 9.72) was identified as *C. fetus subsp venerealis* and another isolate (1.82%, 95% CI 0.05 – 9.72) as *C. lanienae*-like. Comparative 16S rDNA gene sequence analysis revealed 97 - 98% similarity between the *C. lanienae* strain from cattle and the *C. lanienae* from wild boars.

The prevalence of *C. jejuni* was higher in cattle than in wild boar or Iberian ibex ($p < 0.01$ in both cases) and the prevalence of *C. lanienae* in wild boar was higher than in cattle or Iberian ibex ($p < 0.05$ and $p < 0.01$, respectively). Table 14 shows that the predominant *Campylobacter* species in wild boar is *C. lanienae* and *C. jejuni* in cattle. Furthermore, *C. coli* appears to be absent from wild boar and *C. fetus* from cattle.

Table 14. Prevalence and confidence interval of thermophilic *Campylobacter* in wildlife and sympatric livestock.

Host species (sample size)	***C. coli*** (95% CI)	***C. jejuni*** (95% CI)	***C. lanienae*** (95% CI)	***C. fetus*** (95% CI)
Wild boar (150)	2% (0.41 - 5.73)	**0.67%** (0.02 – 3.66)	**12%** (7.27 – 18.3)	0 (0 – 3.63)
Iberian ibex (181)	0 (0 - 3.01)	**0** (0 - 3.01)	**0** (0 - 3.01)	0 (0 - 3.01)
Cattle (55)	0 (0 – 9.55)	**9.09%** (3.02 –19.95)	**1.82**% (0.05 – 9.72)	1.82% (0.05 – 9.72)
Horse (4)	0	0	0	0
Small ruminants (2)	0	0	0	0

*In bold, statistically significant results.

Discussion

Considering species, habitat and geographic similarities, we deem our work comparable to that of Díaz-Sánchez et al (2013), however they found a much higher prevalence of thermophilic *Campylobacter* in wild boar (66%). This can be due to peculiarities in the hunting estates studied by those authors, such as estate fencing, high density of game species and possibly different farming conditions. Further research is needed in order to identify the factors responsible for such differences in the same host in a similar Mediterranean habitat.

Nevertheless, the frequency of *C. lanienae* in wild boars in our study area is surprising. This species has been detected previously in feral pigs from California (Jay-Russell et al, 2012), however, that study found *C. jejuni* to be the most frequent. Despite the importance of the emerging *Campylobacter* species, thermophilic *C. jejuni* and *C. coli* are the most frequently isolated species in food-borne zoonoses in humans (EFSA 2012a), and our study also illustrates a potential risk of *Campylobacter* exposure for hunters during handling and processing of wild boar meat. Emerging Campylobacters isolated from food animals are often strains of species typically associated with livestock, such as *C. lanienae* in cattle and swine (Inglis et al, 2005;

Miller et al, 2012; Oporto and Hurtado, 2011). This species was first described in faeces from healthy humans working in an abattoir and its pathogenicity remains unknown (Logan et al, 2000). In addition, the importance of *Campylobacter fetus* has long been recognized in veterinary medicine. *Campylobacter fetus* subsp. *venerealis* is the causative agent of bovine genital campylobacteriosis, an infectious disease that leads to severe reproductive problems in cattle worldwide. *Campylobacter fetus* subsp. *fetus* is a more general pathogen that causes reproductive problems mainly in sheep although cattle can also be affected (Chaban et al, 2012; Iraola et al, 2012). *Campylobacter* are not always determined to the species level, particularly in wildlife, since many of the *Campylobacter* species isolated are uncommon (e.g. Díaz-Sánchez et al, 2013; Lee et al, 2011 or Wahlstrom et al, 2003), but the identification of such isolates is important since the true prevalence of non-*C. jejuni* and non-*C. coli* campylobacter infections is probably underestimated (Oporto and Hurtado, 2011). In our case, identification to the species level and concurrently sampling of co-habiting wildlife and livestock was crucial for the understanding of the epidemiology of *Campylobacter* in our study area.

This procedure allowed us to discern that only *C. lanienae* was shared between cattle and wild boars from the same canyon in the study area. Three *C. lanienae* isolates came from animals (1 cow, 2 wild boars) that were sampled in the same place within a one-week interval. From a herd of 30 cattle, 9 animals were sampled and one was positive for *C. lanienae*. In the same canyon, three wild boars were hunted six days later and two were positive for *C. lanienae*. These findings suggest that there may be spill-over of *Campylobacter* from cattle to wild boar or viceversa. Nevertheless, this was the only point of space and time that *Campylobacter* was found in livestock and sympatric wildlife. *C. jejuni* was found in both cattle and wild boar, but these were not coincident in space. Similarly, Colles et al (2011) reported differences in the prevalence and the species distribution of *Campylobacter* in wild and domesticated Mallard ducks (*Anas platyrhynchos*) that were temporally and geographically matched; and Colles et al (2008) found only occasionally the same *C. jejuni* ST in lambs and geese gathered on the same pasture and free-range poultry and starlings on the same farm.

The fact that no Iberian ibex was positive for *Campylobacter* suggests that wild ruminants may be not important reservoirs, since studies on cervids also report absence or low prevalence (Díaz-Sánchez et al, 2013; Kemper et al, 2006; Lillehaug et al, 2005; Wahlstrom et al, 2003). Despite the fact that most Iberian ibex faeces had to be frozen and this can affect *Campylobacter* isolation, thirty samples processed without previous freezing were also negative for *Campylobacter* spp., too.

During the completion of this study we faced challenges related to both obtaining of samples and laboratory procedures. Bacteriological culture of *Campylobacter* spp. can be difficult, owing to the fragility of these organisms. Generally, the use of a selective medium is recommended for the recovery from stool and faeces although an ideal single method has not been developed. Moreover, accurate identification of these organisms is known to be problematic (Davis and DiRita, 2008; Engberg et al, 2000; Musgrove et al, 2001). With regards to the sampling, the remoteness of the study area and its steep terrain sometimes hampered hunting success and the location of the livestock herds. This problem frequently limits the research on large wild mammals in mountainous habitats.

Conclusions

Wild boar and cattle appear to have their own predominant *Campylobacter* species, while Iberian ibex do not seem to play an important role in the epidemiology of this microorganism in our study area. However, our findings show a potential spill-over of *C. lanienae* and *C. jejuni*, and thus, further research is needed to quantify the occurrence of inter-specific transmission between free-ranging cattle and sympatric wild boar.

Study V:

Zoonotic pathogens and antimicrobial resistance in urban wild boars in Barcelona, Spain.

Under review.

Abstract

Wildlife is increasingly abundant in urban environments, but little is known about the zoonotic pathogens carried by these populations. Urban wild boars are of particular concern, and thus, we studied selected zoonotic pathogens in urban wild boars in Barcelona, Spain (n=41). *Salmonella enterica* was found in 5% (95% CI 0.61-16.91) and *Campylobacter coli* in 4.88% (95% CI 0.6-16.53) of the animals. *E. coli* O157:H7 and *C. jejuni* were not found, but other thermophilic *Campylobacter* were moderately prevalent (19.51%, 95% CI 8.82-34.87). Additionally, we screened for antimicrobial resistance in indicator bacteria: resistance was most frequent in *Enterococcus faecium* (95% of the isolates were resistant to at least one antimicrobial agent), followed by *Enterococcus faecalis* (50%) and *Escherichia coli* (10%). For the first time resistance to linezolid in bacteria carried by wildlife is reported. These results have implications for public health, and thus, further research is needed on wildlife in urban environments.

Introduction

Urbanization of natural areas is one of the key factors driving disease emergence (Daszak et al, 2000) and in recent decades urban and suburban landscapes have been infiltrated by numerous species previously considered intolerant of human activity (Ditchkoff et al, 2006). Thus, new interactions between animal hosts, zoonotic pathogens and humans may occur due to the increasing urbanization of wild areas.

Wild boar (*Sus scrofa*) is a worldwide distributed species that can thrive in areas that are heavily influenced by human activity (Schley and Roper, 2003). The Western European population has increased over the past several decades and in the USA feral hogs are also in expansion (Massei et al, 2011). Wild boar habituation to urban areas obligate certain cities in countries as diverse as Spain, Germany and Poland to cope with conflicts such as traffic accidents, damage to gardens or occasional attacks on people or pets (Cahill et al, 2010). In the urban and suburban areas of Berlin for example, researchers estimate the wild boar population to be around 5000 (Jansen et al, 2007).

Other problems are those noted by Jansen et al (2007): they found an urban focus of leptospirosis among wild boars in Berlin, but populations in other cities have not been studied for this or other pathogens. In the current work, we aim to contribute to the knowledge of the health status of wild boars from Collserola Natural Park (Barcelona, Spain) by assessing the presence of selected zoonotic pathogens and antimicrobial resistance in this peri-urban wild boar population.

Due to its zoonotic importance in the European Union (EFSA, 2012a), the selected pathogens studied were *Salmonella enterica*, *Campylobacter coli*, *Campylobacter jejuni* and other thermophilic *Campylobacter* species and *Escherichia coli* O157:H7. Indicator bacteria tested for antimicrobial resistance were *Escherichia coli*, *Enterococcus faecium* and *Enterococcus faecalis*. It has been previously stated that among wild mammals, wild boars play an important role in the circulation of resistant *E. coli* isolates (Literak et al, 2010), and that enterococci of the intestinal tract of this species can act as a reservoir for antimicrobial resistance genes or that the bacteria can be transmitted to other animals or even to humans (Poeta et al, 2007). Therefore, it is important to know the prevalence of antimicrobial resistance in saprophytic bacteria in urban wild boars.

Materials and Methods

Study area

The study area is the Serra de Collserola Natural Park in Barcelona (NE Spain), a massif that rises over the Barcelona Metropolitan Area, as can be seen in Figure 7. This is an 8000 ha protected area where forest predominates and is widely frequented by the huge human population (around 3.225.000 in 2010) inhabiting the metropolitan area, since the Natural Park is well-maintained and equipped with leisure areas, information centers, guided walks, and other activities, and is easily accessed by private vehicle or public transport (see Cahill et al (2003) for a detailed description of the Park). The wild boar (*Sus scrofa*) is a very abundant species causing increasing conflicts in the peri-urban area due to its habituation to human and suburban landscapes (Cahill et al, 2010; Llimona et al, 2007). The main reason for habituation as proposed by these studies is artificial feeding (either directly by people or indirectly from bins). This underscores the need for knowledge about this population, since pathogens can be transmitted either through direct contact or indirectly via infected urine or faeces in places frequented by people or pets. Also, transmission may occur from people to wild boars through the consumption of domestic rubbish and other uncontrolled waste.

Animal sampling

Forty-one wild boars from the metropolitan area of Barcelona were necropsied in the facilities of the Veterinary Medicine Faculty (Universitat Autònoma de Barcelona, Spain) from September 2010 to August 2011. Seventeen animals were found dead on the roads by the Collserola Park staff after being hit by a car. The rest of the animals were found seriously wounded or ill, or were posing a danger to citizens, and thus, were anesthetized with Zoletil 0.6 ml and carried to the Veterinary Medicine Faculty, where they were euthanized with Tiobarbital. See locations of the animals in Figure 7. Faeces were taken directly from the rectum and stored in a sterile container. They were placed in refrigeration and sent to the laboratory within the subsequent 24 hours.

Figure 7. Location of the site of capture or death of the wild boars in the Collserola Natural Park.

Microbiological analyses

Isolation and identification of zoonotic agents (*Salmonella enterica,* thermophilic *Campylobacter, Escherichia coli* O157:H7) and indicators (*Escherichia coli*, *Enterococcus faecium* and *E. faecalis*) was performed.

For *Salmonella* culturing we applied the ISO 6579:2002 Annex D (International Organization for Standardization, 2007) method recommended by the EU's CRL (European Union Community Reference Laboratory) for *Salmonella* in faecal and environmental samples. Briefly, samples were cultured in buffered peptone water (BPW, 1/10 dilution) and incubated at 37ºC ± 1°C for 18 h ± 2 h. Next, Modified semi-solid Rappaport-Vassiliadis (MSRV) (Difco) agar plates were inoculated with three drops (a total volume of 0.1 ml) of BPW culture. Plates were incubated at 41.5 ºC ± 1°C for 24h ± 3 h and if negative, they were incubated for an additional 24h ± 3 h. Suspected growth of *Salmonella* was confirmed by plating on both Xylose Lysine Deoxycholate agar (XLD) (bioMérieux) and on chromID^{TM} *Salmonella* agar (SM ID2) (bioMérieux). The plates were incubated for 24h ± 3 h at 37ºC ± 1°C. Colonies of presumptive *Salmonella* were subcultured on Columbia 5% sheep blood agar (bioMérieux) and incubated for 24h ± 3 h at 37ºC ± 1°C. Identity of isolates as *Salmonella* spp. was confirmed by a commercially available biochemical method Enterotube ^{TM} II (BD BBL ^{TM}). Serological typing was performed based on the Kauffmann-White scheme (Grimont and Weill, 2007).

Detection of thermophilic *Campylobacter*, *C. coli* and *C. jejuni* was carried out through direct plating on modified charcoal cefoperazone deoxycholate agar (mCCDA). Presumptive colonies were confirmed by PCR as previously described (Ugarte-Ruiz et al, 2012).

E. coli O157:H7 was determined following the ISO16654:2001 (International Organization for Standardization, 2001), based on immunomagnetic separation. Samples were cultured in tryptone soy broth with novobiocin (20μg/ml) at 41.5ºC up to 18h. After immunomagnetic separation, samples were streaked on SMAC and O157:H7. Presumptive colonies were confirmed by PCR as *E. coli* O157:H7 (Desmarcherlier et al, 1998; Gannon et al, 1997; Heininger et al, 1999).

Indicators were cultured in agar MacConkey at 37ºC for 18-20h (*E. coli*) and M-Enterococcus agar (*Enterococcus* spp.), and one colony per sample and microbial species was selected. Confirmation was based on PCR for *E. coli* (Heininger et al, 1999) and *Enterococcus* (Dutka-Malen et al, 1995).

Antimicrobial susceptibility testing

Escherichia coli and Salmonella enterica

Antimicrobial resistance was tested by an agar disk diffusion method against amoxicillin-clavulanate, cefoxitin, amikacin, apramycin, imipenem and aztreonam while a broth microdilution method was performed to determine the minimum inhibitory concentrations (MIC) of sulfamethoxazole, gentamicin, ampicillin, ciprofloxacin, cefotaxime, ceftazidime, tetracycline, streptomycin, trimethoprim, chloramphenicol, florfenicol, kanamycin and nalidixic acid. Break-points and cut-off values used for *Salmonella* are reported in Study I, while for *E. coli* are reported in Study III.

Enterococcus faecium and *Enterococcus faecalis*

The broth micro-dilution method was performed to determine the minimum inhibitory concentrations (MIC) of streptomycin, gentamicin, chloramphenicol, florphenicol, ampicillin, trimethoprim, ciprofloxacin, vancomycin, erythromycin, quinupristin/ dalfopristin, tetracycline, linezolid and penicillin. Interpretation of antimicrobial susceptibility data was carried out according to the epidemiological cut-off values shown in Table 15.

Table 15. Cut-off values (mg/l) for the interpretation of antimicrobial resistance in *E. faecalis* and *E. faecium*.

Antimicrobial agent	***Enterococcus faecalis***	***Enterococcus faecium***	**Reference**
Ampicillin	>4	>4	EFSA 2008
Ciprofloxacin	>4	>4	EUCAST
Chloramphenicol	>32	>32	EFSA 2008
Erythromycin	>4	>4	EFSA 2008
Streptomycin	>512	>128	EFSA 2008
Florphenicol	>4	>4	EUCAST
Gentamicin	>32	>32	EFSA 2008
Linezolid	>4	>4	EFSA 2008
Penicillin	>8	>8	CLSI
Quinupristin-dalfopristin	>16	>1	EFSA 2012
Tetracycline	>4	>4	EFSA 2012
Vancomycin	>4	>4	EFSA 2008
Trimethoprim	>8	>8	VAV 2005

EFSA 2008: EFSA Journal 2008. 141, 1-44; EFSA 2012: EFSA Journal 2012. 10, 2742; EUCAST:www.srga.org/eucastwt/WT_EUCAST.htm; CLSI: CLSI M100-S21; VAV: http://www.vigilanciasanitaria.es/vav/.

Statistical analysis

The statistical analyses were performed with R Software (R Development Core Team 2.14.0, 2011), specifically with package epiR for obtaining the 95% CI.

Results

Zoonotic pathogens

Results are shown in Table 16. Remarkable findings are the presence of *Salmonella* (although at relatively low prevalence), *Campylobacter coli* and other thermophilic *Campylobacter*.

Indicator bacteria

Table 17 shows the profiles of the resistant strains of indicator bacteria. Percentages of resistance to each antimicrobial agent are summarized below.

Table 16. Enteric zoonotic pathogens isolated from urban wild boars.

Pathogen	Number of positives	Sample size	Prevalence (95% CI)
*S. enterica**	2	40	5 (0.61-16.91)
E. coli O157:H7	0	39	0 (0-13.16)
Campylobacter coli	2	41	4.88 (0.60-16.53)
C. jejuni	0	41	0 (0-12.57)
Other thermophilic *Campylobacter*	8	41	19.51 (8.82-34.87)

*Serotypes Corvallis and Anatum. Antimicrobial susceptibility to all agents tested.

Escherichia coli

All *E. coli* isolates (40) were tested for antimicrobial susceptibility. 10% of the isolates were resistant to at least one antimicrobial agent. Four isolates out of 40 were resistant to tetracycline, 2/40 to sulfamethoxazole, 2/40 to ampicillin, 2/40 to streptomycin, 1/40 trimethoprim and 1/40 to kanamycin.

Table 17. Resistance patterns of indicator bacteria from urban wild boars.

Bacterial species	Resistance pattern (number of isolates)
E. faecium	SYN, TET, STR, SYN, TET, HL-STR (2) SYN, TET, HL-STR,ERY (2) SYN (2) TET, HL-STR SYN, TET, ERY (2) TET (4) TET, HL-STR, ERY SYN, CIP SYN, ERY SYN, TET
E. faecalis	CHL, TET, FFC, HL-GEN, TMP, CIP, HL-STR, ERY, LNZ CHL, TET, TMP, HL-STR, ERY (2) TET, HL-STR, ERY (2) CHL, TET, TMP, ERY
E. coli	SMX, AMP, TET, STR SMX, TET, STR, TMP, KAN TET AMP, TET

SYN= quinupristin/ dalfopristin, TET=tetracycline, STR=streptomycin, HL-STR= High-level streptomycin, ERY=erythromycin, CIP= ciprofloxacin, CHL=chloramphenicol, FFC= florphenicol, HL-GEN= high-level gentamicin, TMP=trimethoprim, LNZ=linezolid, SMX= sulfamethoxazole, AMP=ampicillin, KAN=kanamycin.

Enterococcus faecium

Twenty out of 41 animals were carriers of *E. faecium* (48.78%, CI 95% 32.88 - 64.87). All isolates were tested for antimicrobial susceptibility. Eighteen out of these 20 isolates (90%) showed resistance to at least one antimicrobial agent. Resistance was detected against tetracycline (14/20), quinupristin-dalfopristin (12/20), streptomycin (7/20, of which 6 were at a high concentration (2000 mg/l)), erythromycin (6/20) and ciprofloxacin (1/20).

Enterococcus faecalis

Twelve out of 41 animals were carriers of *E. faecalis* (29.27%, CI 95% 16.13 - 45.54). All isolates were tested for antimicrobial susceptibility. Six out of these 12 isolates (50%) showed resistance to at least one antimicrobial agent. Resistance was detected against tetracycline (6/12), erythromycin (6/12), streptomycin (5/12, all resistant isolates showing resistance at high concentration: 2000 mg/l), chloramphenicol (4/12), trimethoprim (4/12), florphenicol (1/12), ciprofloxacin (1/12), gentamicin (1/12), and linezolid (1/12).

Discussion

Zoonotic pathogens

Campylobacter spp. and *Salmonella enterica* were found in urban wild boars, posing a concern for public health since these are the most frequent zoonoses in the European Union (EFSA, 2012a). In urban wildlife, prevalence of diseases is sometimes greater than that found in rural habitats, due to a greater food availability leading to higher survival, increased density and aggregation, resulting in increased rates of disease transmission (Ditchkoff et al, 2006). On the other hand, prevalence may also be lower: Schielke et al (2009) report a lower detection rate of Hepatitis E Virus in urban wild boars than in their rural counterparts. These authors suggest that the shift from a sylvatic to a synanthropic occurrence of this game species might lead to a future increase of the infection pressure from the Hepatitis E Virus on the human population, although the ecological or biological variations explaining these differences are not known and occur despite the low prevalence in urban wild boars. We agree with this statement and believe this may be the case for the pathogens studied here. In fact, both direct and indirect contact of humans with wild boars has become more likely in urban environments, and thus the exposure of humans to pathogens may be greater in contrast to rural areas. Numerous examples of the proximity of wild boar to settlements are found in the media, especially on-

line: wild boars feed from rubbish dumps or are fed by people, graze in public and private gardens or drink water from swimming pools.

In the case of Barcelona, inferences about how the synanthropization of the wild boars has affected pathogen prevalence are difficult since there are no such data prior to the urbanization of this Natural Park. Studies on *Salmonella* in a wild boar population from another much less anthropized Natural Park in Catalunya have reported a higher *Salmonella* prevalence. Apparently the link to *Salmonella* in free-ranging cattle sharing pastures with wild boar (Navarro-Gonzalez et al, 2012) is the most plausible explanation. The absence of cattle in Collserola Park may be one reason for the lower prevalence of *Salmonella* in wild boar but other hosts like rodents and birds should be investigated, as well as the role of domestic rubbish and sewage in the human-wildlife *Salmonella* cycle.

In regards to *Campylobacter* in wild boar, the only information we could find was from Sweden (12% of positive faecal samples; Wahlstrom et al, 2003), Germany (3 out of 127 wild boar meat samples were positive (Atanassova et al, 2008) and Switzerland (absence in tonsils; Wacheck et al, 2010). Nevertheless, the effect of feeding at refuse sites on *Salmonella* and *Campylobacter* prevalence has been studied in sea gulls from NE Spain: Ramos et al (2010) showed a positive relationship between *Campylobacter* prevalence and refuse consumption. Further research in urban wild boars should address refuse consumption by examining gastric contents, for example, in order to determine its relation to pathogen carriage.

Antimicrobial resistance

Resistant Enterococci have been previously isolated from free-ranging wild boars in Portugal (Poeta et al, 2007) and Belgium (Martin et al, 2007), and resistant *E. coli* have been found in Poland (Mokracka et al, 2012), the Czech Republic (Literak et al, 2010) and Belgium (Martin et al, 2007). On the other hand, Schierack et al (2009) report the absence of resistance in *E. coli* isolates from wild boars in Germany, and furthermore, a comparison with susceptible isolates from piglets show that wild boars carry more sensitive isolates. To our knowledge, this is the first study on antimicrobial resistance in urban wild boars, a situation of great concern, as explained above, and also the first report of a linezolid-resistant microbial isolate in wildlife. As Poeta et al (2007), we detected higher percentages of antimicrobial resistance in *E. faecium* than in *E. faecalis*, and tetracycline- and erythromycin-resistance were the most frequent. It is known that proximity to human activities influences the antibiotic resistance profiles of the gut bacteria of wild mammals (Allen et al, 2010), e.g., Wheeler et al (2012) found a relationship between human proximity and the carriage of resistant isolates in several reptile species in the

Galápagos Islands, or Thaller et al (2010) report two resistant isolates in faeces from land iguanas (*Conolophus pallidus*) on a remote island in this archipelago, whose characteristics and location strongly suggest a human origin. On the other hand, this link may not always be obvious or easily shown since other studies found no evidence for transmission of antibiotic-resistant bacteria from humans to wildlife (e.g., in Lowland Gorillas (Benavides et al, 2012) or in Antarctic penguins (Hernández et al, 2012)). Regarding wild boar, certain particular habits such as their omnivorous diet, the visiting of refuse sites and the consumption of animal waste are assumed to be the source of resistant bacteria for this species (Literak et al, 2010). In the case of our study, despite a lack of absolute certainty on the anthropogenic origin of this antimicrobial resistance, the finding of an *E. faecalis* isolate resistant to linezolid strongly suggests a human origin because resistance to linezolid is still rare in animals (Aslam et al, 2012; Fluckey et al, 2009; Hoelzel et al, 2010; de Jong et al, 2012), as it is used only in human medicine (Aarestrup and Aidara-Krane, 2012) and is a completely synthetic agent (thus, pre-existing resistance is not likely (Meka and Gold, 2004).

At the local scale, our results have public health implications since contact between wild boars and humans is becoming more common and wild boars enter the food chain frequently. This may also be true for other cities and countries. Furthermore, with these results we emphasize the need to pay special attention to certain animal species that are undergoing changes in their habits and ecological traits, as this change may enhance contact rates, disease transmission and human-wildlife interactions.

General discussion and future perspectives

Zoonotic pathogens in wild boar and sympatric cattle

Many studies report the link of *Salmonella* between wildlife and domestic animals, but mainly focusing on an agricultural environment and small-sized wild vectors such as insects, micromammals and/or small birds (e.g. Carlson et al, 2011; Liébana et al, 2003; Skov et al, 2008); hence the originality of our study resides both on the farming system (extensive) and the type of wildlife surveyed (large wild ungulates).

In our study, although it may be difficult to assess the direction in which transmission occurs, prevalence of *Salmonella* is higher in wild boar from areas where cattle co-habit, and probability of infection of a wild boar with *Salmonella* increases with cattle herd size. These results suggest that transmission direction is likely from cattle to wildlife. Similarly, Skov et al (2008) found *Salmonella* in wildlife surrounding farms where and when *Salmonella* was also detected in livestock. The results of Study I are complemented by the PFGE data contributed by Mentaberre et al (in press) where 100% similarities between *Salmonella* Melagridis, *S.* Othmarschen and *S.* Anatum from wild boar and cattle were observed.

However, it should to be emphasized that the cattle herd with the highest prevalence was a bullfighting herd. It has been argued that difficulties in the management of bullfighting herds may be the cause of a greater prevalence of bovine tuberculosis (Boadella et al, 2011), but in the case of *Salmonella* and concisely in our study area we deem this likely to be due to management practices and feeding in particular. However, apart from livestock, other *Salmonella* sources should be considered. Since the prevalence found in wild boar from cattle-grazed areas was higher than prevalence in cattle itself, and some serotypes were found in wild boar but not in cattle, *Salmonella* sources other than cattle may exist, and these are probably linked to cattle presence. For example, other wild hosts (wild birds and rodents) attracted by free access to cattle food or other domestic animals (dogs or cats) could play a role in the epidemiology of *Salmonella* in the study area.

Interestingly, the high *Salmonella* prevalence in wild boars from cattle-grazed areas is similar to that found by Cowled et al (2012). These authors found in wild pigs in Australia, which was higher in populations where infection had been present longer. This leads us to an association with our findings: *Salmonella* infection seems to be endemic in wild boar and cattle in our study area. At this point, it should be considered that transmission may be bi-directional as Bengis et al (2012) defined most disease problems at the wildlife/livestock interface. In such an environment, it is difficult to control this pathogen in this environment, since not only host-to-host transmission but also indirect transmission through contaminated pastures and water,

would complicate control efforts. Bengis et al (2012) emphasize that the presence of one or more maintenance hosts in an ecosystem is epidemiologically significant, and is responsible for the long term persistence of infection in a given ecosystem. Cowled et al (2012) additionally found that ecologically resource-rich areas across the landscape may facilitate the persistence of *Salmonella* in wild pig populations. This means that if we aimed to reduce the *Salmonella* prevalence, a careful consideration of resource distribution across the landscape and spatial targeting of control to those areas of greatest risk would be more efficient than control targeted at wild boar density alone (Cowled et al, 2012), which is the traditional approach.

Our results additionally elucidate the diversity of *Salmonella* serotypes in wild boar. Serotypes found by previous studies on wild boar (Díaz-Sánchez et al, 2013; Millán et al, 2004; Vieira-Pinto et al, 2011; Wacheck et al, 2010) differ from our isolates with the exception of Enteritidis. This reflects a great diversity and spatial variability of *Salmonella* serotypes in wild boar. The large number of serotypes found in our study area and the fact that most serotypes were isolated only once during the study period or in only a single animal, may reflect a scarcity of animal movement (owing to difficulties derived from steep terrain and/or to abundance of food resources) or may be the result of a low intra-specific transmission. The former is supported by the fact that bovine tuberculosis displays a similar pattern in wild boars from the same area (Mentaberre, unpublished data). It has already been suggested that natural barriers cause the separation of both wild boar and their associated pathogens Methner et al (2010) based on the detection of epidemiological groups of *Salmonella* serotype Cholerasuis in only certain regions. This host-adapted serotype, which causes swine paratyphoid, was not isolated in our study area and apparently our wild boar population seems to be unaffected by this disease, unlike farmed wild boars in Spain (Pérez et al, 1999) or free-ranging wild boar populations in Germany (Methner et al, 2010). However, we associate the lower serotype richness observed in cattle to the more homogeneous conditions of farmed animals (e.g. food, water), which leads to homogeneous exposure within the herd. Furthermore, the detection of *Salmonella* serotypes such as Enteritidis or Paratyphi B in wild boar (and not in cattle) displays a certain parallelism with gulls that feed in polluted estuaries and excrete serotypes usually associated with humans (Sánchez et al, 2002; Simpson, 2008). This finding may be explained by the omnivorous diet that could increase exposure in comparison to cattle owing to the utilization of varied sources (which may include human waste) and feeding on the ground.

In conclusion, the *Salmonella*-wildlife-human cycle is complex, and in most cases, outbreaks of human salmonellosis cannot be traced to wildlife, but there are important cross roads

between wildlife, humans and livestock (Hilbert et al, 2012). For this reason, the high prevalence of *Salmonella* in wild boar and the carriage of serotypes associated with salmonellosis in humans is a matter of concern.

Somewhat less clear is the epidemiology of thermophilic *Campylobacter* in the National Game Reserve. The culture and identification of the isolates was challenging, including attempts to achieve a normal growth after storage at -80ºC in order to repeat the techniques that repeatedly failed (PCR, sequencing, etc). Although not mentioned in the manuscript, Api Campy strips were also used in attempts of species identification. However, these yielded unreliable results. Likewise, antibiograms were performed but were ultimately not useful: bacterial growth was inconsistent even in the positive control (which is one of the reasons for not reporting antimicrobial resistance in *Campylobacter* in this Thesis). Nevertheless, the challenging work and repetition was worth the effort. In addition to reporting a considerable *Campylobacter jejuni* prevalence in cattle, our results shed light on the epidemiology of this microorganism. Although not as evident or frequent as *Salmonella enterica*, there may be a spill-over from wild boar to cattle and vice versa, at least in relation to *C. lanienae* and probably in relation to *C. jejuni* as well. Further research into the molecular insights of the *C. jejuni* isolates would clarify questions regarding similarity of *C. jejuni* between herds, for example.

Unlike *Salmonella* and thermophilic *Campylobacter*, *E. coli* O157:H7 was not isolated from free-ranging livestock. Nevertheless, we must emphasize that the findings of this pathogen in wild boar were very limited in time and space; hence we cannot point to wild boar as an important reservoir. Further research in this area should consider the implementation of several changes in the methodology to determine in detail the epidemiology of this microorganism, e. g., sampling could be performed in both domestic and wild populations during summer- autumn, since there may be a seasonal shedding pattern (EFSA, 2009). This would increase the likelihood of finding a positive animal; in fact the EFSA (2009) recommends the sampling of cattle between 1 April and 1 October to make the monitoring programme more cost-effective. Likewise, focusing on young animals, both wild and domestic, would also likely increase the probability of isolating *E. coli* O157:H7. Furthermore, the existence of "supershedders" in wild boar and Iberian ibex populations should be investigated, since in cattle the "supershedders" have been associated with increased prevalence and excretion within pens (Cobbold et al, 2007), and in the wild "suppershedders" would be very important in maintaining this pathogen in the environment.

A further intended analysis explores the frequency of carriage of at least one zoonotic agent including data on other zoonotic agents (tuberculosis, MRSA) that have been found in the same animals used for this Thesis, what providing a deeper insight into the health risks associated with wild boars.

Zoonotic pathogens in Iberian ibex

Very different from wild boar is the picture painted by the Iberian ibex. Such low prevalences of the pathogens studied, as opposed to those found in the wild boar, were unexpected in some instances. The difference in the *Salmonella* prevalence in wild boar concurs with that found in the literature, namely, the wild boars carry *Salmonella* more frequently than wild ruminants (Paulsen et al, 2012). However, we were surprised by the low prevalence of *E. coli* O157:H7 in Iberian ibex, since the ibex is related to small domestic ruminants and known as reservoirs. Although our livestock sampling was not extensive, it is representative of a considerable amount of the total livestock population in the National Game Reserve "Els Ports". Indeed, we sampled a higher proportion of the livestock population than that of the wild ungulates. Thus, we can be assured that the prevalence of *E. coli* O157:H7 in livestock in this study area is very low and not likely to spill-over. The origin of this pathogen in Iberian ibex and its relation to the strains found in wild boar is a question that remains unanswered. PFGE could help in determining if there is a common source, but regardless, further research is needed since basic information on 2 out of the 3 positive ibexes (sex, age, location) was not known, which strongly hinders any further conclusions. The possibility *of E. coli* O157:H7 being pathogenic in Iberian ibex, although remote, should be taken into account: despite the ruminants are symptomless carriers of *E. coli* O157:H7, lesions may form in their gastrointestinal tract (La Ragione et al, 2009). However, in the clinical case mentioned in Study III the most likely cause is a enterotoxic hemorrhagic enteritis (*Clostridium perfringens* type C) (R. Velarde, personal communication).

In spite of the limitations in our ability to draw conclusions from the Iberian ibex data in relation to thermophilic *Campylobacter* spp., we can consistently say that the prevalence is low. To our knowledge, Study IV is the first study on the presence of this microorganism in Iberian ibex, which we consider a valuable addition to the literature. The importance of expanding the knowledge on the epidemiology of thermophilic *Campylobacter* lies both in public health (emerging *Campylobacter* species) and in animal production (as a cause of abortion (Menzies, 2011)). Since domestic small ruminants are known to be reservoirs (La Ragione et al, 2009), the need for assessing its presence in Iberian ibex was real.

Curiously, Iberian ibex is not a carrier of certain pathogens that are highly prevalent in its surrounding environment, as detailed in the discussion of the manuscripts. According to Bengis et al (2002) taxonomic grouping, social organization and behavior, and population densities usually determine the maintenance-host potential of a given species for a specific disease, and most of these conditions are met by Iberian ibex for the pathogens studied. Therefore, further research should be aimed at elucidating the reasons underlying this phenomenon. A first approach could be behavioral, based on the study of resource sharing with cattle and wild boar, predominantly pastures, water sources and mineral blocks. In our opinion, the fact that Iberian ibex is a browser greatly reduces the exposure to pathogens that are typically found in the soil or the grass. Moreover, the percentage of Iberian ibexes that are clinical cases among the total number of ibexes carrying the studied pathogens is surprising: 2 out of 6 (33%).This may indicate that Ibexes are usually not exposed to pathogens, but come into contact with them only in specific situations of weakness or immunodepression, being prone to developing disease, either associated with the studied pathogen (e. g., salmonellosis) or not (i.e., *E. coli* O157:H7 may be rather an indicator of a poor health status than a pathogen itself). It is remarkable that Muñoz et al (2010) report a similar situation in relation to *Brucella*: out of 1086 animals, the only positive was a severely ill male buck.

Antimicrobial resistance in a natural environment

Interestingly, Study III shows how free-ranging livestock are low-significance reservoirs of AMR. This suggests that in the absence of antimicrobial pressure, livestock are not important carriers of resistant bacteria and that extensive farming is environmentally respectful in relation to AMR, and may not be a major force driving the introduction of AMR in a natural environment. Nonetheless, there are further questions that should be asked: why is an *E. coli* resistant to cefotaxime carried by a wild boar? And why do we find *E. coli* resistant to fluoroquinolones in cattle? Ongoing research is focused on these strains, in order to determine the mechanism underlying these resistance patterns. The potentiation test has been performed for the strain resistant to cefotaxime, as it is suspected to be an Extended-Spectrum Beta-Lactamase producer. Additionally, the most frequent mutations and plasmids conferring fluoroquinolone-resistance in Enterobacteriaceae are being sought. *Inter nos*, I believe that poor agricultural practices may explain some of the surprising findings. From our own experience, we know that a certain farmer in the study area is not entirely compliant with the regulations and the implementation of basic biosecurity and hygiene measures. For example, Mentaberrre et al (in press) denounce the use of chicken manure, a by-product that

is banned, as cattle feed. This is one of the irregularities detected but there may be others. It is not surprising that we find a resistance pattern that is not frequent in intensive cattle but is in poultry, for example. Although it is questionable that an *E. coli* other than the host-specific group could colonize cattle or wild boar and that resistance could be transferred horizontally, it may be a topic worth considering in future research.

However, the lack of an association of AMR in wildlife to that in livestock may be true only for commensal bacteria. The finding of a *Salmonella* Mbandaka strain with a resistance profile typically associated with livestock strongly suggests a livestock origin, especially regarding the fact that serotype Mbandaka is the most frequent serotype in Spanish cattle (EFSA, 2012a). The same serotype with the same resistance profile was isolated from a box-trapped piglet in the same location six months earlier (data not shown), which means either that the *Salmonella* source was continually or intermittently present or that this resistant strain persisted in the wild boar population at least during this period. The fact that *S.* Mbandaka was not isolated from co-habiting cattle highlights the need for matched spatial and temporal sampling in order to draw more robust conclusions. Nevertheless, a wild boar from a cattle-free area carried *Salmonella* Enteritidis resistant to fluoroquinolones, thus other resistance sources of equivalent importance cannot be excluded. On the contrary; their presence is highly probable. Concerning *E. coli* O157:H7, although the total susceptibility of wildlife isolates and the lack of isolates from livestock strongly suggest a sylvatic cycle we must further investigate before making strong conclusions.

The need for implementing sound practices in antimicrobial susceptibility testing, such as the use of an internationally accepted procedure, a quality control using a reference strain or the interpretation of the results using the most recent updated criteria, among others, has been previously suggested (Schwarz et al, 2010). These authors also accentuate the correct use of interpretation terms such as “wild-type” and “non-wild-type” when epidemiological cut-off values are used. However, we decided to use the terms “resistant” and “susceptible” because these are the most widely used in the literature and by reference organizations such as the EFSA. Following the suggestions of Schwarz et al (2010), we recognize the need for publishing the full MIC distribution (including MIC_{50} and MIC_{90}) for each species and drug. For this reason, a further manuscript comparing MIC distributions of indicator bacteria of rural and urban wild boar is being prepared for publication with these data. We agree with the proposals of Schwarz et al (2010), and moreover, we deem it useful to agree on a certain methodology and interpretive criteria that allow direct comparison of results from different host species and geographic locations, which would help in understanding the spread of AMR in wildlife and the

environment. Likewise, the monitoring of AMR in a selected sentinel species, as other disciplines like toxicology have been doing for years, would provide valuable information on both local and global trends. Wild boar, as we mention in our manuscript, is a perfect candidate because of its habits, and its nearly worldwide distribution and abundance would enable global AMR monitoring at a relatively low-cost and with low-effort sampling.

The problematics of urban wild boars

A study such as Study V was required as a baseline assessment to compensate for the complete lack of knowledge existing in relation to pathogens in urban wild boars. However, research cannot stop here; current knowledge must be expanded by studying pathogen dynamics in detail and there are still important zoonotic pathogens to be assessed. In the Collserola situation, the next step is to analyze the link between pathogens and AMR in wild boars and the proximity to the sewage treatment plant. Additionally, resistance to linezolid in an *Enterococcus* isolate will be further investigated. In the opinion of Bradley and Altizer (2007), it is necessary to move beyond associational patterns towards experimental and modeling approaches. An interesting work that could serve as an example for future projects in Collserola Park was published by Broadfoot et al (2001); these authors model population data on raccoons (*Procyon lotor*) and striped skunks (*Mephitis mephitis*) to predict the risk of rabies outbreaks in the city of Scarborough (Canada). A more experimental approach has been used to control *Echinoccocus multilocularis* infection in red foxes via anthelmintic baiting (Hegglin et al, 2003). On the other hand, antimicrobial therapy is not a measure that can be considered for the control of the microbial zoonotic agents reported here, but other experimental approaches could be planed based on the alteration of feeding habits, host contact rate, and other factors.

Beyond zoonoses transmission and potential effects on public health, urbanization also plays a role in the transmission and impacts of infectious wildlife diseases (Bradley et al, 2007). However, its effects and consequences are not completely clear since these are variable depending on the level of urban-adaption of the host, the maintenance of the pathogen or the “dilution effect”. Therefore, further research can clarify the mechanisms driving disease dynamics in urban landscapes and contribute to the debate of positive versus negative effects of urbanization on the prevalence and impact of wildlife diseases.

For the near future, we argue that decisions on the management of an urban wild boar population should take into account preferences of the public and seek support of the local

community. Urban and peri-urban inhabitants may be not familiar with or may not agree with management methods such as hunting or culling. One study about roe deer in a peri-urban Scottish area found the most support for other measures such as fencing or changing human behavior to manage "deer problems" (Dandy et al, 2011). The results of Vaske et al (2011) may explain this: they suggest a general shift away from traditional use-oriented beliefs toward a support of the coexistence of humans and wildlife. Simply seeing a wild animal cannot be ignored as a highly-valued experience for many people; thus, successful management strategies should aim at changing these attitudes or adjusting to them.

Closing remarks

In spite of finding zoonotic pathogens in wild ungulates, including both emerging pathogens and emerging host species, we do not wish to cause unnecessary alarm. As stated by Gummow (2010), it must be kept in mind that the magnitude and impact of an emerging disease has very little to do with the fact that that disease comes from wildlife. In this sense, although our findings may appear alarming, the specific consequences and direct implications for human health need to be evaluated, as well as the potential losses in livestock production and wildlife conservation.

Being able to define the wild boar as a carrier and reservoir of zoonotic pathogens, as well as excluding Iberian ibex from a determinant epidemiological role in the National Game Reserve, is a remarkable achievement. It is often challenging to determine the particular epidemiological role of a species in the dynamics of infection. Many studies on wildlife infections do not suggest the epidemiological role of the described species (Martin et al, 2011), partly due to lack of information: data concerning the infection status in livestock and other wild species and the interactions of host species are required but not always available to the authors. In addition, many different and often contradictory definitions of reservoirs exist (Haydon et al, 2002).

From our results we can conclude that factors designating a potential wildlife reservoir of a disease agent are many and unique for each livestock/wildlife situation, as has been noted by Van Campen and Rhyan (2010). A careful study of each interface situation and of the epidemiology of the pathogen in each species is required to control or eliminate interspecies transmission or eradicate the pathogen in a natural environment like the National Game Reserve "Els Ports de Tortosa i Beseit".

From a more general perspective, the presence of zoonotic pathogens in wildlife indicates the existence of uncontrolled sources and cycles of pathogens in the environment. In light of these results, we agree with Thiermann's (2004) statement: "It is important to move from the historical standard such as "disease-free" status, which does not offer absolute guarantees, towards risk-based, scientific and regionalized approaches, since zero risk is impractical". With total certainty, species and habitats do not behave as separate compartments; therefore pathogens cannot be contained either in space or time. This should encourage us to improve our knowledge on the management of pathogens in wild populations, thereby reducing the impact and consequences of diseases that threaten human and animal health, agricultural production and species conservation.

Conclusions

Conclusions

1. Study I provides evidence of the association of *Salmonella* infection in free-ranging wild boar and herd size of sympatric cattle in extensive farming conditions. The presence of antimicrobial resistance in *Salmonella* isolated from wildlife in a protected area is a matter of concern.

2. In light of the results of Study II, Iberian ibex appears to be an accidental or dead-end host of *Salmonella enterica.*

3. As shown in Study III, prevalence of *E. coli* O157:H7 in wild ungulates was low, and may be unrelated to livestock.

4. Free-ranging livestock act like wild ungulates as low-significance reservoirs of antimicrobial resistance. For the purposes of choosing a wild species as an AMR sentinel, wild boar may be a very good indicator of the AMR circulating in a natural environment.

5. For monitoring AMR in wildlife and the environment, there is urgency for the standardization of testing methods and the interpretation of criteria to allow comparisons between species and locations.

6. There is a potential spill-over of *C. lanienae* and *C. jejuni* between wild boar and cattle, despite having their own predominant *Campylobacter* species. Iberian ibex do not play an important role in the epidemiology of this microorganism in our study area.
7. The presence of zoonotic pathogens and antimicrobial resistance anthropic in origin deserves attention and further research.

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