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The multi-parasite/multi-host system occurring at the wildlife ruminant community of Sierras de Cazorla, Segura y Las Villas National Park

El sistema multiparásito-multihospedador de los nematodos parásitos de la población de rumiantes silvestres en el Parque Natural de las Sierras de Cazorla, Segura y Las Villas

Dña. Tessa Carrau Garreta

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"*Living systems are never in equilibrium.*
They are inherently unstable.
They may seem stable, but they're not.
Everything is moving and changing.
In a sense, everything is on the edge of collapse."

Michael Crichton, - Jurassic Park

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Resumen / Summary

Resumen

En esta Tesis Doctoral se ha estudiado la interacción natural que ocurre entre parte de la helmintofauna de cuatro especies de rumiantes silvestres presentes en el sureste de España. En particular, se centra en parte de los nematodos parásitos broncopulmonares y gastrointestinales que se encuentran presentes de forma natural en múltiples hospedadores. Este fenómeno, denominado multihospedador, se encuentra a menudo combinado con el multiparasitismo, o la infección simultánea con varias especies parásitas en un mismo hospedador. Existen multitud de ejemplos en la bibliografía donde se documenta la presencia de especies parásitas compartidas entre distintos miembros de una comunidad de hospedadores silvestres. Estos estudios han demostrado ser particularmente útiles para estudiar la posible influencia de estos patógenos en las dinámicas de poblaciones de animales.

Los fenómenos de multiparasitismo y multihospedador se describen tanto entre especies hospedadoras cercanas entre sí, como entre aquellas taxonómicamente lejanas. Se ha estudiado que los hospedadores que pasan tiempo en diversos hábitats tienen más probabilidades de adquirir y propagar parásitos generalistas, por lo que resulta muy interesante estudiar hospedadores que tienen amplia libertad de movimientos y analizar qué rol juegan cada uno en la transmisión de parásitos generalistas. En el caso de los nematodos gastrointestinales y/o broncopulmonares de los rumiantes silvestres, existen factores extrínsecos que pueden influir en su ecopatología. Un ejemplo de ellos son las condiciones climáticas, que tienen un efecto directo tanto sobre el ciclo de los nematodos, como sobre la disponibilidad de hospedadores intermediarios.

Para poder desarrollar estos estudios es necesario encontrar localizaciones que permitan un abordaje completo los fenómenos de multihospedador y multiparasitismo de forma natural y así poder entender aspectos como la relación ambiente-hospedador-parásito. Un área de estudio óptima para investigar esta dinámica es el "Parque Natural de las Sierras de Cazorla, Segura y Las Villas" (PNSCSV), una zona montañosa de 2.140 km^2 ubicada en la vertiente oriental de la Sierra Bética de Andalucía, en España. El estudio de los nematodos broncopulmonares y gastrointestinales de rumiantes silvestres para profundizar en el conocimiento de las comunidades parasitarias permite evaluar su macroecología (relacionando las características de la población de nematodos con las condiciones ambientales,

geológicas, etc.) y abordar aspectos como el multiparasitismo, combinado con el fenómeno de multi hospedador, y la relación ambiente-huésped-parásito.

El primer hospedador analizado fue la cabra montés (*Capra pyrenaica*), un bóvido que se encuentra en las áreas montañosas de España y norte de Portugal y una especie endémica de la península ibérica. El segundo bóvido incluido en nuestro estudio es el muflón común o europeo (*Ovis orientalis musimon*), ungulado alóctono, introducido en Europa Central con fines cinegéticos. El ciervo (*Cervus elaphus*), especie presente en prácticamente todo el hemisferio norte, y el gamo (*Dama dama*), nativo de la región mediterránea, constituyen los dos cérvidos incluidos en el estudio. Se trata de dos especies nativas con alto valor cinegético en la península ibérica.

Los objetivos principales de esta Tesis Doctoral son:

- Descripción de datos epidemiológicos como son, riqueza, prevalencia, abundancia e intensidad parasitaria, en un contexto multiparásito / multihospedador.
- Discusión del papel epidemiológico de la transmisión de estos parásitos, e intentando explicar la helmintofauna hallada desde una perspectiva ecológica.
- Validación de la técnica de diagnóstico de migración larvaria o método Baermann-Wetzel comparando las muestras pulmonares y fecales obtenidas durante las necropsias usando el análisis Blant-Altman.

En el **Capítulo 1** se estudiaron los nematodos gastrointestinales de los cuatro rumiantes silvestres desde el punto de vista multihospedador. Nuestro objetivo fue abordar el multiparasitismo y fenómeno multihospedador natural de los nematodos gastrointestinales en un escenario simpátrico de rumiantes silvestres presentes en el sureste de España. Para ello, se estudió el tracto gastrointestinal de 252 rumiantes silvestres de cuatro especies diferentes en el PNSCSV. El 81,52% de los animales analizados resultaron estar parasitados por lo menos con una especie de nematodo y se identificaron un total de 29 especies en total. *Spiculopteragia asymmetrica* (55,9%), *Oesophagostomum venulosum* (58,7%) y *Spiculopteragia quadrispiculata* (45,3%) resultaron ser los parásitos más prevalentes en esta parte del estudio. La prevalencia difirió significativamente entre las especies hospedadoras. El gamo presentó la mayor prevalencia de nematodos (n = 109; 91,5%), seguido de los bóvidos silvestres (muflón n = 59,

85,5%; cabra montés *n* = 20, 83,3%) y el ciervo (*n* = 64, 61,4%). Además el muflón presentó la mayor riqueza parasitaria, con hallazgo de hasta 12 especies diferentes. En este sentido nuestro estudio representa un claro ejemplo de un escenario de multiparasitismo ya que la gran mayoría de los nematodos se comparten entre al menos dos especies hospedadoras simpátricas y solo unos pocos parásitos son exclusivos de una única especie hospedadora. En este escenario, además, la estructura de la comunidad de parásitos se vio significativamente influenciada por el hospedador, con la mayor prevalencia y riqueza de parásitos apareciendo consistentemente en muflones. Estos resultados sugieren un punto de dispersión de parásitos originado por el muflón. Además la presencia de patrones específicos de dispersión se realizó mediante el análisis multivariable de abundancia, que demostró diferencias significativas entre las 11 especies de nematodos compartidas por todas las especies hospedadoras. Se observaron patrones claros tanto en las especies hospedadoras (gamo, cabra montés, muflón y ciervo rojo) como en los niveles del grupo hospedador (bóvidos vs cérvidos). Estos hallazgos destacan la capacidad de los parásitos para adaptarse a la comunidad de hospedadores y dar forma a su distribución ecológica de acuerdo con las especies de hospedadoras más adecuadas disponibles.

Siguiendo la línea anterior, en el **Capítulo 2** también se abordó el estudio de los nematodos broncopulmonares de la misma población de rumiantes silvestres desde el punto de vista multihospedador y multiparásito. En este caso se analizaron tanto los parásitos como los factores de riesgo extrínsecos asociados a su presencia en los diferentes hospedadores. Para ello, se examinaron las vías respiratorias de 252 ejemplares pertenecientes a las 4 especies mencionadas anteriormente (ciervo, muflón, cabra montés y gamo). Casi la mitad (48,0%) de los animales estaban infectados con nematodos broncopulmonares. Se identificaron 7 nematodos diferentes, de los cuales 2 géneros (*Protostrongylus* spp. y *Dictyocaulus* spp.) y 3 especies (*Cystocaulus ocreatus, Muellerius capillaris* y *Neostrongylus linearis*) se registraron en al menos dos rumiantes, siendo el muflón el hospedador más común. En este caso, se pudo observar un efecto significativo entre las diferentes áreas de muestreo del PNSCSV. Desde una perspectiva espacial, la mayor abundancia de parásitos se detectó en la parte central del parque. Se encontró un efecto marginal de la edad sobre la abundancia de los parásitos a nivel poblacional. Además, dos especies se hallaron parasitando solo una especie: *Elaphostrongylus cervi* y

Varestrongylus sagittatus; en concreto, se detectó *E. cervi* en la población de ciervos con una alta prevalencia (46,3%), mientras que *V. sagittatus* (13,9%) se encontró únicamente en gamos. Este estudio representa un ejemplo interesante de la estructura y composición de la comunidad de parásitos broncopulmonares en un escenario de multiparasitsimo. Con esta interacción, el muflón vuelve a ser la clave epidemiológica en la red natural de interacciones, mostrando una alta prevalencia y riqueza de nematodos broncopulmonares que son compartidos con las otras especies de rumiantes silvestres simpátricos. Por otro lado, el ciervo parece estar completamente desconectado de la comunidad de nematodos broncopulmonares que comparten las otras 3 especies de rumiantes silvestres presentes. La menor presencia de parásitos que presenten un efecto multihospedador en los cérvidos con respecto al **Capítulo 1** se discute debido a una posible influencia de las condiciones climáticas y ambientales en la comunidad de parásitos. En este caso, la necesidad de hospedadores intermediarios. Además se encontraron los valores más altos de intensidad parasitaria localizados en la parte central del PNSCSV, donde se encuentra la mayor población de muflones.

En el **Capítulo 3** se describe la validación de métodos de diagnóstico de nematodos broncopulmonares. La importancia de este estudio radica en el hecho de que los nematodos broncopulmonares pueden tener un impacto negativo en la conservación de rumiantes silvestres. Por esta razón, el correcto y temprano diagnóstico de nematodos broncopulmonares es una medida de prevención de enfermedades respiratorias asociadas a estos parásitos. La técnica de Baermann-Wetzel es el método más habitual para el diagnóstico de nematodos broncopulmonares y se basa en la migración activa y el movimiento de sus larvas de primer estadio (L1). Las muestras de tejido pulmonar se utilizan con frecuencia para el diagnóstico post mortem de estos parásitos, para saber el estatus parasitario de una población silvestre controlada, pero este tipo de muestra no siempre está disponible y no es fácil de obtener. Las muestras fecales representan una opción accesible para el monitoreo de parásitos, ya que suponen una forma no invasiva de diagnóstico, sin embargo, este tipo de muestra se utiliza sobre todo en rumiantes domésticos, con escasez de estudios en comunidades silvestres. Este trabajo se realizó con el objetivo de evaluar la concordancia entre los resultados obtenidos por la técnica de Baermann-Wetzel cuando se utilizan muestras de parénquima pulmonar o heces de rumiantes silvestres. El estudio demostró que del total de muestras analizadas, el 39,3% (*n* = 98) fueron positivas al utilizar tejido pulmonar y el 38,0% (*n* = 95) a las heces. No obstante, el 45,2% (*n* = 113) de los animales analizados resultaron positivos con al menos un método. Estos números sugieren una sensibilidad similar para ambos métodos (86,7% para tejido pulmonar y 84,1% para heces). En promedio, la carga parasitaria (intensidad parasitaria) fue de 61,4 + 306,1 LPG (larvas por gramo), con valores diferentes según la muestra utilizada: 47,3 + 256,8 LPG en tejido pulmonar y 14,3 + 66,2 LPG en heces. El análisis de las muestras positivas (*n* = 113), identificó 7 géneros diferentes de nematodos broncopulmonares: *Muellerius* spp. (61/113; 53,9%), *Cystocaulus* spp. (54/113; 46,9%), *Neostrongylus* spp. (39/113; 34,5%), *Elaphostrongylus cervi* (34/113; 30,0%), *Protostrongylus* spp. (22/113; 19,4%), *Varestrongylus* spps (14/113; 12,3%) y *Dyctiocaulus* spp. (11/113; 9,7%). Al comparar los dos origenes, tanto general como específicamente, se pudo observar un buen nivel de concordancia, así como una sensibilidad similar entre los dos tipos de muestra, validando el uso de muestras fecales como una alternativa menos invasiva y rentable para el seguimiento de la infección por nematodos broncopulmonares en poblaciones de rumiantes silvestres.

Los hallazgos obtenidos a lo largo de los tres capítulos que conforman esta Tesis Doctoral aportan una valiosa información que permite conocer mejor este sistema natural multihospedador y de multiparasitismo que existe entre estas cuatro especies de rumiantes silvestres en el sureste de la península ibérica, destacando la importancia de cada uno de estos hospedadores en el mantenimiento del ciclo biológico de los nematodos encontrados.

Aunque en pocas ocasiones son tenidos en cuenta, los animales silvestres forman parte de la salud global. Ahora mismo, el foco en la fauna silvestre se encuentra sobre las zoonosis, ya que representan más del 60% de las enfermedades infecciosas emergentes. Por esta razón, la conservación y protección de los animales silvestres a veces pasa a un segundo plano. Es fácil infravalorar o descuidar el estudio de enfermedades que afectan exclusivamente de sanidad animal. Otros autores recomiendan dejar de lado el enfoque tradicional de conservación, basado exclusivamente en el estudio de factores ecológicos y biológicos, y comenzar a integrar el concepto "One Health". De esta forma, sería posible considerar la salud de los animales (domésticos y silvestres), la del medio ambiente y la del ser humano, simultaneando el estudio de las posibles zoonosis y de las enfermedades exclusivamente animales para la conservación de la fauna silvestre. Como veterinarios, dentro de un equipo necesariamente multidisciplinar, es nuestro trabajo comprender este escenario para el futuro de la conservación de especies protegidas. De esta forma, el seguimiento y estudio multifactorial de comunidades silvestres puede servir para reducir el riesgo potencial de aparición de nuevas enfermedades parasitarias.

Además, considerando que algunas especies se encuentran en planes de conservación así como que el muflón supone una especies alóctona en expansión, se evidencia la necesidad de tener en cuenta la presencia de estos hervívoros como un factor de riesgo en la interfaz silvestre-doméstico. Los datos obtenidos podrán servir como base para la elaboración de programas de vigilancia epidemiológica de enfermedades de especial relevancia en la conservación de estos hervíboros o de otras especies, ya sean domésticas o silvestres, con las que pudieran entrar en contacto

Summary

In this Doctoral Thesis, the natural interaction that occurs in the helmitofauna of four species of wild ruminants present in southeastern Spain has been studied. In particular, it focuses on gastrointestinal and bronchopulmonary parasitic nematodes that are naturally present in multiple hosts. This phenomenon, called multi-host, is often found in combination with multi-parasitism (simultaneous infection with several parasitic species in the same host). There are many examples in the literature documenting the presence of parasitic species shared among a community of wild hosts. These studies have proven to be particularly useful in understanding the potential role of these pathogens on the dynamics of animal populations.

Both scenarios, multi-host/multi-parasitism, are described among host species that are close to each other, and among those that are taxonomically distant. It has been demonstrated that hosts that spend time in different habitats are more likely to acquire and spread multi-host parasites, which is why it is very interesting to study hosts that have wide freedom of movement and to analyze what role each one plays, in the transmission and dissemination of parasites. In the case of gastrointestinal and/or bronchopulmonary nematodes of wild ruminants, there are extrinsic factors that influence their ecology. For example, climatic conditions, which have a direct effect on both the nematode cycle and the availability of intermediate hosts.

In order to carry out these studies, it is necessary to find locations that allow to evaluate the multi-host/multi-parasite phenomenon in a natural way. An optimal study area to investigate this dynamic is the "Sierras de Cazorla, Segura and Las Villas Natural Park" (PNSCSV), a 2,140 km^2 mountainous area located on the eastern slopes of the Sierra Bética de Andalucía, in Spain. The study of the bronchopulmonary and gastrointestinal nematodes of wild ruminants to deepen the knowledge of the parasitic communities allows to evaluate their macroecology (relating the characteristics of the nematode population with the environmental, geological conditions, etc.) and to address aspects such as the polyparasitism and the environment-host-parasite relationship.

The first evaluated host was the Iberian ibex (*Capra pyrenaica*), a bovid found in the mountainous areas of Spain and northern Portugal and an endemic species of the Iberian Peninsula. The second bovid

included in our study is the European mouflon (*Ovis orientalis musimon*), an allochthonous ungulate, introduced in Central Europe and in Spain as well for hunting purposes. The red deer (*Cervus elaphus*), species present in the entire northern hemisphere, and the fallow deer (*Dama dama*), native to the Mediterranean region, constitute the last ungulates included in the study. These two species are cervids with high hunting value in the Iberian Peninsula.

The main objectives of this Doctoral Thesis are:

- to describe the epidemiological richness, prevalence, abundance and intensity of parasites, in a multi-parasite / multi-host context.
- to discuss the epidemiological role and transmission of these parasites, trying to explain the parasite community found from an ecological perspective.
- to validate the larval migration technique or Baermann method by comparing parasite presence and load in lung and fecal samples of the same animals, using the Blant-Altman analysis.

In **Chapter 1** the gastrointestinal nematodes of the four wild ruminants are studied from a multi-host point of view. This approach is particularly interesting for investigating the epidemiology behind multi-host/multi-parasite systems, for a better understanding of the complex dynamics that naturally occur in wildlife populations. To do this, the gastrointestinal tract of 252 wild ruminants of four different species in the PNSCSV was evaluated for parasites. Eighty-one point fifty-two of the animals analyzed were positive for parasitic infection and 29 species of nematodes were identified. *Spiculopteragia asymmetrica* (55.9%), *Oesophagostomum venulosum* (58.7%) and *Spiculopteragia quadrispiculata* (45.3%) turned out to be the most frequent parasites in this part of the study. Nematode prevalence differed significantly between host species. Fallow deer had the highest prevalence of nematodes (n = 109; 91.5%), followed by wild bovids (mouflon n = 59; 85.5%; Iberian ibex n = 20; 83.3%) and red deer (n = 64; 61.4%). In addition, the mouflon presented the highest parasitic richness, with the description of up to 12 different species in this host species. In this sense, our study represents a clear example of a multi-parasite/multi-host scenario, since one third of the nematodes are shared between at least two sympatric host species and only a few parasites are exclusive to a single host species. In this scenario, the structure of the parasite community was significantly influenced by the host, with the highest prevalence and richness of parasites consistently appearing in mouflon and fallow deer, suggesting a parasite dispersal point originated by the mouflon. The presence of specific patterns was evaluated by multivariate abundance analysis, which showed significant differences between the 11 species of nematodes shared by all host species. Clear patterns were observed both at the host species (fallow deer, Iberian ibex, mouflon and red deer) and at the host group levels (bovids vs cervids). These findings highlight the ability of parasites to adapt to the host community and shape their distribution according to the host species available.

Following the previously described line, in **Chapter 2**, the bronchopulmonary nematode community of the four wild ruminants are as well studied from a multi-parasite / multi-host point of view. The respiratory tracts of 252 specimens belonging to the same four host species (red deer, mouflon, Iberian ibex and fallow deer) were studied. Almost half (48.0%) of the animals were infected with bronchopulmonary nematodes. Seven different nematodes were identified, of which two genera (*Protostrongylus* spp. and *Dictyocaulus* spp.) and three species (*Cystocaulus ocreatus*, *Muellerius capillaris* and *Neostrongylus linearis*) were recorded in at least two ruminants, the mouflon being the most affected host. A significant effect of the host species and the sampling area was observed. From a spatial perspective, the highest abundance of parasites was detected in the central part of the study area. Additionally, the analysis identified an effect of age, with adult animals more likely to be heavily parasitized. In addition, two species were found parasitizing only one species: *Elaphostrongylus cervi* and *Varestrongylus sagittatus*; specifically, *E. cervi* was detected in the deer population with a high prevalence (46.3%), while *V. sagittatus* (13.9%) was found only in fallow deer. This study represents an interesting example of the structure and composition of the parasite community in a multi-host/multi-parasite setting. On the one hand, the mouflon is once again the epidemiological key in the natural network of interactions, showing a high prevalence and richness of bronchopulmonary nematodes that are shared with other wild ruminant species. On the other hand, the deer seems to be completely disconnected and isolated from the community of bronchopulmonary nematodes shared by the other three host species. The possible isolation of cervids in comparison with **Chapter 1** is probably due to an influence of climatic and environmental conditions, with the highest prevalence in the central part of the PNSCSV, where the largest population of mouflon is located.

Chapter 3 describes the validation of a diagnostic method for the infection with bronchopulmonary nematodes. The importance of this study lies in the fact that bronchopulmonary nematodes can have a negative impact on the conservation of wild ruminants, and the diagnosis of parasite presence is an important measure for disease surveillance and management at population level. The Baermann-Wetzel technique is the most common method for the diagnosis of bronchopulmonary nematodes, and it is based on the active migration and movement of their first stage larvae (L1). Lung tissue samples are frequently used for *post mortem* diagnosis of these parasites, to determine the parasitic infection status of a controlled wild population, but this type of sample is not always available nor easy to obtain. Faecal samples represent a more accessible option for monitoring parasites, since they represent a non-invasive form of diagnosis, however, this type of sample is used mainly in domestic ruminants, with less studies in wild communities. This work was carried out with the objective of evaluating the concordance between the results obtained by the Baermann-Wetzel technique applied to lung parenchyma or feces of wild ruminants. The study showed that of the total samples analyzed, 39.3% (n = 98) were positive when using lung tissue and 38.0% (n = 95) using feces. However, 45.2% (n = 113) of the animals tested were positive with at least one method. These numbers suggest a similar sensitivity for both methods (86.7% for lung tissue and 84.1% for feces). On average, the parasite load (parasitic intensity) was 61.4 + 306.1 LPG (larvae per gram), with different values depending on the sample used: 47.3 + 256.8 LPG in lung tissue and 14.3 + 66.2 LPG in feces . When the data were analyzed by species, from the positive samples (n = 113), 7 different genera of bronchopulmonary nematodes were identified: *Muellerius* spp. (61/113; 53.9%), *Cystocaulus* spp. (54/113; 46.9%), *Neostrongylus* spp. (39/113; 34.5%), *Elaphostrongylus cervi* (34/113; 30.0%), *Protostrongylus* spp. (22/113; 19.4%), *Varestrongylus* spp. (14/113; 12.3%) and *Dyctiocaulus* spp. (11/113; 9.7%). A good level of concordance could be observed, as well as a similar sensitivity, between the two types of samples, validating the use of faecal samples as a less invasive and cost-effective alternative for the follow-up of the infection with bronchopulmonary nematodes in wild ruminant populations. These results support the use and value of

feces as a non-invasive and cost-effective sampling technique for long-term studies, as well as for the contribution of the preservation and conservation of threatened wild ruminant populations.

The findings obtained throughout the three chapters that form this Doctoral Thesis provide valuable information allowing to better understand the natural multi-host and polyparasitic system in the southeast of the Iberian Peninsula, highlighting the importance of each host species in the maintenance of the biological cycle of the nematodes.

Although they are rarely taken into account, wild animals are part of global health. Right now, the focus on wildlife is on zoonoses, as they account for more than 60% of emerging infectious diseases. For this reason, the conservation and protection of wild animals sometimes takes a back seat. It is easy to underestimate or neglect diseases that exclusively affect animal health. Other authors recommend leaving aside the traditional conservation approach based exclusively on the study of ecological and biological factors, and starting to integrate the "One Health" concept. In this way, it would be possible to consider animals' health (domestic and wild), by also protecting the environment and the human health, allowing to simultaneously study possible zoonoses and animal diseases for the conservation of wild fauna. As veterinarians, within a necessarily multidisciplinary team, it is our job to understand this scenario for the future of the conservation of protected species. In this way, the monitoring and multifactorial study of wild communities can serve to reduce the potential risk of the appearance of new infectious diseases.

In addition, considering that some species are included in conservation plans as well as the mouflon is an allochthonous species in expansion, the need to take into account the presence of these herbivores as a risk factor in the wild-domestic interface is evident. The data obtained may serve as a basis for the development of epidemiological surveillance programs for diseases of special relevance in the conservation of these herbivores or other species.

Introduction

The work compiled in this Doctoral Thesis addresses part of the gastrointestinal and bronchopulmonary helminths parasitizing four species of wild ruminants at the Sierras de Cazorla, Segura and Las Villas Natural Park (PNCSV) in Southeast Spain: the Iberian ibex (*Capra pyrenaica*), the European mouflon (*Ovis orientalis musimon*), the red deer (*Cervus elaphus*) and the fallow deer (*Dama dama*). Out of the four species mentioned, two of them were introduced into the park for hunting purposes (the mouflon and the fallow deer) while the Iberian ibex and the red deer are part of the wild autochthonous species. The following introduction will briefly review the ecological role of each ruminant and the importance and consequences of monitoring their parasitic community.

Wildlife and One Health

The environmental health has a direct implication on the health of the human beings, as Hippocrates formulated more than 2,000 years ago in "*On Airs, Waters, Places*" (Jones, 1868). The term zoonosis was later recognized for diseases transmitted from animals to humans, and *vice versa*. In this sense, human and veterinary medicine were unified in the same epidemiological ideology (CDC, 2016). It was *Trichinella spiralis*, transmitted from the pig to humans, the parasite that inspired this term.

However, it was not until almost 150 years later that the relationship between the health of the environment, wildlife and humans was recognized. It was the veterinarian Calvin Schwabe, in 1964, who highlighted the importance of veterinarians in global health, mentioning for the first time the term One Health (Gibbs, 2014). The H5N1 influenza pandemic at the beginning of this century highlighted the need of a common approach of the Food and Agriculture Organization of the United Nations (FAO), the World Organization for Animal Health (OIE) and, the World Health Organization (WHO), to define and guide the One Health concept (Gibbs, 2014).

Many populations of wild animals are threatened by the appearance of new parasitic diseases (Aguirrea & Taborb, 2008). Their health is still a neglected topic in many countries and the prevention and control of parasitic diseases still need significant improvements (Thompson et al., 2010). The One Health concept is a first step, but there is still a need to broaden the ecological perspective of parasitic diseases as well as the relationship between species conservation and environmental health (Thomson et al., 2010), Figure 1.

Figure 1. One Health recognizes the interdependence of human, animal and environmental health, and that a holistic approach to the well-being of all will lead to improved health outcomes and enhanced resilience.
Source: University of Alaska Fairbanks https://www.uaf.edu/onehealth/

The outbreaks of new infectious diseases that put animals' health at risk are related to the introduction of animals and their parasitic communities into new environments (Wyatt et al., 2008). This phenomenon is called "*spillover*" and refers to the transmission of pathogens from one host to a second one that is not the usual host. In wildlife, this phenomenon can occur bi-directionally: from wildlife to domestic animals and humans and *vice versa* (Otranto & Deplazes, 2019; Thompson, 2013). In turn, we speak of "*spillback*" once a new pathogen is established in a given habitat, with reservoir and maintenance, allowing the re-infection of the original host populations (Thomson et al., 2010).

More than 60% of emerging infectious diseases are zoonoses (Mackenzie & Jeggo, 2013). For this reason, conservation and protection of wild animals sometimes takes a backseat due to population's reluctance about wildlife and its role in the transmission and maintenance of zoonoses (Decker et al., 2010). Authors such as Buttke et al. (2015) recommend leaving aside the traditional conservation approach, based exclusively on the study of ecological and biological factors, and starting to integrate the One Health concept. In this way, it would be possible to overlap the health of animals, environment and humans, trying to leave aside the exclusive study of zoonoses as a justification for the conservation of wildlife (Buttke et al., 2015). As veterinarians, in a multidisciplinary approach, it is our role to study this scenario for future biodiversity conservation actions (Decker et al., 2011). Therefore, the monitoring and multifactorial study of wild communities can be useful to conserve wildlife populations, preserve animal health and, reduce the appearance of new zoonoses.

Parasitic helminths

In parasitology, the term helminth is used to refer to parasitic worms that infect other species. In veterinary medical parasitology we can find the terms helminthiasis (worm infection), helminthology (clinical specialty) and anthelminthic (drugs to combat disease) as was first described by Dujardin (1845). It is a concept that encompasses many taxonomic groups, mainly composed by nematodes and flatworms (e.g. trematodes and cestodes).

Gastrointestinal nematodes

Gastrointestinal nematodes are common parasites of herbivores. The life cycle of these helminths is usually direct, with the hatching of eggs and different stages developing within the feces given favorable temperature and moisture conditions. Upon reaching the third larval stage (L3), nematodes migrate to the surrounding vegetation, where they will likely be ingested by grazing herbivores. Nonetheless, sometimes percutaneous infection is also possible in some species. This last scenario is observed, for example, in *Strongyloides stercoralis* (Page et al., 2018). Once inside the final host, larvae will migrate and will molt into the fourth stage (L4) in the abomasal and intestinal mucosa and develop into the adult stage (Taylor et al., 2007).

Gastrointestinal nematodes can exert a negative impact on wildlife. Previous studies have shown that gastrointestinal nematodes reduce food intake in parasitized cervids and/or cause severe lesions that can lead to reduced weight gain (Albon et al., 2002; Arneberg et al., 1996; Coltman et al., 1999; Gulland, 1992; Lavín et al., 1997).

Bronchopulmonary nematodes

Similarly, to gastrointestinal nematodes, bronchopulmonary worms frequently parasitize different animals, including wild ungulates (Panayotova-Pencheva, 2006). The biological cycle of bronchopulmonary nematodes differs depending on whether we are talking about genera belonging to the Dictyocaulidae family or the Protostrongylidae family. These parasites have either a monoxenous (parasites whose development is restricted to a single host species) or heteroxenous development (require an intermediate host, generally a terrestrial mollusk). Briefly, the first larval stage (L1) hatches within the definitive host and is dragged into the trachea where it will be swallowed and finally released with the feces. Outside, the two molts origin a L3, in the case of Dictyocaulidae, or they will penetrate a terrestrial mollusk where they will molt from L1 to L3 in the case of Protostrongylidae. In both cases the L3 are the infectious stages and are ingested by the definitive hosts where they will migrate from the intestinal mucosa to the mesenteric ganglia to make the third molt (to L4). These last larvae will reach the lungs, via the lymphatic and blood streams, and will lodge in the bronchioles, bronchi and trachea (Panuska, 2006).

Bronchopulmonary nematodes are widespread helminthiasis found to parasite different free-ranging wild ungulates (Panayotova-Pencheva & Alexandrov, 2010). Their presence has a direct negative impact on domestic animals and wildlife affecting their health and fitness (Gulland, 1992; Gunn & Irvine, 2003; Hoberg et al., 2001). Bronchopulmonary infections usually course as subclinical parasitism; however, they have also been associated to respiratory disorders (Panayotova-Pencheva and Alexandrov, 2010) and systemic symptoms as weight loss or abortions (Berrag & Urquhart, 1996; Jenkins et al., 2007; Kutz et al., 2001). Moreover, when combined with bacteria, viruses or intrinsic factors, like stress, bronchopulmonary helminthiasis is more likely to cause a pneumonia (Jenkins et al., 2007).

Sierras de Cazorla Natural Park

The PNCSV is the largest protected area in Spain, located in the province of Jaén, with an area of 214,300 ha, which includes 80,000 inhabitants distributed in 23 municipalities. It is one of the most visited natural areas on the Iberian Peninsula due to its scenic beauty and biological wealth[1]. The mountains have a heterogeneous orography integrated into the Prebetic system, approaching Sierra Morena from the west and with a maximum height of 2,107 m in Cerro las Empanadas. They are also the origin of the Guadalquivir and Segura rivers, two of the most important currents in Spain and the Iberian Peninsula (Boletín Oficial de La Junta de Andalucía - Boletín Número 246 de 27/12/2017, 2017; PORN, 2017).

In this last aspect, the hydrography of the mountainous complex is very diverse, with flooded natural caves, numerous streams and waterfalls, as well as various reservoirs. The park's flora includes the largest extension of pine forests in Spain, with an extensive representation of four of the six autochthonous species up to 900 m altitude, from which oak and gall oak forests thrive. In the more humid areas, it can be found yews and specimens of holly and ash trees, willows, poplars, reeds and jays on the riverbanks as illustrated in Figure 2. In addition, the presence of more than 30,000 ha of olive groves with the Sierra de Cazorla are remarked by their designation of origin. This diversity, of more than 1,300 cataloged species, makes the park one of the richest points of flora in the Mediterranean basin, being 34 species endemics to this territory (Boletín Oficial de La Junta de Andalucía - Boletín Número 246 de 27/12/2017, 2017; PORN, 2017).

Finally, this natural area is known for its hunting tradition since it was declared Cazorla-Segura National Hunting Reserve in 1960. Native animals such as deer, wild boar and Iberian ibex stand out above all. Its population has fluctuated in recent decades, remaining relatively stable in recent years. In addition to these, two more ungulates, the mouflon and the fallow deer, were introduced for hunting purposes and represent a large percentage of the herbivores present. The griffon vulture, the golden eagle and the lammergeier are among the birds present and, the Valverde lizard and, the viper stand out among the reptiles. However, the diversity of the park has been diminished by the extinction of autochthonous carnivores and herbivores, such as otters, genets, marten and foxes (Boletín Oficial de La Junta de Andalucía - Boletín Número 246 de 27/12/2017, 2017; PORN, 2017).

[1] In 1983 UNESCO already declared this area as a Biosphere Reserve and in 1987 it was recognized as a Special Protection Area for Birds.

Figure 2. Aerial view of Sierras de Cazorla Natural Park. Source: Junta de Andalucía

Wild ruminant hosts species

Red deer (*Cervus elaphus*)

The red deer, *Cervus elaphus*, (Agnarsson & May-Collado, 2008) is one of the ungulates with the largest natural extension in the northern hemisphere, since its presence ranges from Western Europe to Central Asia, including Corsica, Sardinia and North Africa (Whitehead, 1993) as illustrated in Figure 3. The genus *Cervus*, Linnaeus (1758), has up to 10 species and, within the *Cervus elaphus* species, up to 22 subspecies are known (Geist, 1998).

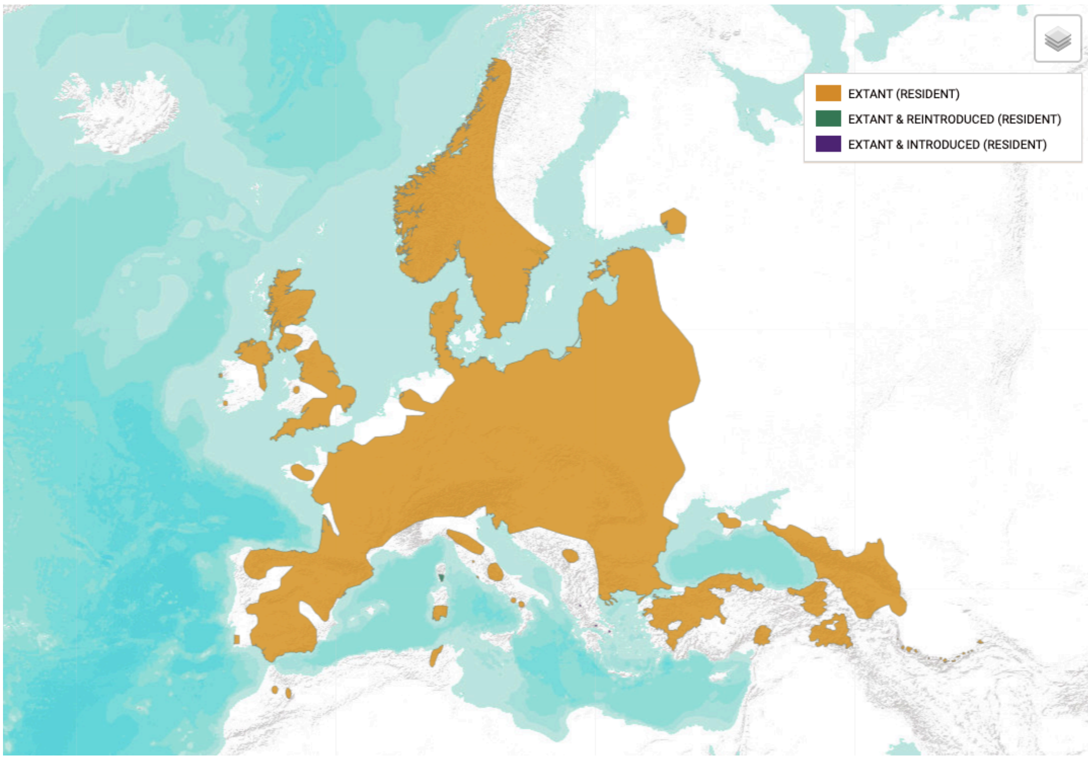

Figure 3. Current distribution of the *Cervus elaphus*. Source: The IUCN Red List of Threatened Species 2018

Cervus elaphus hispanicus (Hilzheimer, 1909) or Iberian deer inhabits the Iberian Peninsula, being a subspecies of the common deer (Dolan, 1988). Morphologically it can be differentiated from the rest of the subspecies by its smaller size, absence of a mane on the neck, grayish coat and anal shield less delimited by black hairs (Dolan, 1988). In addition of its particular morphology, its social organization, reproductive and, feeding behavior provide the Iberian deer enough distinction to justify a different taxonomic classification as subspecies (Carranza, 2004). Nonetheless, all cervids have marked sexual dimorphism in body weight highlighted by the antlers in males (Santiago-Moreno & López-Sebastián, 2010). However, larger body size in males is accentuated in higher latitudes, being significantly less in animals that inhabit the Mediterranean forest, notably influencing their ecology and behavior.

The Iberian subspecies experienced a decline during the first half of the 20th century, mainly due to excessive hunting (De Leyva, 2002), with only a few stable marginal populations left (Crespo-Guerrero, 2013). At the end of the 20th century, the implementation of specific hunting laws allowed this species to repopulate southern Spain, either naturally or through artificial introduction. Currently populations are stable in Spain and their hunting is controlled in order to avoid losses of genetic diversity (Galarza et al., 2015). *Cervus elaphus* and *Cervus elaphus hispanicus* coexist in the PNCSV.

Red deer have gregarious behavior and dusk activity with a social organization based on groups of individuals separated by sex, except during the reproductive season (Recuerda, 1984). In general, the size of the groups is variable with greater number during spring, where the groups of females reach up to 12 individuals, including their offspring (Dolan, 1988; Lincoln & Guinness, 1973). These female-grouped individuals live in a hierarchical matriarchal society (Asher et al., 1993). Although in most of subspecies males control harems, in the Iberian subspecies, due to the particularities of the Mediterranean environment, males defend territories in feeding areas and in the passage areas they intercept the females (Carranza et al., 1995; Carranza & Valencia, 1992). During the first autumnal weeks, males emit bellows to attract the attention of females and challenge other competing males. However, in the Iberian Peninsula, reed deer are all year long sexually active, with females presenting polyoestrous cycle (Caballero, 1985; Sanz & Rodriguez, 1993).

Deer feeding behavior on the Iberian Peninsula varies seasonally. Woody food is the one that is present throughout the year in their diet, reaching the maximum amount in winter and minimum in the warmer months when herbaceous and grasses become available and more important in the diet (Garin et al., 2001).

Fallow deer (*Dama dama*)

The fallow deer, *Dama dama*, is a cervid native to the Mediterranean region and was, therefore, reintroduced on the Iberian Peninsula (Masseti & Mertzanidou, 2008). Currently, its population is not at risk but there are conservation plans adapted to this species. This ruminant is smaller in size than red deer and males have shovel-shaped (palmated) antlers (Reinken, 1990). In addition, this species presents variation of the coat color between seasons, with the characteristic display of a white mottling on a reddish coat in summer that disappears in winter (Braza, 2002).

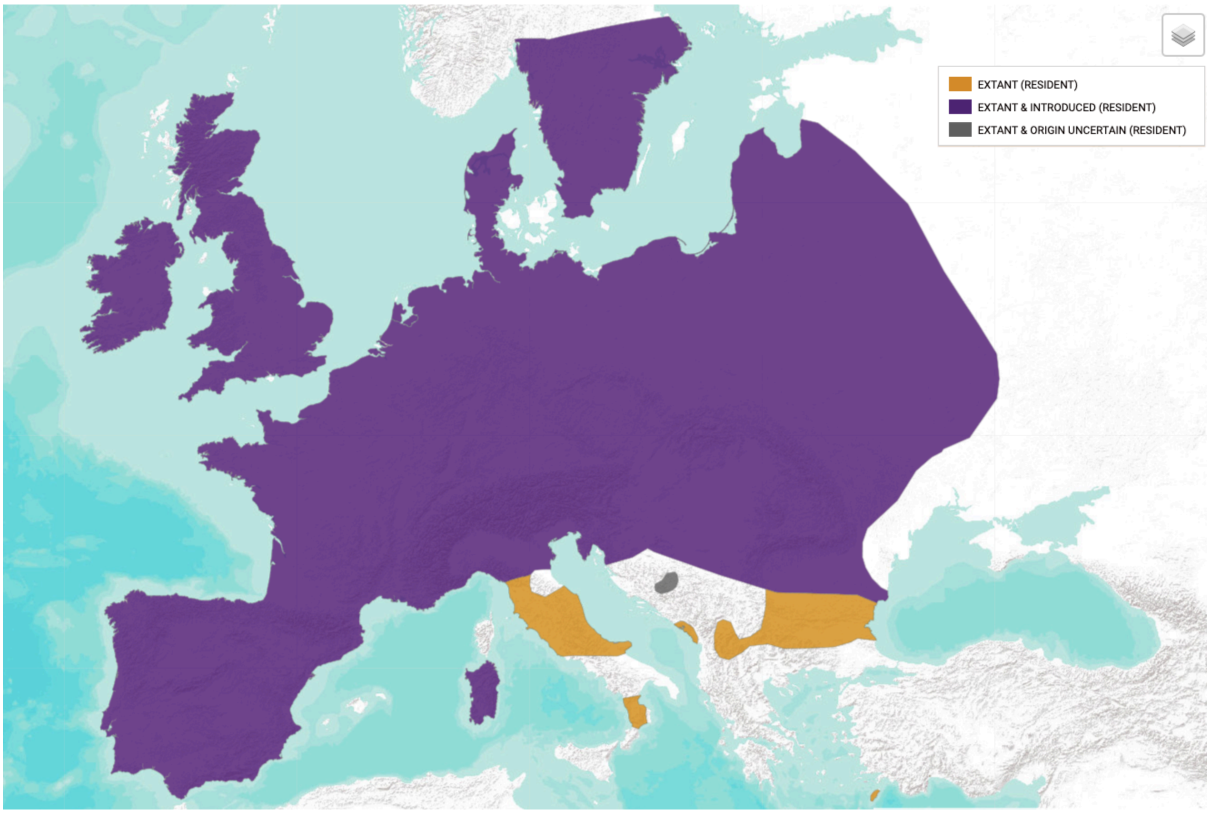

Figure 4 Current distribution of the *Dama dama*. Source: [The IUCN Red List of Threatened Species 2018]

This ruminant has been reintroduced in Europe, as illustrates Figure 4. The latter species, *Dama dama*, is the result of an evolutionary radiation that took place during mid-Pliocene, distributing it throughout Mediterranean and central Europe, including Turkey and Iran (Mena & Molera, 1997; Reinken, 1990). The reason for its extinction in Europe, between 10,000- and 30,000-years BC, has been linked to the Würm glaciation, which led to the migration of the species to warmer areas (Braza, 2002). For their reintroduction in Europe, specimens were transferred from the Mesopotamian region of the Middle East, between the Tigris and Euphrates rivers (Rodríguez-Berrocal, 1993). Currently there are two subspecies, the European fallow deer (*Dama dama dama*) and the Persian fallow deer (*Dama dama mesopotamica*) as described by Reinken et al. (1990).

From 300 BC to the 15th century reintroductions of fallow deer have been recorded, mainly as an ornamental animal, which is why the fallow deer could be attributed as foreigner species in Spain. In 1914 this cervid was exclusively found at the basin of the river Tagus and in the Toledo's forests (revised in Santiago-Moreno and López-Sebastián, 2010). However, it was later distributed in the Doñana National Park, the PNCSV, Cuenca, Navarra and Asturias (Sueve National Reserve), and, Monte de El Pardo (Madrid), as described by Mena & Molera (1997). In recent years, fallow deer have gained greater interest as a game species in Spain, especially as part of managed programs in order to avoid food competition with other populations of wild ruminants. Presently, its census is around 8,000 animals, half of which are located in national or natural parks (Notario-Gómez, 2002; Reinken, 1990). This ruminant lives in groups of less than 10 individuals, although it can also be found alone. Males join these groups during the mating season. Females are mainly seasonally polyestric, photoperiodically regulated (Baker, 1973). Similarly to red deer, their reproductive cycle might vary depending on latitude, in Spain their reproductive seasonality begins in October with a duration of 101-106 days (Mena and Molera, 1997; Santiago-Moreno et al., 2005).

This species lives optimally in deciduous forests, feeding mostly on herbaceous vegetation (up to 95% in spring) and to a lesser extent on leaves of trees and shrubs, and on fruits such as juniper and sloe (Asociación sierra de Baza, 1998).

Mouflon (*Ovis orientalis musimon*)

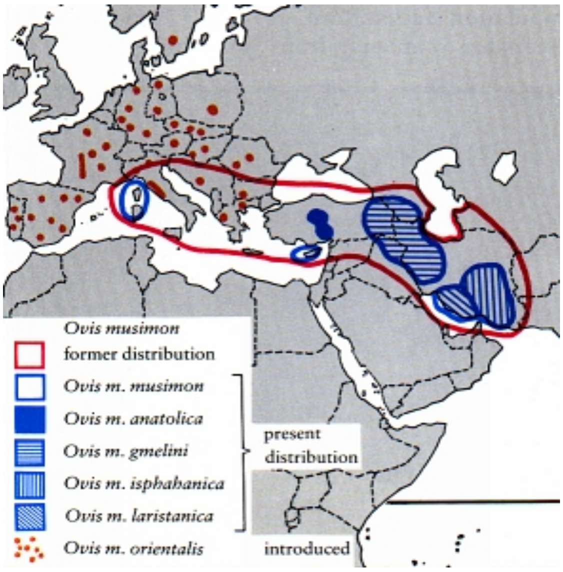

Figure 5. Current distribution of the *Ovis orientalis musimon.* Source: Jagen Weltweit

The genus *Ovis* appeared more than 2 million years ago in Asia and was very common in Eastern Europe, but rare in Central and Western Europe (Kordos, 2000). The presence of fossils has made it possible to identify the mouflon in Europe for 400,000 years, whose extinction was caused by the hunting pressure and the last glaciation. Approximately 8,000 years ago, the *Ovis gmelini gmelini* was introduced in Corsica and Sardinia and from the 18th century, in the European continent (Helmer, 1992; Masala et al., 1991; Vigne, 1992).

The marked polymorphism of this genus complicates the taxonomic situation in this species, for which the European mouflon has received different descriptions: *Ovis musimon, Ovis ammon musimon, Ovis gmelini musimon, Ovis aries, Ovis gmelini musimon* x *Ovis* sp., *Ovis orientalis musimon* (Santiago-Moreno et al., 2004; Rodríguez-Luengo et al., 2002, 2007). Although some authors recommend its name as *Ovis aries* (Valdez, 2008), it has been demonstrated by molecular genetics that the European mouflon is a subspecies of *Ovis orientalis* (Rezaei et al., 2010). Furthermore, as the term "*musimon*" has been widely used, it has been chosen to keep the name *Ovis orientalis musimon* Pallas 1762 (Corbet, 1978).

Currently, mouflon's population is estimated to exceed 100,000 individuals since their introduction in Spain in 1953 (Niethammer, 1963; Weller, 2000). The European mouflon is one of the smallest wild *Ovis* species described[2]. Males have large-spirally horns whereas females are either polled or have small horns (Asdell, 1946; Pfeffer, 1967). The color, length and thickness of the coat varies seasonally, being darker in winter, females have always lighter coat color (Santiago-Moreno & Toledano-Díaz, 2004).

Mouflons show pronounced reproductive seasonality, from October to March with higher activity in the months of October to December (Santiago-Moreno et al., 2001). Females do not reach reproductive maturity until the second year and males until the fifth. Gestation lasts 155 days and usually birth takes place in spring, usually with 1-2 offspring. This animal is diurnal and social, usually living in small family groups of females and their offspring, with males joining groups during the mating season but living lonely the rest of the year (Santiago-Moreno & Toledano-Díaz, 2004).

In winter, they are usually found in open spaces to avoid large amounts of snow. During this season in the PNCSV mouflons feed on grasses, herbs, shrubs and trees, among others. However, in spring, their diet is based on grasses combined with shrubs and trees (Martínez & Fandos, 1989).

[2] It has a height at the withers of 65-75 cm in females with a weight of 30 kg and 70-80 cm in males, weighing 55 kg.

Iberian Ibex (*Capra pyrenaica*)

From an evolutionary point of view, the genus *Capra* appeared in the upper Eocene (Schaller, 1977). Regarding the origin of the *Capra pyrenaica* species, there are two different theories: the first suggests that it appeared after the Würm glaciation, 15,000 years ago, when *C. pyrenaica* differed from *C. caucasus* in the French Pyrenees (Pérez et al., 2002). The second theory is based on *C. ibex* species being the original line, originated from the Alps (Pérez et al., 2002). Nonetheless, it seems possible that the Iberian ibex occupied almost the entire Iberian Peninsula between the Paleolithic and the Neolithic (Alados & Escos, 1985).

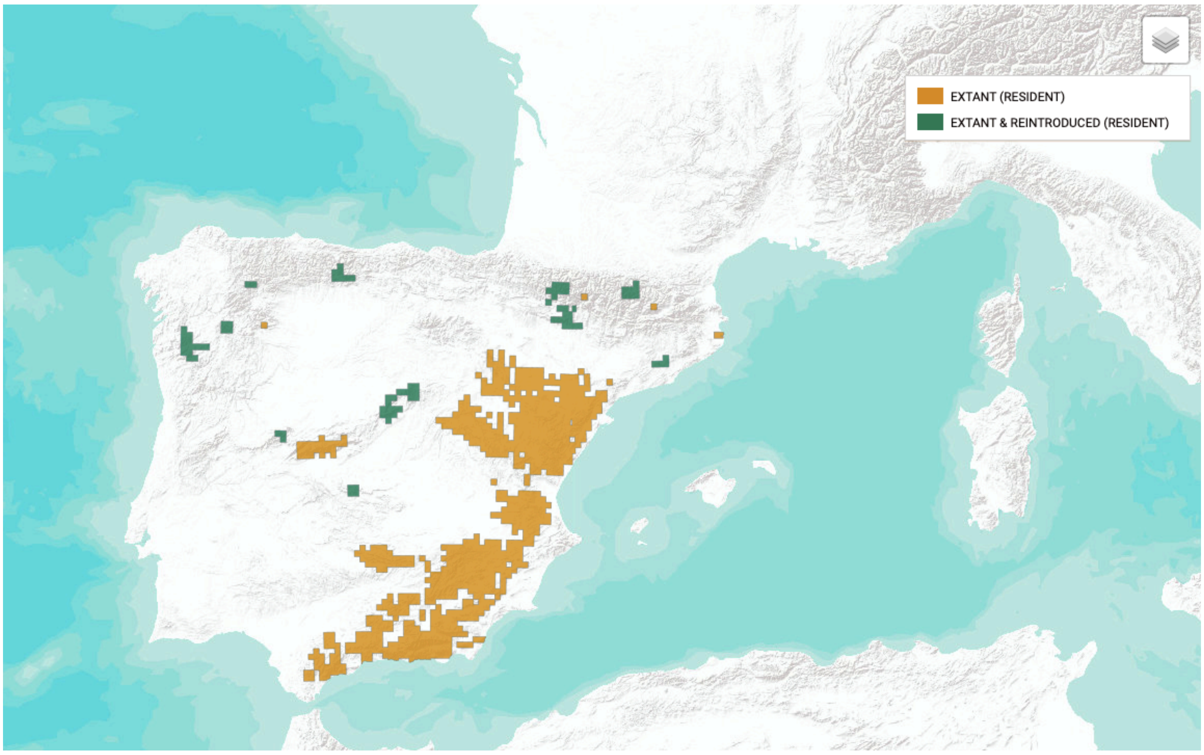

Figure 6. Current distribution of the *Capra pyrenaica*. Source: The IUCN Red List of Threatened Species 2018

In 1911, four subspecies of *C. pyrenaica* were described based on morphological criteria. However, due to the weakness of the argument for this classification (based on environmentally dependent morphotypes), it was later established the presence of a single species with different ecotypes or local variations (Couturier, 1962). The current Iberian ibex population is distributed as illustrated in Figure 6.

In the past centuries, its population decreased significantly due to different reasons: on the one hand, the human hunting pressure, mainly because of its uniqueness constituting a very popular hunting trophy; on the other hand, the sarcoptic mange, an ectoparasitic infection, was a determining factor for the population decline. This disease was particularly decisive in some specific areas, such as the PNCSV (Iacopelli et al., 2020; Sarasa et al., 2010). For this reason, since the beginning of the 19^{th} century conservation plans have been implemented, resulting in an increase of the population, currently estimated in 50,000 animals distributed throughout the mountains and natural parks of the Iberian Peninsula, including the PNCSV.

Similarly, to previously described for the mouflon, Iberian ibex lives in groups of individuals made up of females and their 1 or 2-year-old offspring (Alados and Escós, 1985). Dominance in the group is directly related to the fertility of the individual up to levels in which the most docile females may have ovulatory activity inhibited (Santiago-Moreno et al., 2007). In males, as for the mouflon, reproductive activity begins in October, followed by a fertile period, from December to January (Santiago-Moreno et al., 2007).

The Iberian ibex is mainly found in the mountainous areas of Spain and northern Portugal (Sarasa et al., 2010), especially between 500 and 2,500 m, reaching higher areas during summer. Maximum activity takes time during the early morning or at twilight, although in winter can be seen when the temperature arises. As the rest of the previously described ruminants, their diet varies with seasonal availability, being more herbaceous in spring and shrubbier in winter, when this species digs under the snow to look for food (Granados et al., 2001; Martínez, 1988).

Wild ruminants and parasitic nematode infections

Commonly, a single host is infected by several nematode species. Additionally, it is quite common to find the same parasites in different host species. This might result from faecal egg dispersal and from the same resources being shared by multiple hosts. Thus, parasite transmission and infection rates involve highly stochastic dynamics including intrinsic and extrinsic factors such as host immune status, behavior or climate conditions (Agosta et al., 2010; Agosta & Klemens, 2008; Hoberg & Brooks, 2008).

These interactions have increased interest on how parasite exchanges between host species may affect closely related hosts (Morgan et al., 2004; Obanda et al., 2019), a process that becomes most notorious in natural ecosystems where different host species share the same habitat.

Wild animals are more likely to host a large number of different parasite species compared to domestic animals due to the greater exposure to pathogens, which results from sharing natural spaces with other possible hosts, and to the absence of anti-helminthic treatments (Bordes et al., 2009). From this perspective, the PNSCSV offers a perfect setting for the study of a multi-host/multi-parasite system, since it is a large natural area with four sympatric wild ruminants that can also interact with domestic livestock. Despite this, to date the interactions between parasites in natural populations of different hosts have not been studied in this area. Ecological interactions and their implications in polyparasitism and multi-host coinfection must be well understood in order to establish hypotheses that explain the richness of the parasite species and the relationships established between them and with the host (Craig et al., 2008; Morand, 2015). As a consequence, the importance of interspecific parasite interactions in determining the structure of parasite communities in natural populations remains little explored terrain, although the few available studies highlight the role of various factors (Coltman et al., 1999; Gulland, 1992; Telfer et al., 2010).

The impact of nematodes in wildlife is known, and previous studies have already shown that nematodes exert a wide range negative effect on these animals, spanning from reducing food intake in parasitized cervids to causing serious injuries such as abortions or even death (Albon et al., 2002; Arneberg, 2001; Coltman et al., 1999; Gulland, 1992; Lavín et al., 1997). For this reason, specific studies must be carried out to understand the implications of nematode presence in wild ruminants' populations. Among other reasons, these studies are necessary to understand to what extent nematode infection is involved in population dynamics. A recent example on the same study area is represented by sarcoptic mange in ibex and its long-term implications for this species (Iacopelli et al., 2020).

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Project Goals

The Doctoral Thesis will provide knowledge to implement accurate and species-specific management programs based on the parasite community found in wild ruminants at the PNCSV. The goals involve to study, analyze, evaluate and, ultimately, publish the findings concerning the nematode community and the related ecological patterns of infection of the gastrointestinal and bronchopulmonary nematodes of the wild ruminants at the PNCSV.

First project goal – Epidemiological study of gastrointestinal nematodes

This part of the Doctoral Thesis focuses on the multi-parasite/multi-host phenomenon of the gastrointestinal nematodes. As previously mentioned, this phenomenon usually occurs under natural conditions due to the proximity between host species and the existence of parasitic fauna shared among them. Given the ecological value of studying this phenomenon among naturally parasitized wild ungulates, the goal is to compare the prevalence, intensities and abundances of parasites between hosts, emphasizing the factors related to the closeness of families (bovids and cervids), shared habitat and ecological characteristics.

The results of this study will help to understand the role of wild deer and Bovidae in the transmission and maintenance of some species of parasites under natural conditions. Certain parasites are also shared by domestic ruminants, therefore, this epidemiological study of parasitic species shared between domestic and wild animals may be useful to improve the health security of local livestock as well as the wildlife management of the PNCSV.

Second project goal – Epidemiological study of bronchopulmonary nematodes

After achieving the first objective, the goal is to extend the study to the bronchopulmonary nematodes in the same PNCSV wild ruminant community. The gastrointestinal system is one of the most commonly analyzed, but of equal relevance is the bronchopulmonary one. Comparison between gastrointestinal and bronchopulmonary parasite community will be made to offer a wider perspective of the multi-parasite/multi-host phenomenon. The evaluation of extrinsic risk factors will be added in order to evaluate the possible interaction host-parasite-environment.

Third project goal – Comparison and concordance analysis of the sensitivity of the Baermann-Wetzel technique

Due to the wide availability of lung and faecal samples, the third goal is to evaluate the concordance between the results of the larval migration technique as it is elaborated with samples of faecal or pulmonary origin, using the correlation of the larval intensities found within the margin of the statistical set using the Bland-Altman analysis. This part of the project represents a novel approach to understand the sensitivity of the technique based on sample availability, with the impact that this may have on parasitic monitoring and control programs.

Chapter 1: Epidemiological approach to nematode polyparasitism occurring in a sympatric wild ruminant multi-host scenario[3]

Abstract

The epidemiology behind multi-host/multi-parasite systems is particularly interesting to investigate for a better understanding of the complex dynamics naturally occurring in wildlife populations. We aimed to approach the naturally occurring polyparasitism of gastrointestinal nematodes in a sympatric wild ruminant scenario present in south-east Spain. To this end, the gastrointestinal tract of 252 wild ruminants of four different species (red deer, *Cervus elaphus*; mouflon, *Ovis aries musimon*; Iberian ibex, *Capra pyrenaica* and fallow deer, *Dama dama*) were studied in Cazorla, Segura y Las Villas Natural Park (Andalusia, Spain). Of the analysed animals, 81.52% were positive for parasite infection and a total of 29 nematode species were identified. Out of these, 25 species were detected in at least two host species and 11 parasitized all ruminant species surveyed. The multi-host interaction between these nematodes and

[3] This work has been published in the Journal of Helminthology. **CARRAU, T.**, Martínez-Carrasco, C., Garijo, M., Alonso, F., Vizcaíno, L., Herrera-Russert, J., Tizzani, P., Ruiz de Ybáñez, R. (2021). Epidemiological approach to nematode polyparasitism occurring in a sympatric wild ruminant multi-host scenario. *Journal of Helminthology, 95*, E29. doi: https://doi.org/10.1017/S0022149X21000183

Chapter 2: Multivariate abundance analysis of multi-host/multi-parasite lungworms in a sympatric wild ruminant population [4]

Abstract

In the analysis of a multi-host/multi-parasite system and its associated risk factors, it is particularly interesting to understand the natural dynamics among pathogens, their hosts, and the environment in wildlife populations. This analysis is particularly feasible in a scenario where multiple overlapping host populations are present in high densities, along with a complex community of parasites. We aimed to describe and analyze the naturally occurring lungworm polyparasitism in a wild ruminant community in Southeast Spain. The respiratory tracts of 250 specimens belonging to four different species (red deer, mouflon, Iberian ibex, and fallow deer) were studied. Almost half (48.0%) of the animals were infected with bronchopulmonary nematodes. Seven different nematodes were identified of which two genera (*Protostrongylus* spp. and *Dictyocaulus* spp.) and three additional species (*Cystocaulus ocreatus*, *Muellerius capillaris*, and *Neostrongylus linearis*) were recorded in at least two ruminants, with the mouflon as the commonest host. Our study shows a significant effect of host species and sampling area, plus a marginal effect of age, on parasite multivariate abundance at the host population level. Mouflon and adults of all hosts appear to carry the highest parasite load on average. From a spatial perspective, the highest parasite abundance was detected at the central part of the park.

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[4] This work has been published in the Journal of Helminthology. **CARRAU, T.,** Martínez-Carrasco, C., Garijo, M., Alonso, F., Ruiz de Ybáñez, R., & Tizzani, P. (2021). Multivariate abundance analysis of multi-host/multi-parasite lungworms in a sympatric wild ruminant population. *Diversity*, *13*(6) 227. https://doi.org/10.3390/d13060227

Chapter 3: Evaluation of the Baermann–Wetzel method for detecting lungworm larvae in wild ruminants from faecal samples[5]

Abstract

Lungworms can exert a negative impact on wild ruminant fitness; for this reason, the diagnosis of the associated diseases is an important prevention measure. The Baerman–Wetzel technique is the most usual method for the diagnosis of bronchopulmonary nematodes and is based on the active migration and movement of their first-stage larvae. Pulmonary tissue samples are frequently used for the post-mortem diagnosis of these parasites, but this kind of sample is not always available and easy to obtain. Faecal samples represent a more accessible choice for parasite monitoring. This work aimed to evaluate the agreement between the results obtained by the Baermann–Wetzel technique when samples of lung parenchyma or faeces from wild ruminants are used. A good level of agreement as well as a similar sensitivity between the two types of sample were observed, validating the use of faecal samples as a less invasive and cost-effective alternative for the monitoring of lungworm in wild ruminant populations.

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General discussion

Although wild animals are rarely taken into account in surveillance and monitoring programs, they belong to global health. Currently, the main focus on surveillance in wildlife is on zoonoses, since they represent more than 60% of the emerging infectious diseases in humans (Mackenzie & Jeggo, 2013). For this reason, sometimes, the conservation and protection of wild animals takes a back seat; in particular the study of diseases that exclusively affect animal health is often underestimated or neglected (Decker et al., 2010). Under a biodiversity conservation perspective, there is a need of leaving aside the traditional approach, based exclusively on the study of ecological and biological factors, and starting to integrate the “One Health” concept. In this way, it would be possible to consider as a whole the health of animals (domestic and wild), environment and humans, including in a unique perspective the study of both zoonotic diseases of public health importance and animal diseases relevant for biodiversity conservation (Buttke et al., 2015).

As previously discussed, wild animals are more likely to be affected by a larger number of pathogens, including parasites, compared to domestic animals due to greater exposure and the absence of pharmacological treatments (Bordes et al., 2009). The work compiled in this Doctoral Thesis offers a novel perspective on pathogens surveillance in wildlife, based on the multi-host/multi-parasite system of PNSCSV, a large natural area with four sympatric wild ruminants interacting with livestock. **Chapter 1** described that 29 gastrointestinal nematode species were found and 81.52% of the analyzed ruminants were parasitized at least in one gastrointestinal section. From these species, 11 were shared among all host species. **Chapter 2**, focused on the bronchopulmonary findings, describing 7 nematode genera with a prevalence of 48.0%. Also in this case multi-host parasites were described, with up to 5 being present in more than one host. In both gastrointestinal and bronchopulmonary scenarios, the mouflon seemed to act as common link between host species, being important in the dissemination of the different parasites in the habitat.

“*Spill over*” events of infectious and emerging diseases that threat animal health are usually related to the introduction or reintroduction of new animal species and their pathogens in an area, against which autochthonous wildlife might be more susceptible (Wyatt et al., 2008). An example is represented by the introduction of Asian ruminant the Sika deer (*Cervus nippon*) in Europe, along with their respective parasitic community that infected autochthonous wildlife. The exotic *Ashworthius sidemi* nematode, hosted by Asian ruminants is now commonly found in the European bison from the Bialowieza forest (Demiaszkiewicz et al., 2009). Additionally, the introduced host species are excellent reservoir and transmitters of "local" parasites, since they are no longer exposed to their "original" parasitic species (Dunn et al., 2012; Osinska et al., 2010).

This phenomenon has been studied and highlighted by the results described in the **Chapters 1** and **2** of this Doctoral Thesis, in the PNSCSV mouflon population. This wild ruminant was introduced in Spain in 1975 for hunting purposes (Cassinello & Salvador Milla, 2017). When animal species are introduced into a new ecosystem, their reproductive success, the colonization of a new habitat, and the occupation of existing ecological niches can threat local biodiversity (Hulme et al., 2009; Kelly et al., 2009). The mouflon has well adapted to its new habitat in the Iberian Peninsula (Cassinello and Salvador Milla, 2017) and shares pastures in winter and spring with other wild bovids, such as the ibex (Martínez & Fandos, 1989), and in summer with wild cervids, such as red deer (Miranda et al., 2012). In our study area the mouflon might act as a crucial node in the maintenance of the parasitic network, acting as a link between wild and domestic ruminants housed in small farms located in the peripheral zone of the PNSCSV. Nonetheless, further and more exhaustive analysis must be carried out in order to draw more robust conclusions on the role of the mouflon in the local parasitic dynamics.

The multi-host/multi-parasite system and parasite richness described for the gastrointestinal and bronchopulmonary nematode communities have shown to be different. While up to 29 different nematode species were identified in **Chapter 1** only 7 were found in **Chapter 2**. Additionally, one third of the species were shared among all hosts for the gastrointestinal parasites while most of the bronchopulmonary nematodes were shared only between bovids. This notable difference between gastrointestinal and bronchopulmonary polyparasitism might be due to several factors affecting directly or indirectly parasite richness. On the one hand, feeding behavior play a role in the infection, since considering the life cycle of bronchopulmonary nematodes, it modulates the risk of animals to enter into contact with the infective larvae. Under this perspective, it should be noted that the mouflon is the only of the four wild ruminant hosts classified as a grazer, while the other host species are classified as “intermediate feeders” (Redjadj et al., 2014). Therefore, the access to potentially infected intermediate hosts is different. The type of feeder factor affects equally the infection with gastrointestinal nematodes, as a dichotomic pattern between cervids and bovids was also observed in **Chapter 1**. On the other hand, this phenomenon might be accentuated by the climatic and ecologic conditions needed for the lungworm development (Alasaad et al., 2009; Cabaret et al., 1980), that can be even more intensified when gastropods are involved in parasite life cycles (e.g. protostrongylids) (Handeland & Slettbakk, 1994; Vicente et al., 2005, 2006). For this reason, prevalence and intensity of infection of *N. linearis*, *M. capillaris* and *C. ocreatus* increases when relative humidity and rainfall increase, and decreases when the temperature decreases (Cabaret et al., 1980). This pattern was studied in **Chapter 2** by evaluating the differences among sampling sites, that shown the presence of clusters in parasite abundance distribution, with the highest values localized in the central part of PNSCSV, suggesting an influence of the climatic and environmental conditions on the parasite community.

Bronchopulmonary and gastrointestinal nematode presence can imply negative impact on domestic and wild animals (Gulland, 1992; Gunn & Irvine, 2003; Hoberg et al., 2001). Gastrointestinal nematodes may reduce food intake, hence reducing weight gain (Albon et al., 2002; Arneberg et al., 1996; Coltman et al., 1999; Gulland, 1992; Lavín et al., 1997). Bronchopulmonary nematode infection usually presents subclinical, however it has also been associated with respiratory disorders (Panayotova-Pencheva & Alexandrov, 2010) and systemic disorders such as weight loss or abortions (Berrag & Urquhart, 1996; Gunn & Irvine, 2003; Lavín et al., 1997). Risk of concomitant bacterial infection has already been reported when wild ruminants are infected with protostrongylid. Jenkins et al. (2007) described parasitic protostrongylid infection in wild Dall's sheep associated with bacterial pneumonia: *Arcanobacterium pyogenes*, *Pasteurella* sp., and *Mannheimia* sp.. **Chapter 3** compared the performance of the Baermann–Wetzel technique applied to two types of sample (pulmonary tissue and feces). In the literature, many studies in wildlife are based on results obtained using the Baermann–Wetzel technique on lung tissue samples while the diagnosis of lungworm infection in livestock is usually carried out using faecal samples (Díez-Baños et al., 1994; Panadero et al., 2001; Pyziel, 2014; Viña et al., 2013).

The objective of this chapter was to evaluate the possibility to use a less invasive sampling approach for the monitoring of bronchopulmonary parasites in wildlife. In **Chapter 3,** results showed the accuracy and agreement of the results obtained with the two types of samples. The approach described in the previously mentioned chapter combined both kinds of sample from the same animal. Results presented here are helpful to understand when and under which circumstances the Baermann–Wetzel technique can be applied in non-invasive sampling schemes to evaluate the status and dynamics of lungworms in free roaming ruminant populations. In general, the results highlighted a consistent agreement in the diagnostic results obtained with the two sampling approaches. In particular, this agreement was highlighted for two lungworm genera, *Neostrongylus* spp. and *Muellerius* spp., suggesting that for these two genera in particular it might be possible to use both pulmonary and faecal samples for a proper sanitary surveillance. These results agree with Diez-Baños & Campillo (1990) who reported similar finding for the chamois.

To sum up, to date the interactions between parasites in natural populations of different hosts have not been studied in this area. Ecological interactions and their implications in polyparasitism and multi-host coinfection must be well understood in order to establish hypotheses that explain the richness of parasite species and the relationships established at parasite community, with the host and the environment (Craig et al., 2008; Morand, 2015). The possible implications of parasite presence and dynamics for wildlife conservation can be studied through a proper monitoring, including the diagnosis of lungworm and gastrointestinal nematodes. The work highlighted in **Chapter 3** shows the possibility to use non-invasive and cost-effective sampling technique for parasite surveillance. Future work focusing on long-term investigation of spatial and temporal dynamics of parasite communities should be carried out. Nonetheless, the work presented in this Doctoral Thesis represent a baseline to be considered for the planning and implementation of wild ruminant management projects.

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Conclusions

FIRST. High prevalence and abundance of gastrointestinal nematodes is present in the wild ruminant community in the PNSCSV. Approximately one third of the isolated nematodes was present in all the hosts, showing a significantly host-influenced dichotomous distribution pattern between bovids and cervids. Similarly, lungworm presence, prevalence and abundance within the same wild ruminant community in the PNSCSV was described in almost half of the surveyed community. Lungworms also presented dichotomous distribution pattern between bovids and cervids being significantly host-influenced.

SECOND. Mouflon presented the highest richness and prevalence of isolated and identified nematodes (for both bronchopulmonary and gastrointestinal parasites) sharing its parasite community with bovids and cervids. This wild ruminant might act as a crucial node in the maintenance of the parasitic network in the PNSCSV.

THIRD. There is an agreement of pulmonary versus faecal samples for the Baermann-Wetzel diagnostic methods for lungworm infection, indicating that both types of samples can be used without losing sensitivity and accuracy. Therefore, feces screening is a valuable, non-invasive and cost-effective sampling technique for long term studies as well as for the preservation and conservation of threatened wild ruminant populations.

Conclusiones

PRIMERA. Existe una alta prevalencia y abundancia de nematodos gastrointestinales en la comunidad de rumiantes silvestres del PNSCSV. Aproximadamente un tercio de los nematodos aislados se hallaron en todos los hospedadores evaluados, lo que muestra un patrón de distribución dicotómico entre los bóvidos y los cérvidos que está significativamente influenciado por el hospedador. La presencia, prevalencia y abundancia de nematodos broncopulmonares dentro de la misma comunidad de rumiantes silvestres en el PNSCSV se describió en casi la mitad de la comunidad analizada. Los nematodos broncopulmonares también presentaron un patrón de distribución dicotómico entre los bóvidos y los cérvidos que con diferencias significativas entre hospedadores.

SEGUNDA. El muflón presenta la mayor riqueza y prevalencia de nematodos aislados e identificados (para parásitos broncopulmonares y gastrointestinales) compartiendo su comunidad de parásitos con bóvidos y cérvidos. Este rumiante silvestre podría ser un nodo crucial en el mantenimiento de la red parasitaria en el PNSCSV.

TERCERA. Existe concordancia entre las muestras pulmonares versus las fecales para el método de diagnóstico de Baermann-Wetzel utilizado para detección de larvas de nematodos broncopulmonares, lo que indica que ambos tipos de muestras pueden usarse sin perder sensibilidad y precisión. Por lo tanto, el examen de heces es una técnica de muestreo valiosa, no invasiva y rentable para estudios a largo plazo, así como para la preservación y conservación de poblaciones de rumiantes silvestres amenazadas.