Ecoepidemiology of Rabbit Haemorrhagic Disease (RHD) in the European Wild Rabbit (*Oryctolagus cuniculus* L.) in the Central West Iberian Peninsula

Ecoepidemiología de la enfermedad hemorrágica del conejo (EHC) en el conejo de monte (Oryctolagus cuniculus L.) en el Centro Oeste de la Península Ibérica

TESIS DOCTORAL

Universidad de Extremadura
Centro Universitario de Plasencia
Dpto. Ingeniería del Medio Agronómico y Forestal

TOMÁS MERCHÁN SÁNCHEZ

2014

UNIVERSIDAD DE EXTREMADURA

DEPARTAMENTO DE INGENIERÍA DEL MEDIO AGRONÓMICO Y FORESTAL

GRADO EN INGENIERÍA FORESTAL Y DEL MEDIO NATURAL

CENTRO UNIVERSITARIO DE PLASENCIA

TESIS DOCTORAL:

Ecoepidemiología de la enfermedad hemorrágica del conejo (EHC) en el conejo de monte (*Oryctolagus cuniculus* L.) en el Centro Oeste de la Península Ibérica

Ecoepidemiology of Rabbit Haemorrhagic Disease (RHD) in the European Wild Rabbit (Oryctolagus cuniculus L.) in the Central West Iberian Peninsula

Tesis presentada porTomás Merchán Sánchez para optar al título de
Doctor en Veterinaria con Mención Doctor Europeo
por la Universidad de Extremadura.
Dirigida por Dr. Gregorio Rocha Camarero

Thesis submitted by Tomás Merchán Sánchez for the degree of Doctor of Philosophy in Veterinary Science at Universidad de Extremadura (Spain) and the "European Doctorate" label (Doctor Europaeus).
Directed by Dr Gregorio Rocha Camarero

Vº Bº Director de Tesis — Doctorando

2014

Dr. Gregorio Rocha Camarero — Tomás Merchán Sánchez

A mi familia,
a lo extremeño.

Y un día percibí
emocionadamente
que las hierbas, los pinos
las rocas, las arenas,
el mar y el cielo,
todos a la vez, eran
mi verdadera consciencia,
yo mismo.
Y no había forma de prescindir
de algo sin romperlo todo.
Era la unidad de todos los seres.
La Gran Nada

Gassho
Soko Daido

CONTENIDOS
CONTENTS

ACKNOWLEDGMENTS

ACKNOWLEDGMENTS

A phone call from from Extremadura some years ago gave me the welcome opportunity to return to Cáceres to work on a research project on the European wild rabbit. Gregorio Rocha Camarero gave me the chance to return to university and work with wildlife, and this is why these acknowledgments begin with my heartfelt gratitude to Gregorio, who, apart from being my thesis director, is also a great friend. We spent endless hours in each other's company and our relationship was based not only on work but also on friendship. I thank Gregorio for his constant encouragement in the preparation of this thesis. His example and assistance were the backbone of my training in research, and his unconditional friendship has been a gift. From receiving that phone call to completing this thesis (which had its rough patches), many people had a smaller or larger role in helping to see the thesis through its various stages from conception to maturity. Naming some of these many individuals in this list is just a small gesture of public recognition. Some had a direct role in the work of the thesis: in fieldwork, the laboratory, revision and editing, and helping with the writing. Their contribution was both necessary and rewarding. Others shared moments of their lives with me, and in some cases it was enough to know they were there during the hard times. All of these people have my most heartfelt thanks. I apologise to those who took part in the thesis or felt they were part of it but are not mentioned in these few lines. I'm sure they'll understand that any omission is unintentional.

Some of the people who took part in the origins of the "Rabbit Project", where this thesis was born, are the interns and other assistants who were students at the Faculty of Veterinary Science in Cáceres, as well as the anonymous individuals who sent samples, helped to publicise the project or made their land available for our studies, including Serafín Mayoral and the owners of Finca la Rusal and Finca las Golondrinas. The Extremadura Hunting Federation helped with biological sample collection. I'm grateful to Alberto Rocha, César Martín, and other "residents" of the Biology Department of the Faculty of Veterinary Science, in Cáceres, including Francisco Cid, Carlos Zahínos, José Luis and Guadalupe Arenas. Thanks also to Sebastian Hidalgo de Trucios, lecturer and friend, and everyone else who worked with me on the Rabbit Project: Francisco Galo, Francisco Gómez, Chema Vadillo, Gloria Cortázar and Sara Serrano. Thanks also to our field expert Francisco Ordiales, with whom I spent countless hours in the field. I thank Ester Sansinforiano for her willingness to help at all times, and Alberto Pagés, Carlos Diez Valle and Fernando Alda for their practical approaches and friendship.

I thank my relatives who took part in or listened to tales of mice and rabbits over the years for encouraging me and patiently understanding and accepting that I wasn't always around. Thank you to my grandfather Tomás Merchán, for his fortitude and for being the first person to show me what a wild rabbit was. For their tireless affection, I thank my maternal grandparents Josefa and Valeriano, with whom I enjoyed every summer spent in their village listening to stories of animals. I thank my parents, Tomás and Esperanza, for their dedication and ceaseless love, and my sisters, Esperanza and Beatriz, for their unconditional affection and large doses of patience when the only photos that were taken were of "critters". I'm grateful for the constant support and highly positive influence of Richel, Bote, Bego, Massimiliano, Sergio, Lina and Patricia. Thanks to Pedro Luis, a source of knowledge and inspiration about the natural world. I'm grateful for the patience of my colleagues and good friends Angela, Mario, Gladis, Jelem and Yoli, from Arenas de San Pedro. Thanks also to Silvia Igual, Oscar and Marta for their friendship and support, and to Mario and Georgina, who also played a part in all of this. I thank Mónica, who was so much more than an inspiration from start to finish, and Pedro Valencia and Ana for their spiritual friendship. Thanks to Silvia for her honest, refreshing encouragement, and to Jane McGrath for her hard work and thoroughness in translating this thesis.

I'd also like to thank the University of Extremadura and the Regional Government of Extremadura. Through collaboration agreements between these institutions I was able to receive numerous grants and sufficient means to conduct the studies properly. Support for our projects was provided by individuals at these institutions, including Isidro Lázaro Santos, Head of the Extremadura Regional Government's Forestry, Hunting and Fishing Service, who at the time had the necessary faith for the "Rabbit Project" to go ahead; forestry expert Javier Caldera; and environmental rangers Alberto Britos, Fabian and Jesús Montero, whose participation made it possible to carry out the introduction project in Granadilla. Lastly, I'd like to thank the Antonio María Concha Cultural Foundation, in Navalmoral, for allowing me to use the library meeting room for talks with my thesis director at the "halfway" stage.

Metta bhavana

ABSTRACT IN SPANISH

ABSTRACT IN SPANISH

El conejo de monte, *Oryctolagus cuniculus*, es un endemismo ibérico de incalculable valor ecológico ya que actúa como especie clave multifuncional. Si bien son muchos los aspectos sobre la biología y ecología de la especie que han sido estudiados, aún se desconocen en profundidad otros aspectos que inciden sobre la dinámica poblacional de la especie. Este es el caso de las enfermedades víricas y en concreto, cuestiones de índole epidemiológico de la enfermedad hemorrágica vírica (RHD). Esta tesis se ha enfocado en comprobar cómo ratones de campo (*Apodemus sylvaticus*) y ratones morunos (*Mus spretus*) que conviven de forma natural con los conejos de monte, contienen las mismas cepas del virus de la enfermedad hemorrágica vírica del conejo (RHDV). Además, se ha estudiado el trasiego de virus de conejos infectados a roedores, así como el tiempo de permanencia y la viabilidad de estos virus en el interior de los roedores. Este estudio ha requerido un ensayo de experimentación en laboratorio. En este mismo estudio, se comprueba no sólo que el virus no afecta a los roedores, sino que además, después de un tiempo, es capaz de provocar una respuesta inmune en conejos susceptibles que entran en contacto con roedores.

También se ha estudiado el porcentaje en la naturaleza de conejos seropositivos y con presencia del virus. Estos datos sobre animales portadores y persistentemente infectados nos ofrecen una información sobre la situación de inmunidad natural elevada de los animales en la naturaleza y pueden servir como herramienta de gestión para las repoblaciones. Este alto porcentaje de animales seropositivos ha sido detectado en diferentes momentos del año (durante la caza en verano y en otoño) y es un indicador de cierto equilibrio con el virus. Altas densidades de conejos está relacionado con altas prevalencias de anticuerpos. Estas poblaciones densas muestreadas tampoco han presentado episodios recientes de brotes de RHD.

Por otra parte, se ha estudiado la interacción entre la enfermedad hemorrágica con la mixomatosis. Se ha comprobado cómo no existe correlación entre estas enfermedades víricas con el sexo y sí con la edad de los conejos. Se determina que las titulaciones positivas de RHDV presentan complementariedad en relación a las titulaciones positivas de mixomatosis. Además las titulaciones medias son más altas en el caso de la mixomatosis, hecho que podría explicarse por la mayor antigüedad de la enfermedad. También se ha observado como los tratamientos sanitarios como las vacunaciones tienen un efecto sobre los títulos positivos frente a la mixomatosis. En esta tesis se han estudiado las características morfométricas de muestras de conejos de Valladolid y de Badajoz. Se ha comprobado como los individuos norteños presentan mayor peso y longitud que los del sur pero

estos muestran mayor condición corporal con respecto a los del norte. Estos resultados no sólo pueden explicarse por las diferencias latitudinales sino por la propia subespecificidad, ya que ambas poblaciones muestreadas se corresponden al linaje *O.c. algirus* (sur) y al linaje *O.c. cuniculus* (norte).

Por último se aportan resultados del seguimiento de una repoblación atendiendo a criterios no sólo de manejo del hábitat, sino genéticos e inmunológicos. De esta monitorización se obtuvieron unos resultados que han permitido elaborar recomendaciones de tipo técnico para otras repoblaciones. Cabe destacar, por afectar positivamente el curso de la repoblación, el efecto de la utilización de animales de zonas geográficas compatibles desde el punto de vista genético así como el uso de animales con alto estatus inmunológico previo. Por último, se comprobó el efecto de la hiperinmunización sobre la supervivencia de los conejos.

ABSTRACT IN ENGLISH

ABSTRACT IN ENGLISH

The European wild rabbit, *Oryctolagus cuniculus*, is an Iberian endemism of immeasurable ecological value because it acts as a multifunctional keystone species. Although many aspects of the biology and ecology of this species have been studied, little is known about other factors that affect its population dynamics. This is the case of viral diseases and, more particularly, epidemiological issues of rabbit haemorrhagic disease (RHD). This thesis explores how wood mice (*Apodemus sylvaticus*) and Algerian mice (*Mus spretus*) naturally cohabiting with wild rabbits host the same strains of rabbit haemorrhagic disease virus (RHDV). Virus transmission from infected rabbits to rodents was studied, as well as the viability and persistence of the virus inside rodents. Laboratory experimentation required for the study showed not only that the virus does not affect rodents, but after some time it is also capable of producing an immune response in susceptible rabbits that come into contact with infected rodents.

The percentage of seropositive rabbits with the virus living in the wild was also studied. These data on carrier and persistently infected rabbits provide information on the high natural immunity status of animals in the wild and can be used as a management tool for repopulation. A high percentage of seropositive animals was detected at different times of year (during summer hunting and in autumn), indicating a certain degree of equilibrium in the virus. High rabbit densities are related to a high prevalence of antibodies. The dense populations surveyed showed no recent episodes of RHD outbreaks.

A further study analysed the interaction between haemorrhagic disease and myxomatosis. No correlation was found between these viral diseases and gender, although a correlation to rabbit age was observed. It is shown that RHDV positive titres present complementarity to MV positive titres. Average titres are also higher in the case of myxomatosis, which could be due to the longer time this disease has existed. Health treatments such as vaccinations were similarly shown to have an effect on positive titres against myxomatosis.

Morphometric characteristics of rabbit samples from Valladolid and Badajoz were analysed, confirming that northern individuals show greater weight and length than individuals in the south, although southern rabbits show better body condition than northern rabbits. These results can be explained not only by latitudinal differences, but also by subspecificity, as the two populations sampled were *O.c. algirus* lineage (south) and *O.c. cuniculus* lineage (north).

Lastly, the thesis provides the results of monitoring a repopulation project based not only on habitat criteria, but also on genetic and immunologic criteria. These results enable technical recommendations to be made for other repopulation projects. Highlights include elements that positively affect repopulation, such as the effect of using animals from compatible geographical areas from a genetic point of view, and using animals with high prior immunologic status. The effect of hyperimmunisation on rabbit survival is also demonstrated.

INTRODUCTION

INTRODUCTION

1. Origin and distribution of the wild rabbit

The European wild rabbit, *Oryctolagus cuniculus* L., is one of the most emblematic animals in the Iberian Peninsula. The species as we know it today originated around 900,000 years ago, and the earliest fossil of the *Oryctolagu*s genus found in Spain dates from the Miocene (López-Martínez, 1977). During the Quaternary glaciations, eight to 10 million years ago, ancestral populations of the *Oryctolagus* genus (*O. laynensis* and *O. lacosti*) dated in the Middle Pliocene disappeared from the Iberian Peninsula, although *O. laynensis* is considered the origin of the present-day wild rabbit (López-Martínez, 1989). During the Holocene, a natural expansion of *O. cuniculus* towards the south of France occurred, in which climate and vegetation factors were the main reasons for the distribution of the species (Callou, 1995). Rabbits may also have become restricted to this area because they had no competition from the *Cricetus, Spermophilus* or *Clethrionomys* genera (Corbet, 1994).

Humans have played an important role in introducing the species to other parts of the world, to the extent that it is now distributed worldwide with the exception of Antarctica. It occurs in central and northern Europe, the eastern Mediterranean, North Africa and North America, and is rapidly expanding in some regions of South America (Corbet, 1986; Callou, 1997). The European wild rabbit is distributed in more than 800 islands in Oceania (Flux & Fullagar, 1992) and has become a pest in Australia and New Zealand (Myers *et al.,* 1994). This situation was mostly caused by humans and began with the Romans, who domesticated the northern populations in the Iberian Peninsula, specifically the subspecies *cuniculus*, the origin of all domestic breeds (Reumer & Sanders, 1984; Hardy *et al*., 1994). The greatest dispersal of rabbits in Europe occurred during the Middle Ages (Rogers *et al.*, 1994) and they are now found throughout France, the United Kingdom, Ireland, Germany and other European countries as far from their original area as the Ukraine. Recent molecular studies have confirmed that these historical introductions occurred primarily from wild rabbits (Branco *et al*., 2000) and how, for example, French rabbits originated from free-living rabbits in the south of France and United Kingdom rabbits originated from French rabbits (Flux, 1994).

2. Genetics

Two subspecies of wild rabbits have been distinguished in the Iberian Peninsula: *Oryctolagus cuniculus cuniculus*, distributed in the eastern and central areas of the Iberian Peninsula and the rest of Europe, and *Oryctolagus cuniculus algirus*, found in the most southwestern area of the Peninsula

and in North Africa. The two subspecies were established through the study of polymorphisms in their mitochondrial DNA, which made it possible to identify the maternal lineages, known as A and B (Ennafaa *et al*., 1987; Biju-Duval *et al*., 1991). It has been confirmed that lineage A corresponds to *algirus* and lineage B to *cuniculus*. From a geographical point of view, an overlap between the two lineages has been demonstrated, crossing the Iberian Peninsula from the northwest to the southeast (Ferrand *et al*., 1998; Branco *et al*., 2000) (Figure1). The differences between these large groups were identified through studies using nuclear DNA markers (Ferrand, 1995).

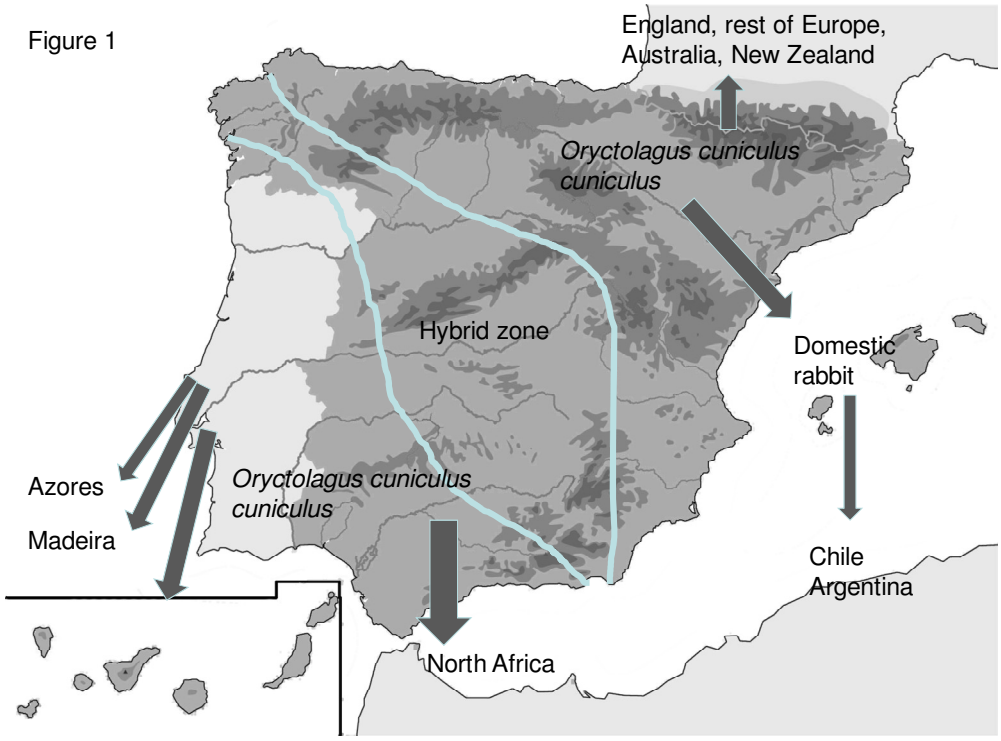


Figure 1: The map proposed by Ferrand *et al*., 1998 shows the distribution of the two subspecies and the hybrid zone between them.

The *cuniculus* subspecies has spread virtually worldwide with human assistance and given rise to all domestic breeds (Biju-Duval *et al.*, 1991). The two subspecies appear to be morphologically distinguishable, as stated in Chapter 6 of this thesis and mentioned by other authors (Castro *et al*., 2011). From a genetic point of view, most authors agree that *O. c. cuniculus* and all domestic varieties and European populations have less genetic variability than *O. c. algirus* (Branco *et al*., 2000). The great diversity and complex genetic and evolutionary structure of European wild rabbits in the Iberian Peninsula (Queney *et al*., 2001; Branco *et al*., 2002; Geraldes *et al*., 2006) has resulted in a high ability to adapt to diverse ecological conditions, as well as high productivity (Soriguer, 1981).

This circumstance has developed despite such important historic phenomena as glaciations and such high impact events as viral diseases.

In Extremadura, a study based on polymorphic restriction fragments in mitochondrial DNA of a representative sample of wild rabbits to describe populations (Cortázar *et al.*, 2005) indicated that although much of the territory is in a hybrid zone, *O. c. algirus* clearly predominates over *O. c. cuniculus* (Figure2).

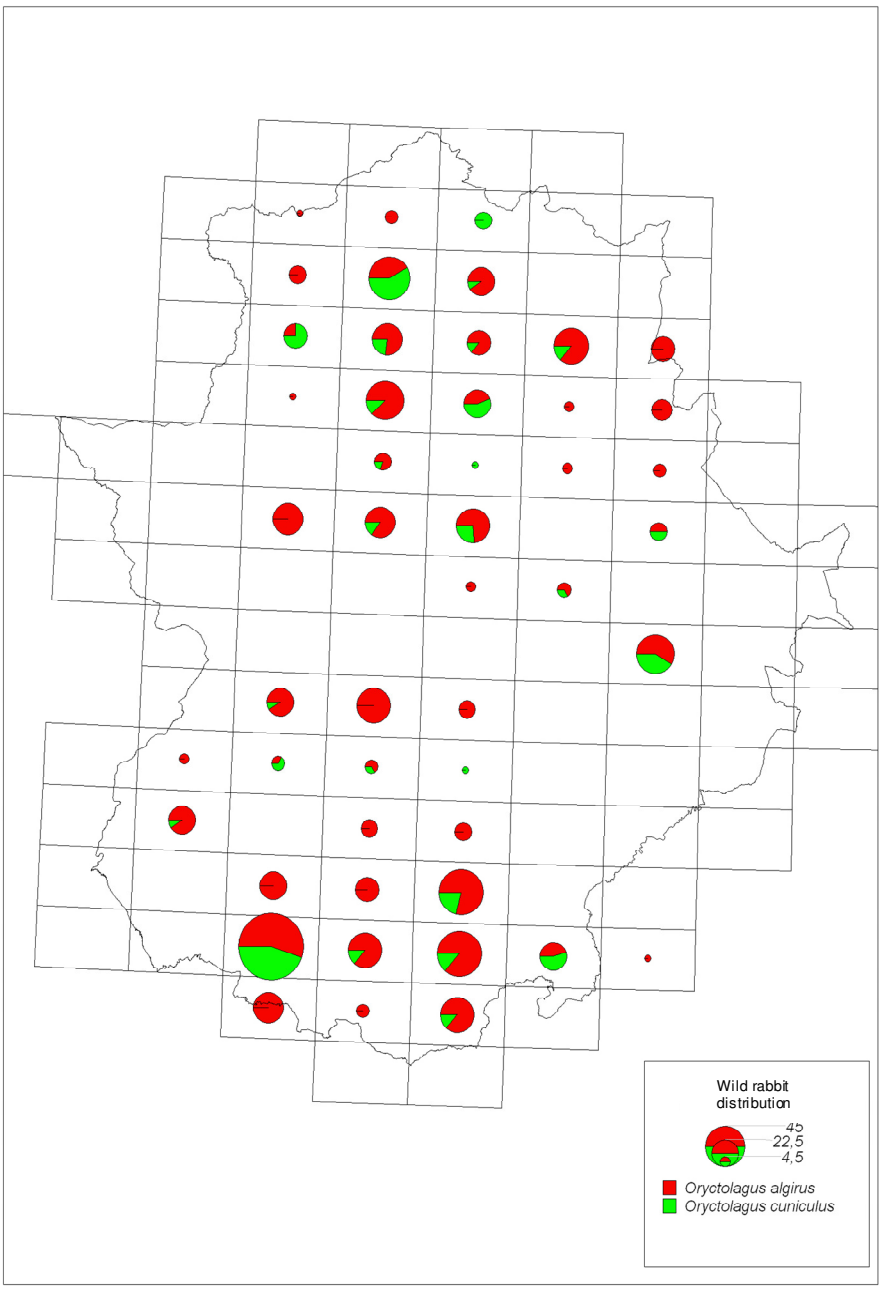


Figure 2: Map using pie charts to show the distribution of *O. c. algirus* and *O. c. cuniculus* according to grids from regional mapping; scale 1:50000 (Cortázar *et al*., 2005).

3. Wild rabbit bioecology

The wild rabbit belongs to the Lagomorpha order and the Leporidae family, and although it is from the same family as 10 rabbit genera, none of these occurs in the Iberian Peninsula. One genus that does occur in the Iberian Peninsula is *Lepus* (hares), although rabbits and hares have notable anatomical differences (both have long, straight ears but rabbit ears are shorter and have no black tip) and in particular major ecological differences. Male and female rabbits have been found to be similar in weight, averaging from 0.90-1.34 kg (*O. c. algirus*) and 1.50-2.00 kg (*O. c. cuniculus*), with a head-to-tail length of 34-35 cm (Villafuerte, 2002). The Iberian rabbits are the smallest of the species, compared to French, English, Swedish and Australian wild rabbits (Anderson *et al*., 1979). A full examination of the external genital organs is required to distinguish genders, as there is no external sexual dimorphism to differentiate bucks from does simply by sight. In young rabbits, the difference is further complicated by the lack of fully developed sexual organs. Age is calculated by feeling the external area of the distal end of the ulna. Rabbits less than nine months have a lump of cartilage that older individuals lack as a result of ossification of the ulnar epiphysis (Biadi & Le-Gall, 1993; Kauhala &Soveri, 2001; Sáez de Buruaga *et al*., 2001). For greater accuracy in assessing rabbit age, eye lens weight must be determined. In vertebrates, this lens grows throughout the individual's lifetime without shedding cells (Bloemendal, 1977), and through a formula proposed by Dudzinski and Mykytowycz (1959) used in Mediterranean climates, rabbit age can be estimated.

Wild rabbits prefer open areas, cropland and meadows close to forests, scrubland and other refuge areas. They keep to environments at low-mid altitude (less than 900 metres) with warm temperatures and low precipitation (Blanco & Villafuerte, 1993). They are highly capable of adapting and generally choose areas with dry, loose soil. When soil conditions are suitable (neither too hard to dig nor too soft to risk collapse), rabbits prefer to dig warrens. The size of their warrens depends on the characteristics of the immediate habitat: warrens are larger in open habitats than in enclosed habitats, where the greater plant cover provides shelter for the rabbits. In abundant scrubland, rabbits tend to use warrens simply as breeding chambers rather than shelters, as the vegetation acts as refuge (Soriguer, 1981). This type of life in scrubland is frequent among wild rabbits in mountain areas and common in the north of the province of Cáceres, in Extremadura, and is also typical of limited, fragmented populations (personal observation).

Warrens tend to be located in ecotone areas underneath elements that provide physical protection, such as rocks, shrubs and trees (Gea-Izquierdo *et al*., 2005). The wild rabbit is a polygenic species that lives in groups of two to five adults (Biadi & Le-Gall, 1993). Wild rabbits are territorial

and are organised in hierarchies. Each group has one to three dominant females responsible for raising the young in the warren. Non-dominant females dig smaller breeding warrens nearby, known as nests (Mykytowycz, 1959). Social organisation in small groups and the use of nests are anti-predatory strategies of the wild rabbit (Villafuerte & Moreno, 1997; Hudson *et al*., 1996). The group's main area of activity centres around the warren, within a radius of 150-300 metres (Cooke, 1981) in territories with an average size of 2.5 hectares (Calvete *et al.*, 1997), although at times they may move further, e.g., during food shortages and particularly when the young disperse at the age of three to five months. Dispersal may be more extensive at the beginning and the end of the reproductive period, especially in males, to distances of 1,500 metres from the original warren (Parer, 1982). This distance may be shorter if cover and shelter are plentiful (Vitale, 1989).

Rabbits spend an average of four hours a day on activities related to food. The rest of the time is used for reproduction, grooming, social interaction and resting. Antagonistic interactions among adults are more frequent among males, either from the same social group or another group, and are related to hierarchy. They are less frequent among females and are associated with the search for a site to have their litter (Cowan, 1987; Roberts, 1988). As herbivores, wild rabbits take advantage of all plants according to their availability throughout the year. The biological success of rabbits is largely based on this circumstance. Their physiological adaptation to nutrient assimilation and detoxification is highly efficient, allowing them to survive under the harshest conditions. Rabbits also have an extraordinary adaptation that acts as an added bonus to the efficient use of plant material. This is cecotrophy, where plant material passes through the digestive tract twice, initially producing soft faeces known as cecotrophs, which rabbits then reingest for final digestion. Cecotrophs are rich in vitamins and proteins of microbial origin which are easily absorbed after reingestion (Hirakawa, 2001).

The rabbit mating system is based on polygyny (Surridge *et al*., 1999), where bucks mate with several does. As opportunistic reproducers, rabbits take advantage of periods with abundant food for breeding. Reproduction takes place from October or November, with the first autumn rain, until July, when grass becomes scarce, demonstrating the high sensitivity of rabbits to environmental conditions (Wheeler & King 1985; Gonçalves *et al*., 2002). Although reproduction may drop in winter, mating ceases temporarily in summer in Mediterranean environments. Gestation lasts 28-33 days and does can become pregnant again shortly after giving birth. Rabbit litters are large and a single doe can have several litters during the reproductive season (Rogers *et al.*, 1994).

In the south of Spain, an average of 2.3 litters per doe per year has been calculated, with Mediterranean litters producing three or four kits (Soriguer, 1981). Does have developed

reproductive and maternal behaviour designed to minimise the impact of predation, consisting of giving birth quickly and then ingesting the placenta and cleaning the young to remove olfactory traces (Hudson *et al*., 1996).

4. Importance of wild rabbits for ecological functionality

The European wild rabbit is a species with immeasurable ecological value. It is a multifunctional keystone species, to the point that it can shape the landscape simply by its presence (Gálvez, 2008; Gálvez *et al*., 2009), providing shelter to a large number of vertebrates and invertebrates. Rabbit grazing, which includes a significant variety of woody species (Soriguer, 1988) and nuts (Zamora *et al*., 1985), has been shown to alter the composition of plant species, creating open areas in scrubland and contributing to plant diversity (Delibes-Mateos *et al*., 2008). This landscape shaping, in which large plant species are controlled through grazing (Crawley & Weiner, 1991), could also affect animal species and help to maintain ecotones and mosaic habitats (Van der Wal *et al*., 2000; Delibes-Mateos *et al*., 2008).

Rabbits are important seed dispersers, to the extent that their activity is considered more important than that of other, larger herbivores (Malo *et al*., 2000). Although rabbit dung can contain an average of approximately 2.5% seeds (Dellafiore *et al*., 2006), the number of seeds dispersed by a dense population is very high, as each individual can produce more than 300 faeces a day (Wood, 1988). The possibility of spores of certain fungi eaten by rabbits being spread through faeces has also been reported (Alves *et al*., 2006). In addition to producing a large amount of faeces, rabbits habitually deposit dung in the same place, forming latrines that have a social role and tend to be used for years (Gibb, 1993). Latrines are abundant in enclaves where population density is high, and latrine counts have been associated with rabbit abundance (Gálvez, 2008) and used to describe wild rabbit populations in Spain (Guzmán *et al*., 2004). Rabbit latrines alter the composition, density and biomass of the vegetation in the surrounding area and increase levels of nitrogen and phosphorous, affecting soil fertility (Petterson, 2001; Willot *et al*., 2000). It has also been confirmed that latrines can be a trophic resource for some coprophagous invertebrates such as *Onthophagus latigena* and *O. emarginatus,* which commonly feed on rabbit faeces (Galante & Cartagena, 1999; Sánchez-Piñero & Ávila, 1991).

The major digging activity of rabbits creates cover and shelter for other species of vertebrates, such as the Iberian ribbed newt (*Pleurodeles waltl*), the Montpellier snake (*Malpolon monpessulanus*), the garden dormouse (*Eliomys quercinus*), the European badger (*Meles meles*) and the Iberian lynx (*Lynx pardinus*)(Blázquez & Villafuerte, 1990; Palomares & Delibes, 1993; Revilla *et*

al., 2001). This circumstance was verified in the case of *Apodemus sylvaticus* and *Mus spretus* during the sampling of mice required for this thesis, as both species were found inside or near rabbit warrens, which they use as shelter.

5. Socioeconomic importance of wild rabbit hunting

Countless historical records indicate that wild rabbits have been hunted since ancient times. They were initially captured with the aid of ferrets, nets, traps and various other devices (Biadi & Le-Gall, 1993), but it was not until weapons came into use that rabbit hunting became widespread, particularly in the countries where the wild rabbit originally occurred (Rogers *et al*., 1994). The wild rabbit is one of the most important small game hunting species in the Iberian Peninsula and is subject to intense hunting pressure. It is captured primarily in hunting grounds, which cover more than 70% of the Peninsula (Villafuerte *et al*., 1998). Rabbits have traditionally been valued both for their meat and their skins, although their importance now lies in their value as a hunting species. Approximately 3,543,000 rabbits are hunted each year in Spain (Virgós *et al*., 2005; Spanish Ministry of Agriculture, Fisheries and Food, 2006). The number of hunting permits issued in 2007 was 898,036, and even though this figure is much higher than the 500,000 permits issued in the 1960s (EU REGHAB Project, 2002), hunters are decreasing. The emergence of rabbit viral disease has redirected hunting pressure to the partridge in several regions of Spain, leading in turn to overhunting of galliforme populations (Blanco-Aguiar *et al*., 2004; Blanco-Aguiar, 2007). Although some authors have shown how wild rabbit populations may be hunted to nearly 87% of their total to stop population growth (Hone, 1999), these are undoubtedly populations in good condition and therefore this figure is not applicable to most fragmented or declining populations, where overhunting has been shown to be an additive mortality factor (Marboutin & Peroux, 1995; Bro *et al*., 2000). Low-density populations are more affected by any stochastic phenomenon and once a destabilising factor is established, the population gradually declines, often with no possibility of recovery when a minimum density threshold is exceeded. Hunting low-density populations tends to encourage rarefaction (Bennet & Robinson, 2000; Sutherland, 2001).

Hunters adopt measures and agreements on catch restrictions by estimating the abundance of their catches, although these restrictions tend to be related to hunting activity rather than population abundance (Angulo & Villafuerte, 2003). This makes it very important to have legal regulations consistent with existing scientific information, as much useful information for the management of wild rabbit populations has been produced. As far as the ideal hunting season is concerned, there is some controversy about whether rabbits should be hunted in summer or autumn

in terms of species management. In Extremadura, rabbits are hunted in summer (culling) for a maximum of four days, usually from July 15th to August 15th, after hunters obtain a permit that is issued taking into account population densities, catches in previous seasons and damage prevention. Hunting is also permitted during the ordinary closure period in autumn, from October 12th to January 6th. Summer hunting affects a fraction with a higher percentage of young rabbits and therefore has less effect on the reproducing fraction than autumn hunting. Rabbits that have survived disease are hunted in autumn, which is not advisable. However, some authors consider that autumn hunting subjects rabbits to differential selection by individuals, in which adult females are not affected, as they stray less for grazing and are therefore hunted less (Angulo & Villafuerte, 2003; Calvete *et al*., 2005).

In Extremadura, hunting is a source of income providing approximately 295 million euros (Spanish Hunting Federation 2007, in press). This income is associated not only with the sale of meat (relatively minor) and hunting-related activities, but also with the hotel and catering industry, clothing, weapons and ammunition, hunting dogs, etc. It is important to note that in some rural areas of Extremadura, hunting is the main economic activity. Calculations based on Spanish Hunting Federation studies show 80,000 permits were issued for some 3,357 hunting grounds in 2007. The number of rabbits hunted in Extremadura in 2007 was 115,000 (Spanish Hunting Federation 2007, in press), showing a decline. Some sources estimate a 20% drop between the 2000-2001 season and the 2005-2006 season (Ecologists in Action, in press).

6. Prey species

The European wild rabbit is one of the main species of fauna in Iberian ecosystems, as it is the highest consumer of herbaceous vegetation (Soriguer, 1988) and the prey base of a large number of predators. It is part of the diet of 40 species of vertebrates in the Iberian Peninsula (Soriguer & Rogers, 1979) and is an irreplaceable base in the nutrition of more than 20 of these species (Delibes & Hiraldo, 1981), including such threatened and emblematic species as the Spanish imperial eagle (*Aquila adalberti*) and the Iberian lynx (*Lynx pardinus*). The decline in most wild rabbit populations (Virgós *et al*., 2005) has directly affected the Iberian lynx (MMA -Spanish Ministry of the Environment-, 1999), resulting in it being considered the world's most endangered feline species (Nowell & Jackson, 1996). The interaction between the presence of the lynx and its influence on mesopredators that prey on wild rabbits has been studied (Valverde, 1963; Palomares *et al*., 1995; Virgós & Travaini, 2005), including the interactions between the abundance of rabbit, lynx, fox and a variety of mesopredators such as the Egyptian mongoose, the common genet and the mustelids. The

complex network of interrelationships among all these animals has been demonstrated (Linnel & Strand, 2002). In Extremadura, there appears to be a clear connection in the distribution of Egyptian mongoose and rabbit distribution and abundance (Figure 3), as higher rabbit abundance appears to determine a greater likelihood of finding Egyptian mongoose (Figure4). However, in addition to the presence of rabbits, the disappearance of the lynx as a controller of the Egyptian mongoose may be a significant factor in the emergence of this situation (Hidalgo *et al*., 2005).

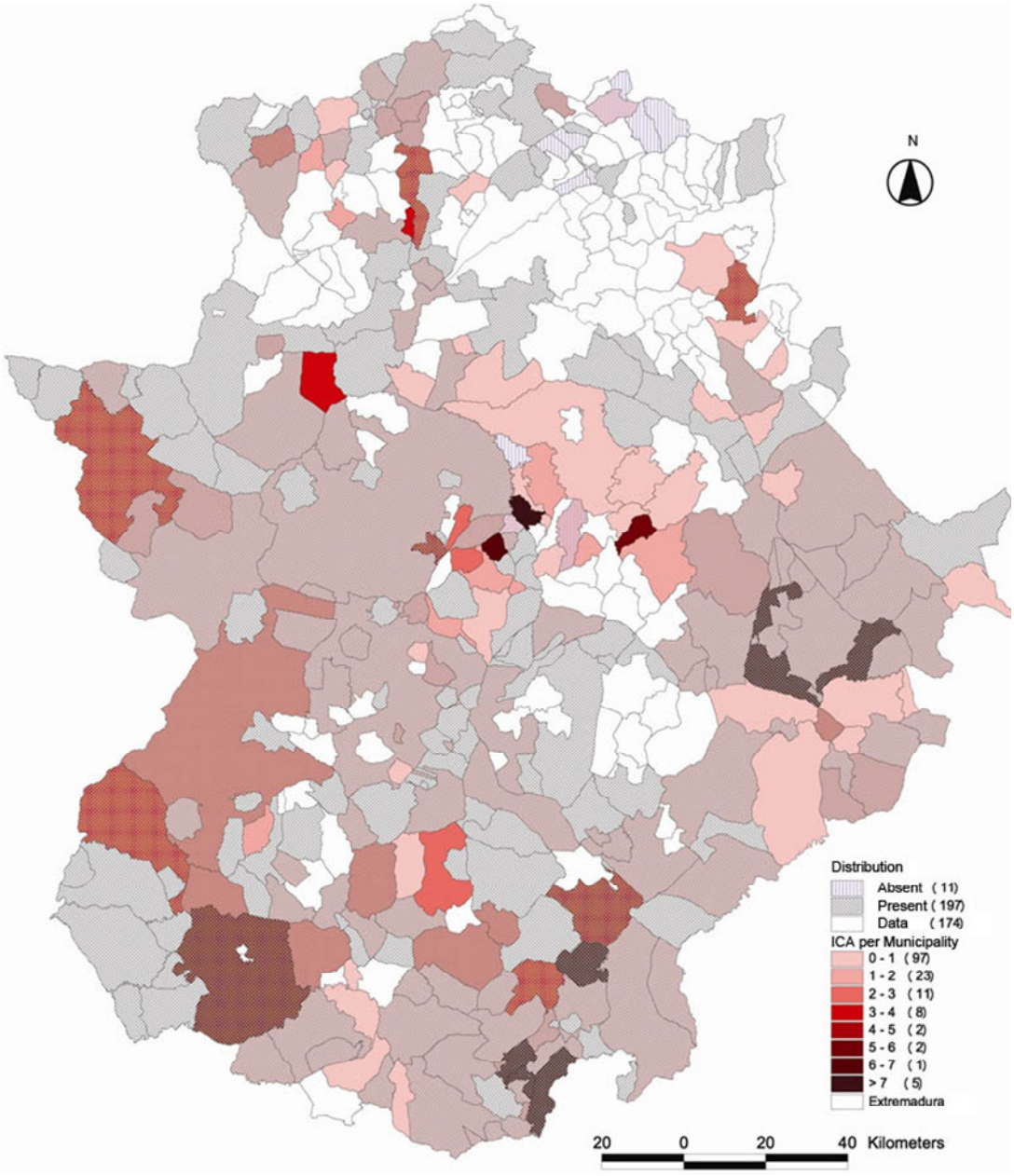


Figure 3: Wild rabbit abundance in Extremadura, expressed in the Hunting Abundance Index (ICA) by municipality, compared to the distribution of the Egyptian mongoose (Hidalgo *et al*., 2005).

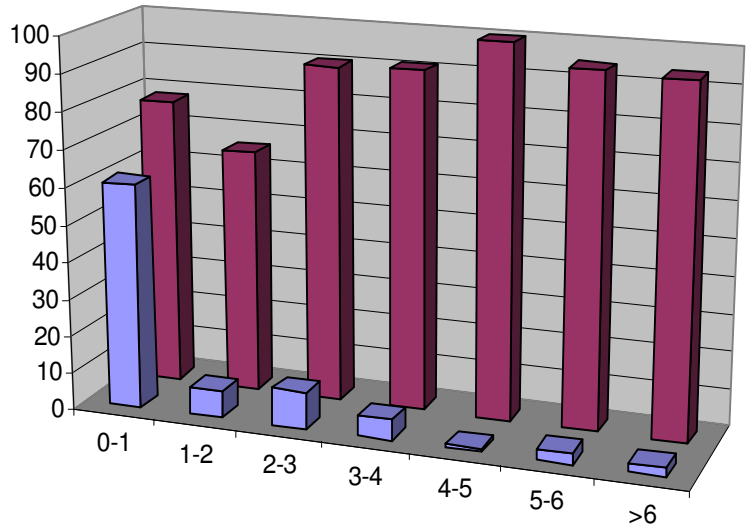


Figure 4: Bar graph showing the wild rabbit Hunting Abundance Index (ICA) distributed by range and the area covered by rabbits in Extremadura (blue), compared to the area covered by Egyptian mongoose within each range (red). (Graph prepared using Hidalgo *et al*., 2005).

7. Destabilising factors of wild rabbit populations. The role of diseases

The European wild rabbit is a keystone species and an Iberian endemism (Thompson & King, 1994) of vital importance in Mediterranean ecosystems. However, until quite recently, no further in-depth studies were conducted to obtain information to help mitigate the trend towards a general decline in the abundance of this species (Moreno & Villafuerte, 1995; Villafuerte *et al*., 1998). Rather than responding to increasingly alarming figures on rabbit decline, management of the species (now based on its conservation) was prompted by recovery programmes for endangered species such as the Spanish imperial eagle and the Iberian lynx, the driving forces behind new approaches (Ferrer & Negro, 2004; Guzmán *et al*., 2004; MARM -Spanish Ministry of Agriculture, Food and the Environment-, 2008). Ironically, the wild rabbit is considered a pest species for agriculture and is fought against as such, not only in countries where it was introduced, but also in some parts of Spain (Thompson & King, 1994; Twigg *et al.*, 2000).

The current situation of a significant part of the Iberian Peninsula populations is a worrying decline, with high variation between zones. Although rabbits are thought of as being widespread, this should not be confused with abundance, as their presence is obviously patchy (Blanco & Villafuerte, 1993) and shows a trend towards scarcity and regression. This situation is caused by the disappearance of suitable habitats for the development of the species, inadequate hunting management leading in many cases to excessive hunting pressure, movements of animals of dubious

genetic and health quality, proliferation of generalist predators and, above all, the major impact of viral diseases.

As in most of the Iberian Peninsula, population densities in Extremadura are unstable and highly sensitive to these factors, which can cause major variations in their annual evolution, from one season to another. Data on the presence of wild rabbits was obtained from 687 hunting grounds in Extremadura, in areas where rabbit presence was shown using UTM grids (10x10). This study revealed the presence of rabbits in 398 of the 518 grids covering the area of Extremadura, with no rabbits detected in the other 120 grids (Gómez *et al*., 2005). This result is more extensive than in the Spanish mammal distribution map, where the species was detected in only 181 grids.

Questionnaires were used in Extremadura to assess the population trend in 687 hunting grounds. The result shows a population trend of regression in 65% of the grounds surveyed (449), no population in 15% (101), population maintenance in 8% (58) and an increase in only 27% (4) (Gómez *et al*., 2005). The survey also assessed the situation of wild rabbits in terms of hunting and population density using the Hunting Abundance Index (ICA, from the Spanish). The ICA is defined as the number of catches per hunter per year. It is a measure of the normalised population abundance, which enables a comparative analysis to be made. The distribution frequency by the ICA in Figure 5 was obtained by grouping the hunting ground information within each municipality of Extremadura. This is shown in the map in Figure 6.

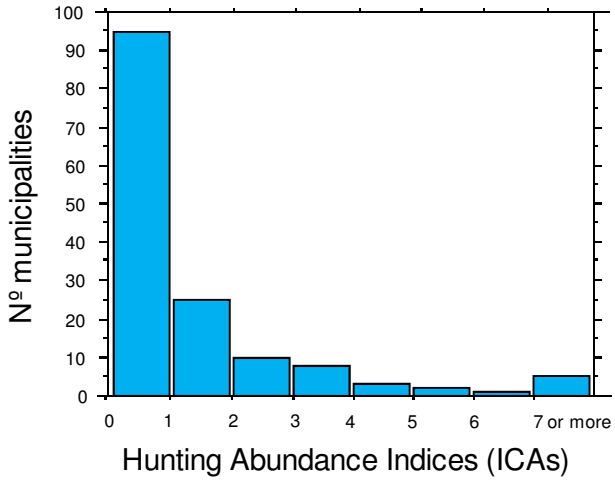


Figure 5: Distribution frequency by Hunting Abundance Indices (ICAs) in Extremadura (Gómez *et al*., 2005).

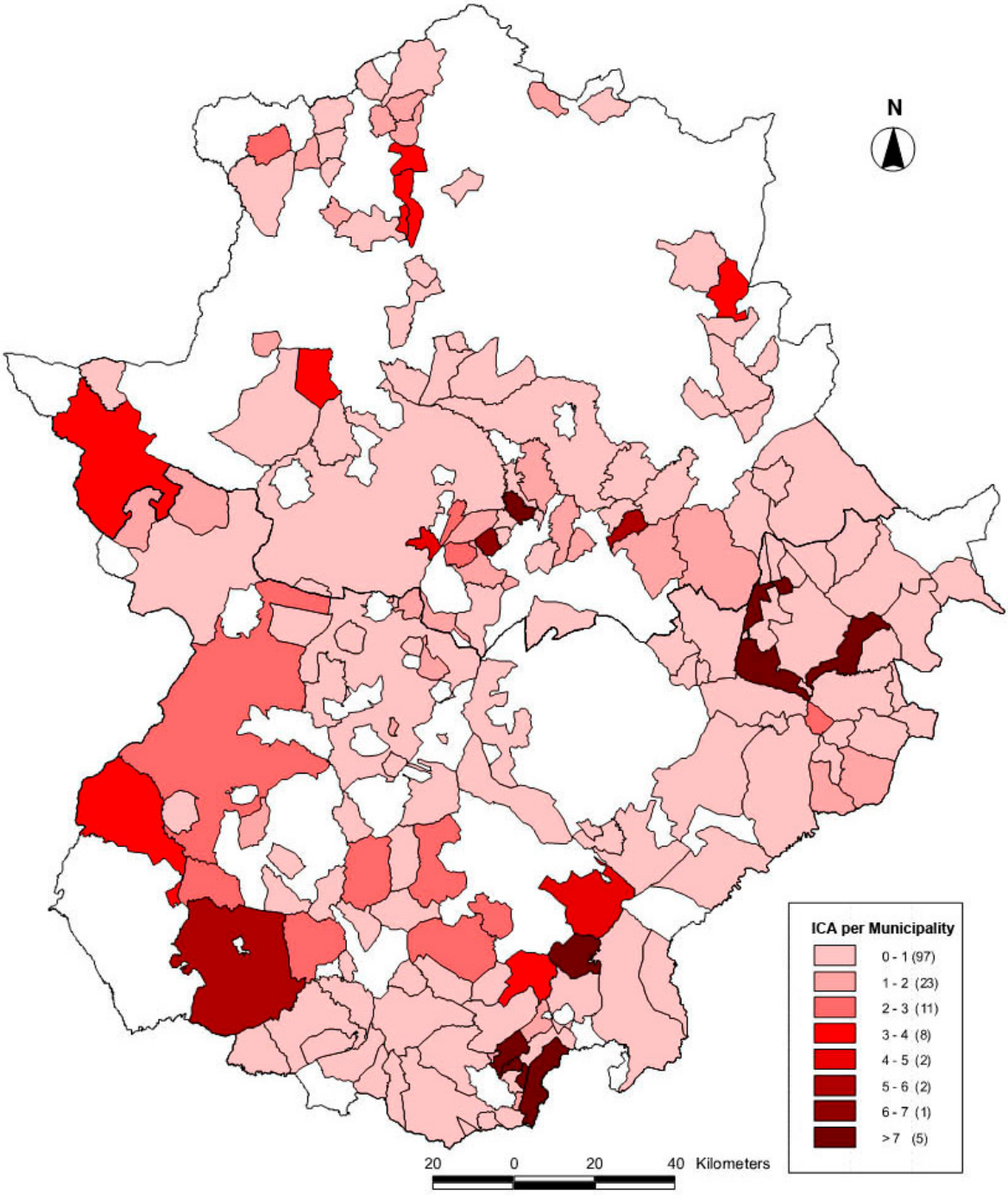


Figure 6: Hunting Abundance Indices (ICAs) grouped by municipality (Gómez *et al*., 2005).

8. Significance of the loss of suitable habitats

The patchy spatial distribution of wild rabbits (Kolb, 1991; Lombardi *et al.*, 2003) shows how larger patches correspond to ecotone areas between scrubland and grassland, the edges of cropland, and other border areas such as ditches along roadways. In these areas, where the soil is easier to dig

and more trophic resources are available, rabbits find suitable habitats. With the exception of roadside ditches, these favourable habitats have decreased due to land-use change processes in Spain in recent decades (primarily the proliferation of intensive agriculture and reforestation). Land-use changes have affected wild rabbit population dynamics to varying degrees, particularly as far as reproduction is concerned. The drop in the quality and amount of food available in a habitat, which is influenced by weather conditions, could affect the viability of patchy populations (Moreno & Villafuerte 1995; Villafuerte *et al*., 1997; Palma *et al*., 1999). Rabbit survival is also significantly affected when habitats provide insufficient shelter. Even though some habitats have high rabbit abundance and limited shelter (such as land easy to dig for warren building), a suitable habitat should ideally provide sufficient shelter and areas for reproduction and feeding (Angulo, 2004). Spatial connection of populations distributed in patches through recovery and maintenance of optimum habitats is one of the prerequisite and necessary measures for managing the species. These measures include creating plots of herbaceous vegetation and scrub (Moreno & Villafuerte, 1995). Measures to improve habitats and build and protect warrens have also proven effective (Aubineau *et al.*, 1985), in addition to supplying extra food and water resources when necessary.

9. Predation

As well as hunting and diseases, which will be addressed later in this introduction, predation is a factor with a high impact on rabbit mortality. Predation has a limiting effect only in cases where rabbit population abundance is low (Trout *et al*., 2000), leading populations to a situation known as a "predator trap", which is a contributing factor to decline but not the actual cause. In abundant populations, predation is a widely studied natural occurrence (Villafuerte *et al*., 1994; Calzada, 2000). Not all age groups show the same predation level: adult rabbits have higher survival rates than kits (Villafuerte, 1994). Young rabbits are the target of a high predation rate, mainly from foxes and wild boars, even when in the nest. This type of mortality has one of the highest impacts in rabbit population dynamics (Mykytowycz, 1959). The types of impact caused by predators are well known, depending on their trophic range. The Iberian lynx, rabbit specialist *par excellence*, needs only one rabbit a day to subsist, but has less impact than generalist predators (Aldama, 1993). The presence and abundance of these types of predators are not related to rabbit density, as they adjust their diet to the most abundant prey at the time and therefore their impact is greater in areas with low rabbit abundance. Through the phenomenon known as "interspecific killing", specialist predators can encourage the presence of their prey (Palomares *et al*., 1995), although unfortunately few areas still have large predators to perform this natural task of controlling generalist predators. In specific circumstances, predator controls are performed (mainly of foxes, dogs, feral cats and wild boars), but

they do not always result in an increase in rabbit populations, as other factors that negatively affect lagomorph population dynamics have not been limited or remedied.

10. Inadequate hunting management: the effect of hunting and restocking

Hunting affects the reproduction and survival of individuals in a population. Although theory recommends hunting only populations that are developing positively (Covisa, 1998), hunting could actually cause rarefaction and a decline in the abundance of many populations. This trend would be accentuated above all in low density populations, as they are under greater influence from variations in abundance, which are exacerbated by hunting (Angulo & Villafuerte, 2003).

Hunting is therefore an activity that should be self-managed if it is to be truly efficient. It is essential to determine the populations that can be hunted and those where hunting should not be permitted. Hunting grounds with suitable landscape heterogeneity and optimum rabbit abundance could be appropriate for hunting. Similarly, stochastic phenomena can occur in these areas (mainly due to diseases), making them areas where hunting should not be allowed. It is therefore imperative to monitor wild rabbit populations to observe changes associated with pressure from the hunting that actually occurs. The wild rabbit is a species likely to undergo processes that could end in extinctions or the loss of its essential role, and overhunting could lead to dense, unstable populations (Hidalgo, 2001).

In addition to aspects related to hunting seasons and methods, which should be adjusted to the possibilities for hunting, it is very important to establish catch quotas and create reserve areas. Hunting can be managed by limiting the number of hunting days, reducing the number of hours when hunting is permitted, restricting the number of hunters, and of course by limiting the number of catches allowed per hunter. Reserves should be created on patches of rabbit populations in optimum state of conservation.

Rabbit restocking and translocation are among the most commonly used management methods, although they are not always advisable. Translocation is increasingly used by hunters (Letty *et al.*, 2000; Calvete & Estrada, 2004; Angulo, 2004). It is sometimes the only option to solve major conservation problems of endangered species or manage populations (Biadi & Le-Gall, 1993; Calvete *et al*., 1997; Letty *et al*., 1998; Cotilla *et al*., 2003), but at other times the risks outweigh the benefits.

These methods may be encouraging the introduction and circulation of viruses (Calvete *et al*., 2002) or aiding gene pool loss through the introduction of genetically unsuitable individuals. Genetic problems consist of standardising the genetic structure and losing local adaptations, which can occur

when rabbits come from uncontrolled commercial farming where domestic rabbits crossed with wild rabbits are used to increase productivity and improve control. These rabbits usually have serious problems due to poor adaptation to the natural environment (Alves *et al.*, 1998; Ferrand *et al*., 1998; Calvete *et al*., 1995).

Although rabbit restocking has been studied (Calvete *et al*., 1997; Calvete & Estrada, 2004), the results address short term survival, with little information on the reproductive success of translocated rabbits (Letty *et al., 2*002). However, when translocated rabbits are from the wild, they have been studied in greater depth than rabbits from breeding farms used for restocking (Ceballos *et al*., 1997). In Extremadura, studies have shown that in areas where rabbits have been relocated, the proportion of the *algirus* lineage is lower than the *cuniculus* lineage, even though the region corresponds mostly to the area of lineage A (*algirus*) (Cortázar *et al*., 2005) (Figure 7). These findings could be explained not only by the type of origin (wild or farm rabbits, hybridised to a greater or lesser extent), but also by the geographic origin of the rabbits, as they are often from very distant areas and include individuals from a different lineage to the relocation area lineage. Figure8 shows the highly diverse origin of 10,178 rabbits released in a single year in Extremadura with public authorisation (Cortázar *et al*., 2005).

Wild rabbit farms in Spain are very successful, despite difficulties in control and breeding (González Redondo, 1995; 1998; 2001). Production methods include semi-intensive systems with mating rabbits kept in cages (Roca, 1994; González Redondo, 1994), semi-extensive breeding in parks, where rabbits are kept in small enclosures (Ñudi, 1998), and extensive or controlled release breeding, where rabbits complete their life cycles in large fenced areas.

Before any restocking is conducted, the variables that led to extinction and the limiting factors for recovery must be taken into consideration. The corresponding standards and protocols should be implemented (Calvete *et al*., 1994; González Redondo, 1996; Alves *et al*., 1998; Calvete, 2002), but in each specific case it is always necessary to assess both the type of release to be used and how the release will be monitored.

Pressure from predators is a constant factor in all restocking and if it becomes excessive, this pressure can cause any restocking attempt to fail (Calvete *et al*., 1997). Even though it is very difficult to counteract, this type of pressure is necessary to some extent, as it favours selective pressure on diseased or less suitable animals (Henning *et al*., 2005). To avoid excessive pressure from predators, release locations that naturally favour defence from predation are usually sought beforehand or created through habitat control (Calvete, 2002; García, 2005). Open restocking is performed in these

areas, although this is normally less advisable, as translocated animals disperse and are exposed to high predation rates (Calvete *et al.*, 2005a). These measures also tend to fail if artificial warrens have not been created before release (Villafuerte *et al*., 1997).

Animal releases into permanent enclosures significantly reduce terrestrial predation (Linhart *et al*., 1982; Ruíz-Olmo *et al*., 2003; Shivik *et al*., 2003) and allow control, capture and health monitoring. When fenced areas become overcrowded due to reproduction, individuals are released using natural gates or through successive translocations in the vicinity (Myers & Poole, 1959; Myers, 1964).

It is also very important to take the time of release into account, as it is always more advisable to release animals before the mating season begins (Calvete, 2002). Among other aspects, it has been shown that most animals are adults and have higher immunity levels and therefore restocking will be more successful (Letty *et al.*, 2003).

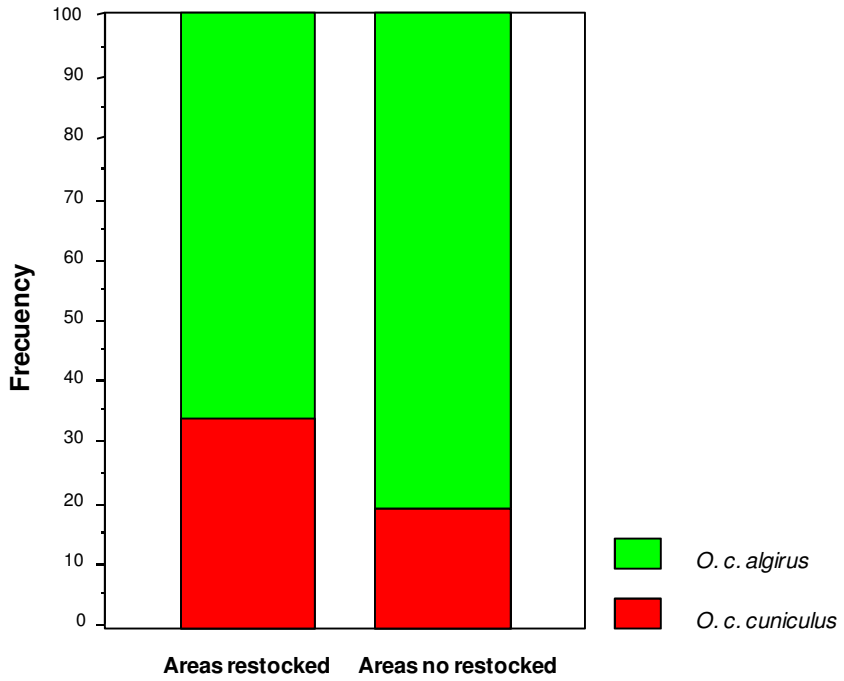


Figure 7: Frequencies of the occurrence of rabbit lineages in restocked areas and in areas without regular restocking (Cortázar *et al*., 2005).

Figure 8: Origin of authorised restocking conducted in the province of Cáceres from January 2002 to June 2003 (in percentages) from a total of 10.178 wild rabbits (Cortázar *et al*., 2005).

11. Viral diseases: myxomatosis and rabbit haemorrhagic disease

Viral diseases - myxomatosis and rabbit haemorrhagic disease - have been responsible for the decline in rabbit populations in Europe in recent decades (Muñoz-Goyanes, 1960; Villafuerte *et al*., 1995; Fenner & Fantini, 1999).

The myxoma virus entered Spain in 1953 and its rapid spread caused drastic changes in rabbit distribution and had a very negative effect on wild rabbit abundance. The pathogenic agent that causes myxomatosis is a very resistant virus of the *Leporipoxvirus* genus, which belongs to the Poxviridae family (Aragaõ, 1927). The virus genome comprises a double-stranded DNA molecule with a size of 163 Kb. It is a virus that contains little information at the molecular level and shows low variability (Fenner & Fantini, 1999). The myxoma virus originated in South American rabbits of the *Sylvilagus* genus, which are affected subclinically.

The original strain (Lausanne) was isolated in Brazil and later intentionally released in Australia. It was then introduced into France and spread throughout Europe (Bárcena *et al.*, 2000). During the first phases after introduction of the virus, mortality was approximately 99% in the first two years according to some authors (Hudson *et al*., 1955), although most consider mortality to have been around 90% (Queney *et al.*, 2000). The impact of myxomatosis is now selective because although it causes the death of many adults, it kills 40-50% of young rabbits, which greatly affects reproduction (Osácar & Lucientes, 1997). The virus is transmitted through the bites or stings of arthropod vectors such as mosquitoes and fleas. This system of transmission determines the phenology of outbreaks, which drops significantly during the coldest months and is activated in warm, rainy periods, when these types of arthropods proliferate.

With the emergence of less pathogenic strains and the existence of populations with some genetic resistance (Fenner & Ratcliffe, 1965; Best & Kerr, 2002), the disease has become enzootic (Fenner & Ross, 1994) and seasonal. Its pathogenic effect and mortality are variable, although generally less pronounced, with some recovery observed from the 1950s to the 1970s. In England, the virus has been shown to become more virulent as rabbit resistance increases (Ross *et al*., 1989).

Rabbit population status suffered an even greater setback in 1988 with the arrival in Spain of rabbit haemorrhagic disease (RHD), which had spread widely in Europe by 1989 (Morisse *et al*., 1991). This disease affects both domestic and wild rabbits and is now well established throughout Europe (Cooke, 2002). Various theories on the origin of the disease have been proposed. The most widespread of these places the earliest detection of the virus in China, in 1984 (Liu *et al*., 1984). Recent phylogenetic and serological studies have determined its origin in Europe, where the virus was present in rabbits in non-virulent forms for decades before the major epidemics of the 1980s (Moss *et al*., 2002; Boots *et al*., 2004; Forrester *et al.*, 2006). This supports the theory that in addition to the source of spread in China, more than one source could have existed at different times (Forrester *et al.*, 2006).

The etiological agent of RHD is an RNA virus of the *Calicivirus* group (Calvete & Estrada, 2000) which is maintained both in the environment and in rabbits, and is specific to rabbits (Olinger & Thiel, 1991). Mice hosting the virus and living sympatrically with wild rabbits have recently been discovered (Merchán *et al*., 2011). This article leads to a reopening of the debate on the origin of rabbit haemorrhagic disease virus (RHDV), as some authors consider it could be a case of an intestinal virus that replicated itself in another organ, causing hepatic-haemorrhagic disorders (Ueda, 1992; Park *et al*., 1995). RHDV is spread horizontally, by direct or indirect transmission, and through all routes (Xu, 1991). The etiological agent of RHD is a single-stranded RNA virus of 7.4 Kb, from the Caliciviridae

group. The impact of the disease in Spain was very pronounced in the first phases, with 55% mortality according to some authors (Peiró & Seva, 1991) and 75% according to others (Villafuerte *et al*., 1995).

RHD evolution is tending towards stability in some populations (Blanco & Villafuerte, 1993; Marchandeau *et al*., 2000; Calvete *et al*., 2002) and theories have been proposed to explain this trend. One of these focuses on the emergence of high immunity levels in the wild, associated in particular with high density populations (Marchandeau *et al*., 1998; Calvete *et al*., 2002). This circumstance is detailed in some of the chapters of this thesis. Another theory describes the existence of non-pathogenic forms in the wild that could aid the emergence of high immunity levels (Nagesha *et al*., 2000). The disease has been maintained as enzootic, with annual outbreaks occurring during the coldest seasons, generally in winter and spring. Reports have been made of a connection between the emergence of outbreaks and several environmental factors, such as sudden changes in weather conditions, food shortages and increased population density (Calvete *et al*., 1995a; Muzquiz *et al*., 1997).

The role of epidemiological factors in the spread of RHD is yet to be discovered, including the location of viruses between enzootic outbreaks. Studies have focused on whether viruses can persist inside warrens (Calvete & Estrada, 2000), if rabbits themselves are virus reservoirs (Cancelloti & Renzi, 1991), and the role of recently studied vertebrates (Merchán *et al.*, 2011). It is known that some species of scavengers and predators feeding on rabbits that have died from or been infected by the RHD virus not only spread the virus, but may even develop an immune response (Simón *et al*., 1994; Leighton *et al*., 1995; Dedek & Frölinch, 1997; Frölich *et al*., 1998). Through experimentation, it has been confirmed that when dogs are infected with the virus, they can act as a source of infection (Simón *et al*., 1994). Transportation of RHDV in the claws, feathers and digestive tract of birds scavenging off rabbits that have died from the disease has been described as the source of RHD outbreaks in England (Chasey, 1994). Little is known about the epidemiological role of rabbits themselves in the transmission of the virus, although kits could play a very important role (Calvete, 2011).

More is known about the possible role of invertebrates from an epidemiological perspective and therefore this zoological group is not addressed to the same extent. Flies of the *Calliphora* and *Chrysomya* genera have been shown to retain viable viruses some days after ingesting fluids from rabbits that have died from or been infected by RHDV (Asgari *et al.*, 1998). Other species of hematophagous insects shown to infect susceptible rabbits are *Spilopsyllus cuniculis*, *Xenopsylle*

cunicularis and *Culex annulirostris* (Lenghaus *et al*., 1994). In Australia, 13 different species of insects in which RHDV was detected have been reported (Westbury, 1996).

Vaccination against RHD is a relatively effective preventive measure (Calvete *et al*., 1995a), although it is impossible to vaccinate all individuals in a population *en masse* and therefore the pathogenic agents persist and systematically reappear. Research is currently under way to prepare a new vaccine to effectively protect wild rabbits from two diseases (including myxomatosis). The effects of the vaccine will be transferable and will propagate naturally. The final result will be a recombinant vaccine (Bárcena *et al*., 2000; Torres *et al.,* 2001) that would provide acceptable immunity to myxomatosis and RHD but have limited transmissibility. The line of study developing this vaccine currently appears to have been discontinued.

Consequently, in light of the difficulties of mass vaccination, natural regeneration of populations should be encouraged using appropriate management to encourage environmental factors that have proven to enhance resistance to both enzootic diseases. The current situation must therefore be accurately diagnosed to establish appropriate management by determining which variables provide natural resistance to specific rabbit populations.

To preserve and appropriately manage rabbit populations, it is necessary to have detailed information that is normally collected through extensive, costly fieldwork. However, a first-rate source for collecting biological data is available and was used in this thesis. Animals killed through hunting provide real, highly accurate information on various biomedical parameters. The combination of these data, the information provided by RHDV hosts, and other ecological variables made it possible to prepare this thesis as a way to extend knowledge on essential aspects of RHDV epidemiology that could be useful for improving the management of this species.

12. References

- Aldama, J., 1993. Ecología energética y reproductiva del lince ibérico (*Lynx pardina* Temminck, 1824) en Doñana. Tesis Doctoral, Universidad Autónoma de Madrid. España.
- Alves, P.C., Gonçalves, H., Ferrand, N., 1998. Biologia do coelho-bravo (*Oryctolagus cuniculus algirus*). IV Técnicas de repovoamento. Direcçao-Geral das Florestas, 1-10. Portugal.
- Alves, J., Vingada, J., Rodrigues, P., 2006. The wild rabbit (*Oryctolagus cuniculus*) diet on a sand dune area in central Portugal: a contribution towards management. Wildlife Biology in Practice 2: 63-71.
- Anderson, M., Dahlback, M., Meurling, P., 1979. Biology of the wild rabbit, *Oryctolagus cuniculus*, in southern Sweden. I. Breeding season. Viltrevy 11: 103-127.

- Angulo, E., 2004. Factores que afectan a la distribución y abundancia del conejo en Andalucía. Tesis Doctoral, Universidad Complutense de Madrid.
- Angulo, E., Villafuerte, R., 2003. Modelling hunting strategies for the conservation of wild rabbit populations. Biological Conservation 115: 291-301.
- Aragaõ, H.B., 1927. Myxoma of rabbits. Memorias do Instituto Oswaldo Cruz (Rio de Janeiro), 20: 225-247.
- Asgari, S., Hardy, J.R., Sinclair, R.G., Cooke, B.D., 1998. Field evidence for mechanical transmission of rabbit haemorrhagic disease virus (RHDV) by flies (Diptera: Calliphoridae) among wild rabbits in Australia. Virus Research 54: 123–132.
- Aubineau, J., Biadi, F., Tesson, J.L. 1985. Le lapin de garenne: exemple d'aménagement et de gestion d'un territoire de chasse en milieu bocager (Deux-Sèvres). Bulletin Mensuel de l'Office National de la Chasse 89: 21-23.
- Bárcena, J., Morales, M., Vázquez, B., Boga, J.A., Parra, F., Lucientes, J., Pagès-Manté, A., Sánchez-Vizcaíno, J.M., Blasco, R., Torres, J.M., 2000. Horizontal Transmissible Protection against Myxomatosis and Rabbit Hemorrhagic Disease by Using a Recombinant Myxoma Virus. Journal of Virology, 74 (3): 1114-1123.
- Bennett, E.L., Robinson, J.G., 2000. Hunting for the Snark. In: Hunting for sustainability in tropical forests (Eds Robinson, J.G. & Bennett E.L.), pp. 13-30. New York, USA: Columbia University press.
- Best, S.M., Kerr, P.J., 2002. Coevolution of host and virus: The pathogenesis of virulent and attenuated strains of myxoma virus in resistant and susceptible European rabbits. Virology 267: 36-48.
- Biadi, F., Le Gall, A., 1993. Le lapin de garenne. Vie, Gestion et Chasse d'un gibier authentique. Office National de la Chasse. Ed Hatier. Paris.
- Biju-Duval, C., Ennafaa, H., Dennebouy, N., Monnerot, M., Mignotte, F., Soriguer, R., Gaaïed, E., Hili, A.E., Mounolou, A., 1991. Mitochondrial DNA evolution in lagomorphs: origin of systematic heteroplasmy and organization of diversity in European rabbits. Journal of Molecular Evolution 33: 92-102.
- Blanco-Aguiar, J.A., Virgós, E., Villafuerte, R., 2004. Perdiz Roja (*Alectorisrufa*). In: Libro Rojo de las Aves de España. (EdsMadroño, A., González, C. & Atienza, J.C.), pp. 182-185. Dirección General para la Biodiversidad-SEO/BirdLife, Madrid, España.
- Blanco-Aguiar, J.A., 2007. Variación espacial en la biología de la perdiz roja (*Alectoris rufa*): una aproximación multidisciplinar. Tesis Doctoral, Universidad Complutense de Madrid, España.
- Blanco, J.C., Villafuerte, R., 1993. Factores ecológicos que influyen sobre las poblaciones de conejos. Incidencia de la enfermedad hemorrágica. ICONA. Madrid.
- Blázquez, M., Villafuerte, R., 1990. Nesting of the Montpellier snake (*Malpolon monspessulanus*) inside rabbit warrens at Doñana National Park (Spain): phenology and a probable case of communal nesting. Journal of Zoology 222: 692-693.

- Bloemendal, H., 1977. The vertebrate eye lens. Science 197(4299): 127-138.
- Boots, M., Hudson, P.J., Sasaki, A., 2004. Large shifts in pathogen virulence relate to host population structure. Science 303: 842-844.
- Branco, M., Ferrand, N., Monnerot, M., 2000. Phylogeography of the European rabbit (*Oryctolagus cuniculus*) on the Iberian Peninsula inferred from RFLP analysis of the cytochrome b gene. Heredity 85: 307-317.
- Branco, M., Monnerot, M., Ferrand, N., Templeton, A.R., 2002. Postglacial dispersal of the European rabbit (*Oryctolagus cuniculus*) on the Iberian Peninsula reconstructed from nested clade and mismatch analyses of the mitochondrial DNA genetic variation. Evolution, 56: 792-803.
- Bro, E., Sarrazin, J., Clobert, J., Reitz, F., 2000. Demography and the decline of the grey partridge *Perdixperdix* in France. Journal of Applied Ecology 37: 432-448.
- Callou, C., 1995. Modifications de l'aire de répartition du lapin (*Oryctolagus cuniculus*) en France et en Espagne, du Pléistocène à l'époque actuelle. État de la question. Anthropozoologica 21: 95-110.
- Callou, C., 1997. Biogeographic history of the rabbit (*Oryctolagus cuniculus*) since the late glaciation: new data. Proceedings of the XIIth Lagomorph Workshop, Clermont-Ferrand, France, 8-11, July, 1996. Gibier Faune Sauvage 14(3): 501-502.
- Calvete, C., 2002. Biología y gestión del conejo silvestre. Cuadernos de caza y pesca de Aragón. Gobierno de Aragón. Zaragoza.
- Calvete, C., 2011. ¿Es posible hiperinmunizar a las poblaciones de conejos frente a la enfermedad hemorrágica? Quercus 309: 18-22.
- Calvete, C., Lucientes, J., Osácar, J., Villafuerte, R., 1994. Conejo silvestre. ¿Cómo repoblar? Mundo cinegético 1: 50-55.
- Calvete, C., Estrada, R., Osácar, J.J., Lucientes, J., 1995. Conejos de repoblación. In: La hibridación pone en peligro la pureza de nuestras especies. Trofeo 305: 18-32.
- Calvete, C., Estrada, R., Villafuerte, R., Osácar, J.J., Lucientes, J., 1995a. Primeros resultados en campo sobre la enfermedad hemorrágica del conejo. Trofeo 304: 22-28.
- Calvete, C., Villafuerte, R., Lucientes, J., Osácar, J.J., 1997. Effectiveness of traditional wild rabbit restocking in Spain. Journal of Zoology 241: 271-277.
- Calvete, C., Estrada, R., 2000. Epidemiología de Enfermedad Hemorrágica (VHD) y Mixomatosis en el Conejo de monte en el Valle Medio del Ebro. Herramientas de Gestión. Publicaciones del Consejo de Protección de la Naturaleza de Aragón. Serie Investigación. 175 pp.
- Calvete, C., Estrada, R., Villafuerte, R., Osácar, J.J., Lucientes, J., 2002. Epidemiology of viral hemorrhagic disease and myxomatosis in a free-living population of wild rabbits. Veterinary Record 150: 776-782.

- Calvete, C., Estrada, R., 2004. Short-term survival and dispersal of translocated European wild rabbits. Improving there lease protocol. Biological Conservation 120: 507-516.
- Calvete, C., Angulo, E., Estrada, R., 2005. Conservation of European wild rabbit populations when hunting is age and sex selective. Biological Conservation 121: 623-634.
- Calvete, C., Angulo, E., Estrada, R., Moreno, S., Villafuerte, R., 2005a. Quarantine length and survival of translocated European wild rabbits. Journal of Wildlife Management 69(3): 1063-1072.
- Calzada, J., 2000. Selección de presa e impacto de depredación del lince y el zorro sobre el conejo en el Parque Nacional de Doñana. Tesis Doctoral, Universidad de León.
- Cancellotti, F.M., Renzi, M., 1991. Epidemiology and current situation of viral haemorrhagic disease of rabbits and the European brown hare syndrome in Italy. Revue Scientifique et Technique. Office International des Epizooties, 10 (2): 409-418.
- Castro, F., Ramírez, E., Ferreira, C., Aparicio, F., Álvaro, P.J. Manners, RE., Redpath, S., Villafuerte, R., 2011. ¿*Algirus* o *cuniculus*? Pequeñas y grandes diferencias. In: Díaz-Portero, M.A., Sánchez, J.F. Robles, M., (Eds). II Congreso internacional sobre el conejo de monte. Ponencias y comunicaciones. Proyecto LIFE+ 07 NAT/E/000742 "Priorimancha". Toledo 2011.
- Ceballos, O., Leránoz, I., Urmeneta, A., Albizu, C., 1997. Estudio del ciclo biológico del conejo de monte en Navarra. Boletín de Información Técnica sobre Especies Cinegéticas 4.
- Chasey, D., 1994. Possible origin of rabbit haemorrhagic disease in the United Kingdom. Veterinary Record 135: 496-499.
- Cooke, B.D., 1981. Rabbit control and the conservation of native mallee vegetation on roadsides in South Australia. Australian Wildlife Research 8: 627-636.
- Cooke, B.D., 2002. Rabbit haemorrhagic disease: field epidemiology and the management of wild rabbit populations. Revue Scientifique et Technique. Office International des Épizooties 21: 347-358.
- Corbet, G. B., 1986. Relationships and origins of the European lagomorphs. MammalReview 16 (3/4): 105-110.
- Corbet, G.B. 1994. Taxonomy and origins. The European Rabbit: the History and Biology of a Successful Coloniser (Eds Thompson, H.V. & King, C.M.), 1-7. Oxford Science Publications, Oxford, UK.
- Cortázar, G., Gómez, F., Merchán, T., Serrano, S., Hidalgo de Trucios, S.J., Rocha, G., 2005. Caracterización genética del conejo de monte en Extremadura. In: López Caballero, J.M. (Ed.): Conservación de la naturaleza en Extremadura. Comunicaciones en Jornadas y Congresos 2002-2004. Consejería de Agricultura y Medio Ambiente. Junta de Extremadura. Mérida. Pp. 11-22.
- Cotilla, I., Villafuerte, R., 2003. Empleo de modelos para la mejora de los traslados de conejo. Libro de resúmenes de las VI Jornadas de la Sociedad Española para la Conservación y Estudio de los Mamíferos. Ciudad Real, 50.

- Covisa, J., 1998. Ordenacióncinegética: Proyectos de Ordenación y Planes Técnicos. Auryn, S.A. Madrid.
- Cowan, D.P., 1987. Aspects of the Social Organisation of the European Wild Rabbit (*Oryctolagus cuniculus*). Ethology 75: 197-210.
- Crawley, M.J., Weiner, J., 1991. Plant size variation and vertebrate herbivory: winter wheat grazed by rabbits. Journal of Applied Ecology 28: 154-172.
- Dedek, J., Frölich, K., 1997. Serological survey for calicivirus antibody among free-living red foxes in Mecklenburg-Vorpommern, Germany. [in German]. Tierärztliche Umschau 52, 149-152.
- Delibes, M., Hiraldo, F., 1981. The rabbit as prey in the Mediterranean Ecosystem. In: Myers, K. & Mclnnes, C.D., (Eds). Proceedings of the World Lagomorph Conference. Guelph, Ontario, pp. 601-613.
- Delibes-Mateos, M., Delibes, M., Ferreras, P., Villafuerte, R., 2008. Key role of European rabbits in the conservation of the Western Mediterranean basin hotspot. Conservation Biology 22: 1106–1117.
- Dellafiore, C.M., Muñoz Vallés, S., Gallego Fernández, J.B., 2006. Rabbits (*Oryctolagus cuniculus*) as dispersers of *Retama monosperma* seeds in a coastal dune system. EcoScience 13: 5-10.
- Dudzinski, M.L., Mykytowycz, R., 1961. The eye lens as an indicator of age in the wild rabbit in Australia. CSIRO. Wildlife Research 6(2): 156 – 159.
- Ennafaa, H., Monnerot, M., Gaaied, A., Mounolou, J.C., 1987. Rabbit mitochondrial DNA: preliminary comparison between some domestic and wild animals. Genetique, Selection, Evolution 19(3): 279-288.
- Fenner, F., Ratcliffe, F. N., 1965. Myxomatosis. Cambridge University Press, Cambridge, England.
- Fenner, F., Ross, J., 1994. Myxomatosis. The European rabbit: the history and biology of a successful colonizer (Eds Thompson, H.V. & King, C.M.), 205-239. Oxford University Press, Oxford.
- Fenner, F., Fantini, B., 1999. Biological control of vertebrate pests: the history of myxomatosis, an experiment in evolution. Oxon, UK: CABI Publishing.
- Ferrand, N., 1995. Variçaogenética de proteinasempoulaçoes de coelho (*Oryctolagus cuniculus*). Unpublished doctoral thesis, Universidade do Porto.
- Ferrand, F., Gonçalves, H., Alves P.C., 1998. Biologia do coelho-bravo (*Oryctolagus cuniculus algirus*). III Identificaçao da proveniència de coelhos utilizados emrepovoamentos. Ed. DirecçaoGeral de Forestas. 9 pp.
- Ferrer, M., Negro, J.J., 2004. The Near Extinction of Two Large European Predators: Super Specialists Pay a Price. Conservation Biology 18: 344-349.
- Flux, J.E.C., Fullagar, P. J., 1992. World distribution of the rabbit *Oryctolagus cuniculus* on islands. Mammal Review, 22(3/4): 151-205.

- Flux, J.E.C., 1994. World distribution. In: Thompson, H.V., King, C.M., (Eds). The European rabbit: the history and biology of a successful colonizer. Oxford University Press, Oxford. Pp 8-21.
- Forrester, N.L., Trout, R.C., Turner, S.L., Nelly, D., Boag, B., Moss, S., Gould, E.A., 2006. Unravelling the paradox of rabbit haemorrhagic disease virus emergence, using phylogenetic analysis; possible implications for rabbit conservation strategies. Conservation Biology 131: 296-306.
- Frölich, K., Klima, F., Dedek, J., 1998. Antibodies against Rabbit haemorrhagic disease virus in free-ranging red foxes from Germany. Journal of Wildlife Diseases 34: 436-442.
- Galante, E., Cartagena, M.C., 1999. Comparison of Mediterranean dung beetles (Coleoptera: Scarabaeoidea) in cattle and rabbit dung. Environmental Entomology 28: 420-424.
- Gálvez, L., 2008. El conejo europeo (*Oryctolagus cuniculus*) como especie ingeniera de ecosistemas. Tesis doctoral, Universidad de Alcalá, Madrid.
- Gálvez, L., Belliure, J., Rebollo, S., 2009. European rabbits as ecosystem engineers: warrens increase lizard density and diversity. Biodiversity and Conservation 18: 869-885.
- García, J.F., 2005. Manual técnico para el fomento de las poblaciones de conejo. Informe inédito. Dirección General para la Biodiversidad. Ministerio de Medio Ambiente. Madrid.
- Gea-Izquierdo, G., Muñoz, J., San Miguel, A., 2005. Rabbit warren distribution in relation to pasture communities in Mediterranean habitats: consequences for management of rabbit populations. Wildlife Research 32: 1-9.
- Geraldes, A., Ferrand, N., Nachman, M.W., 2006. Contrasting patterns of introgression at X-linked loci across the hybrid zone between subspecies of the European rabbit (*Oryctolagus cuniculus*). Genetics 173: 919-933.
- Gibb, J.A., 1993. Sociality, time and space in a sparse population of rabbits (*Oryctolagus cuniculus*). Journal of Zoology 229: 581-607.
- Gómez, F., Cortázar, G., Merchán, T., Rocha, G., Serrano, S. Hidalgo de Trucios, S.J., 2005. Distribución y abundancia del conejo de monte (*Oryctolagus cuniculus*) en Extremadura. In: J.M. López Caballero (Ed.). Conservación de la naturaleza en Extremadura. Comunicaciones en Jornadas y Congresos 2002-2004. Pp. 11-22. Consejería de Agricultura y Medio Ambiente. Junta de Extremadura. Mérida.
- Gonçalves, H., Alves, P.C., Rocha, A., 2002. Seasonal variation in the reproductive activity of the wild rabbit (*Oryctolagus cuniculus algirus*) in a Mediterranean ecosystem. Wildlife Research 29: 165-173.
- González Redondo, P., 1994. Cómo criar conejos de monte en cautividad. Federcaza 108: 93-96.
- González Redondo, P., 1995. Expedición y transporte de conejos de monte para repoblación cinegética. Boletín de Cunicultura 79 (18, 3): 48-50.
- González Redondo, P., 1996. Cómo reintroducir conejos de monte. Federcaza 123: 10-17.
- González Redondo, P., 1998. ¿Criar conejos de monte en cautividad? Federcaza 154: 22-26.

- González Redondo, P., 2001. Reproducción en cautividad del conejo de monte genéticamente puro destinado a la repoblación cinegética. 1er Congreso Internacional sobre el conejo de monte como recurso cinegético y ecológico. Cáceres.
- Guzmán, J.N., García, F.J., Garrote, G., Pérez de Ayala, R., Iglesias, M.C., 2004. El Lince ibérico (*Lynx pardinus*) en España y Portugal. Censo-diagnóstico de sus poblaciones. DGCN, Ministerio de Medio Ambiente, Madrid.
- Hardy, C., Vigne, J.D., Casane, D., Dennebouy, N., Mounolou, J. C., Monnerot, M., 1994. Origin of European Rabbit (*Oryctolagus cuniculus*) in A Mediterranean Island - Zooarchaeology and Ancient DNA Examination. Journal of Evolutionary Biology 7: 217-226.
- Henning, J., Meers, J., Davies, P.R., Morris, R.S., 2005. Survival of rabbit haemorrhagic disease virus (RHDV) in the environment. Epidemiology and Infection, 133(4): 719-730.
- Hidalgo de Trucios, S., 2001. Revisando la situación del conejo. Trofeo, 379: 44-52.
- Hidalgo de Trucios, S.J., Merchán Sánchez, T., Gómez Correa, F., Cortázar Hurtado, G., Rocha Camarero, G., Serrano Pérez, S., 2005. Distribución del meloncillo (*Herpestes ichneumon*) en Extremadura. In: López Caballero, J.M. (Ed.). Conservación de la naturaleza en Extremadura. Comunicaciones en Jornadas y Congresos 2002-2004. Pp. 11-22. Consejería de Agricultura y Medio Ambiente. Junta de Extremadura. Mérida.
- Hirakawa, H., 2001. Coprophagy in leporids and other mammalian herbivores. Mammal Review 31: 61-80.
- Hone, J., 1999. On the rate of increase (r): patterns of variation in Australian mammals and the implications for wildlife management. Journal of Wildlife Management 36: 709- 718.
- Hudson, J.R., Thompson, H.V., Mansi, W., 1955. Myxoma virus in Britain. Nature 176: 783.
- Hudson, R., Schaal, B., Bilko, A., Altbäcker, V., 1996. Just three minutes a day: The behaviour of young rabbits viewed in the context of limited maternal care. Proceedings of the 6th World Rabbit Congress. Toulouse (2): 395-403.
- Kauhala, K., Soveri, T., 2001. An evaluation of methods for distinguishing between juvenile and adult mountain hares *Lepus timidus*. Wildlife Biology 7: 295-300.
- Kolb, H.H., 1991. Use of burrows and movements of wild rabbit (*Oryctolagus cuniculus*) in an area of hill grazing and forestry. Journal of Applied Ecology 28: 282-905.
- Leighton, F., Artois, M., Capucci, L., Gavier-Widen, D., Morisse, J., 1995. Antibody response to rabbit viral hemorrhagic disease virus in red foxes (*Vulpes vulpes*) consuming livers of infected rabbits (*Oryctolagus cuniculus*). Journal of Wildlife Diseases 31(4): 541-544.
- Lenghaus, C., Westbury, H., Collins, B., Ratnamohan, N., Morrissy, C., 1994. Overview of the RHD project in Australia. In 'Rabbit Haemorrhagic Disease: Issues in Assessment for Biological Control'. (Eds Munro, R.K. &. Williams, R.T.). Pp. 104-129. (Bureau of Resource Sciences, Australian Government Publishing Service: Canberra).

- Letty, J., Marchandeau, S., Clobert, J., 1998. Étude expérimentale de différents facteurs affectant la réussite des repeuplements de lapin de garenne (*Oryctolagus cuniculus*). Gibier Faune Sauvage. Game and Wildlife 15: 453-464.
- Letty, J., Marchandeau, S., Clobert, J., Aubineau, J., 2000. Improving translocation success: an experimental study of antistress treatment and release method for wild rabbits. Animal Conservation 3: 211-219.
- Letty, J., Marchandeau, S., Reitz, F., Clobert, J., Sarrazin, F., 2002. Survival and movements of translocated wild rabbits (*Oryctolagus cuniculus*). Game and Wildlife Science 9 (1): 1-23.
- Letty, J., Aubineau, J., Marchandeau, S., Claubert, J., 2003. Effect of translocation on survival in wild rabbit (*Orycyolagus cuniculus*). Mammalian Biology 68: 250-255.
- Linhart, S.B., Roberts, J.D., Dasch, G.J., 1982. Electric fencing reduces coyote predation on pastured sheep. Journal of Range Management 35(3): 276-281.
- Linnel, J.D.C., Strand, O., 2002. Interference interactions, co-existence and conservation of mammalian carnivores. Diversity and Distributions 6: 169-176.
- Liu, S.J., Xue, H.P., Pu, B.Q., Quian, N.H., 1984. A new viral disease in rabbits. Animal Husbandry and Veterinary Medicine 16: 253-255.
- Lombardi, L., Fernández, N., Moreno, S., Villafuerte, R., 2003. Habitat-related differences in rabbit (*Oryctolagus cuniculus*) abundance, distribution, and activity. Journal of Mammalogy 84: 26-36.
- López-Martínez, N. 1977. Nuevos lagomorfos del neogeno y cuaternario español. TrabajosNeogeno-Cuaternario 8: 7-45. In: Thompson, H. V., King, C.M. (Eds). The European rabbit. The history and biology of a successful colonizer. Oxford Science Publications.
- López Martínez, N., 1989. Revisión sistemática y bio-estratigráfica de los Lagomorpha (Mammalia) del Terciario y Cuaternario inferior de España, 1 edn. Memoria Museo Paleontológico. Universidad de Zaragoza, 3, Zaragoza.
- Malo, J.E., Jiménez, B., Suárez, F., 2000. Herbivory dunging and endozoochorous seed deposition in a Mediterranean dehesa. Journal of Rangeland Management 53: 322–328.
- Marboutin, E., Peroux, R., 1995. Survival pattern of European hare in a decreasing population. Journal of Applied Ecology 32: 809-816.
- Marchandeau, S., Ricci, J.C., Chantal, J., 1998. Taux de prévalencesérologique du virus de la maladie viral hémorragique (VHD) du lapin de garenne (*Oryctolagus cuniculus*) et de ses formes apparentées au sein de différentes populations sauvages de France. Mammalia 62: 95-103.
- Marchandeau, S., Chaval, Y., Le Goff, E., 2000. Prolonged decline in the abundance of wild European rabbits *Oryctolagus cuniculus* and high immunity level over three years following the arrival of rabbit haemorrhagic disease. Wildlife Biology 6: 141-147.

- MARM., 2008. Estrategia Nacional para la conservación de lince ibérico. In: Dirección General para la Conservación de la Naturaleza, Ministerio de Medio Ambiente, Madrid.http://www.catsg.org/iberianlynx/20_il-compendium/home/index-compatibility.htm
- Merchán, T., Rocha, G., Alda, F., Silva, E., Thompson, G., de Trucios, S.H., Pagés, A, 2011. Detection of rabbit haemorrhagic disease virus (RHDV) in nonspecific vertebrate hosts sympatric to the European wild rabbit (*Oryctolagus cuniculus*). Infection, Genetics and Evolution 11(6): 1469-74.
- Ministerio de Agricultura, Pesca y Alimentación, 2006. Anuario de estadística agroalimentaria (en www.mapya.es).
- MMA., 1999. Estrategia para la conservación del lince ibérico. Ministerio de Medio Ambiente, Madrid. 34 pp.
- Moreno, S., Villafuerte, R., 1995. Traditional management of scrubland for the conservation of rabbits *Oryctolagus cuniculus* and their predators in Doñana National Park, Spain. Biological Conservation 73: 81-85.
- Morisse, J.P., Le Gall, G., Boilletot, E., 1991. Hepatitis of viral origin in Leporidae: Introduction and aetiological hypothesis. Revues cientifique et technique (International Office of Epizootics) 10: 283-295.
- Moss, S.R., Turner, S. L., Trout, R. C., White, P. J., Hudson, P. J., Desai, A., Armesto, M., Forrester, N. L., Gould, E. A., 2002. Molecular epidemiology of Rabbit haemorrhagic disease virus. Journal of General Virology 83: 2461-2467.
- Muñoz-Goyanes, G., 1960. Anverso y reverso de la mixomatosis. Dirección General de Montes, Caza y Pesca Fluvial. Ministerio de Agricultura, Pesca y Alimentación. Madrid.
- Muzquiz, J.L., Alonso, J.L., Simón, M.C., Ortega, C., 1997. Estudio epidemiológico de la mixomatosis y de la enfermedad Vírica Hemorrágica del conejo de monte (VHD) en la Comunidad Foral de Navarra. Universidad de Zaragoza (Ed.) In: Boletín de Información Técnica Sobre Especies Cinegéticas nº 4. Estudios sobre el conejo silvestre. Gestión de la población en Navarra. Navarra. Dirección General de Medio Ambiente. Gobierno de Navarra, pp: 47-68.
- Myers, K., Poole, W.E., 1959. A study of the biology of the wild rabbit, *Oryctolagus cuniculus* (L.), in confined populations. I. The effects of density on home range and the formation of breeding groups. CSIRO Wildlife Research 4: 14-26.
- Myers, K., 1964. Influence of density on fecundity, growth rates and mortality in the wild rabbit. CSIRO Wildlife Research 9: 134-137.
- Myers, K., Parer, I., Wood, D. H., Cooke, B. D., 1994. The rabbit in Australia. The European rabbit: the history and biology of a successful colonizer (Eds Thompson, H.V. & King, C.M.), 108-157. Oxford University Press, Oxford.
- Mykytowycz, R., 1959. Social behaviour of an experimental colony of wild rabbits, *Oryctolagus cuniculus* (L.). II. First breeding season. CSIRO Wildlife Research 4: 1-13.

- Nagesha, H.S., McColl, K.A., Collins, B.J., Morrissy, C.J., Wang, L.F., Westbury, H.A., 2000. The presence of cross-reactive antibodies to rabbit haemorrhagic disease virus in Australian wild rabbits prior to the escape of virus from quarantine. Archives of Virology 145: 749-757.
- Nowell, K., Jackson, R.M., 1996. Wild Cats: Status Survey and Conservation Action Plan. UICN, Berna, 762 pp.
- Ñudi, J.I., 1998. Cómo recuperar los conejos de un coto. Trofeo 362: 28- 34.
- Olinger, V.F., Thiel, H.J., 1991. Identification of the viral haemorrhagic disease of rabbits as a calicivirus. Revue scientifique et technique de l'Office international des épizooties 10: 311-323.
- Osácar, J.J., Lucientes, J., 1997. Estudio de los vectores de la mixomatosis del conejo de monte en Navarra. En Dirección General de Medio Ambiente. Gobierno de Navarra (Eds). Boletín de información técnica sobre especies cinegéticas. Estudios sobre el conejo conejo de monte. Gestión de la población de Navarra. Navarra. 4, pp: 24-45.
- Palma, L., Beja, P., Rodrigues, M., 1999. The use of sighting data to analyse Iberian lynx habitat and distribution. Journal of Applied Ecology 36: 812-824.
- Palomares, F., Delibes, M., 1993. Key habitat for Egyptian Mongooses in Doñana National Park (southwestern Spain). Journal of Applied Ecology 30: 752-758.
- Palomares, F., Gaona, P., Ferreras, P., Delibes, M., 1995. Positive effects on game species of top predators by controlling smaller predator populations: an example with lynx, mongooses, and rabbits. Conservation Biology 9: 295-305.
- Parer, I., 1982. Dispersal of the wild rabbit, *Oryctolagus cuniculus*, at Urana in New South Wales. Australian Wildlife Research 9: 427–441.
- Park, J.H., Lee, Y.S., Itakura, C., 1995. Pathogenesis of Acute Necrotic Hepatitis in Rabbit Hemorrhagic-Disease. Laboratory Animal Science 45: 445-449.
- Peiró, V., Seva, E., 1991. Maladie Haemorrhagique Virale du Lapin de garenne au sud-est de l'Espagne. XXth Congress of the International Union of Game Biologists, Godollo, Hungary, August 21-26: 752-758.
- Petterson, D., 2001. The effects of the wild rabbit (*Oryctolagus cuniculus*) on soils and vegetation in semi-arid, south-eastern Spain. Doctoral thesis. University of Leeds, United Kingdom.
- Queney, G., Ferrand, N., Marchandeau, S., Azevedo, M., Mougel, F., Branco, M., Monnerot, M., 2000. Absence of a genetic bottleneck in a wild rabbit (*Oryctolagus cuniculus*) population exposed to a severe viral epizootic. Molecular Ecology 9: 1253-1264.
- Queney, G., Ferrand, N., Weiss, S., Mougel, F., Monnerot, M., 2001. Stationary distributions of microsatellite loci between divergent population groups of the European Rabbit (*Oryctolagus cuniculus*). Molecular Biology and Evolution 18: 2169-2178.
- REGHAB, 2002. Reconciling gamebird hunting and Biodiversity. V Forework Program of the European Union. Proposalnumber: EKV-2000-00637. Project Coordinator: J. Viñuela.

- Reumer, J.W.H., Sanders, E.A.C., 1984. Changes in vertebrate fauna of Menorca in prehistoric and classical times. Zeitschriftfür Säugetierkunde 49: 321-325.
- Revilla, E., Palomares, F., Fernández, N., 2001. Characteristics, location and selection of diurnal resting dens by Eurasian badgers (*Meles meles*) in a low density area. Journal of Zoology 255: 291-299.
- Roberts, S.C., 1988. Social influenceonvigilance in rabbits. Animal Behaviour 36: 905-913.
- Roca, T., 1994. Rentabilidad de la explotación del conejo de monte. Boletín de Cunicultura 71 (17, 1): 37-42.
- Rogers, P.M., Arthur, C.P., Soriguer, R.C., 1994. The rabbit in continental Europe. In: Thompson, H.V. & King, C.M. (Eds). The European rabbit: the history and biology of a successful colonizer, pp. 22-63. Oxford University Press, Oxford.
- Ross, J., Tittensor, A.M., Fox, A.P., Sanders, M.F., 1989. Myxomatosis in farmland rabbit populations in England and Wales. Epidemiology and Infection 103: 333-357.
- Ruíz-Olmo, J., Blanch, F., Vidal, F., 2003. Relationships between the Red Fox and Waterbirds in the Ebro Delta Natural Park, N.E. Spain. Water birds, 26(2): 217-225.
- Sáez de Buruaga, M., Lucio, A., Purroy, F., 2001. Reconocimiento de sexo y edad en especies cinegéticas. Edilesa. 127 pp.
- Sánchez-Piñero, F., Ávila, J.M., 1991. Análisis comparativo de los Scarabaeoidea (Coleoptera) coprófagos en las deyecciones de conejo (*Oryctolagus cuniculus* L.) y de otros mamíferos: estudio preliminar. Eos 67: 23-24.
- Shivik, J. A., Treves, A., Callahan, P., 2003. Nonlethal Techniques for Managing Predation: Primary and Secondary Repellents. Conservation Biology 17(6): 1531-1537.
- Simón, M.C., Muguruza, R., Alonso, J.L., Muzquiz, J.L., Gironés, O. Haffar, A., 1994. Recherche du virus de la maladie hémorragique virale du lapin (RHD) chez le renard et rôle des canidés domestiques dans la transmission de la maladie. Recueil de Médecine Vétérinaire 170 (12): 841-845.
- Soriguer R. C., 1981. Biología y dinámica de una población de conejos (*Oryctolagus cuniculus* L.) en Andalucía Occidental. Doñana Acta Vertebrata 8(3): 1-378.
- Soriguer, R., 1988. Alimentación del conejo (*Oryctolagus cuniculus* L. 1758) en Doñana. SO, España. Doñana Acta Vertebrata 15: 141-150.
- Soriguer, R., Rogers, P.M., 1979. The European wild rabbit in Mediterranean Spain. In Myers, K. MacInnes, C.D. (Eds). Proceedings of the World Lagomorph Conference held in Guelph. University of Guelph, Ontario, pp: 601-613.
- Surridge, A. K., Bell, D. J., Hewitt, G.M., 1999. From population structure to individual behaviour: genetic analysis of social structure in the European wild rabbit (*Oryctolagus cuniculus*). Biological Journal of the Linnean Society 68: 57-71.

- Sutherland, W.J., 2001. Sustainable exploitation: a review of principles and methods. Wildlife Biology, 7: 131-140.
- Thompson, H.V., King, C.M., 1994. The European rabbit: the history and biology of a successful colonizer. Oxford University Press, Oxford.
- Torres, J.M., Ramírez, M.A., Morales, M., Bárcena, J., Vázquez, B., Espuña, E., Pagès-Manté, A., Sánchez-Vizcaíno, J.M., 2001. Safety evaluation of a recombinant myxoma-RHDV virus inducing horizontal transmissible protection against myxomatosis and rabbit haemorrhagic disease. Vaccine 19: 174-182.
- Trout, R.C., Langton, S., Smith, G.C., Haines-Young, R.H., 2000. Factors affecting the abundance of rabbits (*Oryctolagus cuniculus*) in England and Wales. Journal Zoological London 252: 227-238.
- Twigg, L.E., Lowe, T.J., Martin, G.R., Wheeler, A.G., Gray, G.S., Griffin, S.L., O'Reilly, C.M., Robinson, D.J., Hubach, P.H., 2000. Effects of surgically imposed sterility on free-ranging rabbit populations. Journal of Applied Ecology 37: 16-39.
- Ueda, K., 1992. Pathology of Rabbit Hemorrhagic-Disease (RHD) - Pathology of Disseminated Intravascular Coagulation (DIC). Japanese Journal of Veterinary Research 40: 64.
- Valverde, J.A., 1963. Información sobre el lince español. Servicio Nacional de Pesca Fluvial y Caza, Madrid, 42 pp.
- Van der Wal, R., Van Wijnen, H., Van Wieren, S., Beucher, O., Bos, D., 2000. On facilitation between herbivores: how Brent Geese profit from brown hares. Ecology 81: 969-980.
- Villafuerte, R., Calvete, C., Gortázar, C., Moreno, S., 1994. First epizootic of rabbit hemorrhagic disease in free living populations of *Oryctolagus cuniculus* at Doñana National Park, Spain. Journal of Wildlife Disease 30: 176-179.
- Villafuerte, R., Calvete, C., Blanco, J. C., Lucientes, J., 1995. Incidence of viral haemorrhagic disease in wild rabbit populations in Spain. Mammalia 59: 651-659.
- Villafuerte, R., Lazo, A., Moreno, S., 1997. Influence of food abundance and quality on rabbit fluctuations: conservation and management implications in Doñana National Park (SW Spain). Revista Ecología. (Terre Vie) 52: 345-356.
- Villafuerte, R., Moreno, S., 1997. Predation risk, cover type, and group size in European rabbits in Doñana (SW Spain). Acta Theriologica 42 (2): 225-230.
- Villafuerte, R., Viñuela, J., Blanco, J.C., 1998. Extensive predator persecution caused by population crash in a game species: the case of red kites and rabbits in Spain. Biological Conservation 84: 181-188.
- Villafuerte, R., 2002. "*Oryctolagus cuniculus*" in Atlas de los Mamíferos Terrestres de España, pp: 464-467, Dirección General de Conservación de la Naturaleza-SECEM-SECEMU. Madrid.
- Virgós, E., Travaini, A., 2005. Relationship between Small-game Hunting and Carnivore Diversity in Central Spain. Biodiversity and Conservation 14: 3475-3486.

- Virgós, E., Cabezas, S., Lozano, J., 2005. El declive del conejo en España. Quercus 236: 16-21.
- Vitale, A.F., 1989. Pattern of dispersion of young wild rabbits, *Oryctolagus cuniculus* L., in relation to burrows. Ethology 83(4): 306-315.
- Westbury, H., 1996. Field Evaluations of RCD under Quarantine. Final Report of Project CS. 236, Meat Research Corporation, Sydney, Australia.
- Wheeler, S.H., King, D. R., 1985. The European Rabbit in South-western Australia II. Reproduction. Australian Wildlife Research 12: 197-212.
- Willot, S.J., Miller, A.J., Incoll, L.D., Compton, S.G., 2000. The contribution of rabbits (*Oryctolagus cuniculus* L.) to soil fertility in semi-arid Spain. Biology and Fertility of Soils 31: 379-384.
- Wood, D.H., 1988. Estimating rabbit density by counting dung pellets. Australian Wildlife Research 15: 665-671.
- Xu, W.Y., 1991. Viral haemorrhagic disease of rabbits in the People's Republic of China: epidemiology and virus characterisation. Revue scientifique et technique de l'Office international des épizooties 10: 393-408.
- Zamora, M., Peinado, E., Sánchez, M., Gallego, B., Mata, C., 1985. Consumo de bellota por conejos en pastoreo continuo. Archivos de Zootecnia 34: 257-264.

THESIS STRUCTURE AND OBJECTIVES

THESIS STRUCTURE AND OBJECTIVES

1. Thesis structure

The European wild rabbit is a species of immeasurable value, as indicated in the Introduction. The situation in Extremadura, as in the rest of the Iberian Peninsula except the central area, parts of Navarre and the Levante and specific areas where rabbits are pests, can be considered irregular, with a tendency towards decline. IUCN criteria and the findings of studies in the Peninsula based on hunting statistics indicate a drop in most wild rabbit populations in Spain, which could lead to classification of the species as "Endangered". However, the current situation is quite complex and wild rabbits are fought against in areas where they are a threat to agriculture. Protecting the wild rabbit is no easy task, as many factors now come into play in rabbit management. Whether populations are conserved or exterminated depends on the area where they occur, and in between these extremes, it is possible to find highly divergent opinions and interests depending on which sector is consulted.

Nonetheless, management of this species, regardless of the reasons behind it, must rely on knowledge as a starting point. Many key aspects in rabbit management are known, such as the importance of maintaining suitable habitats for rabbit development, the positive effect of the existence of apex predators, the impact of hunting management, and issues associated with the importance of maintaining the gene pool. However, little is known about the epidemiological variables that affect, in particular, the most recently emerged viral disease, or whether current knowledge can be integrated with the considerations mentioned above as a way to achieve more adequate population control and protect rabbit populations.

This ECO-EPIDEMIOLOGY thesis therefore focuses on the study of the diverse factors involved, with a higher or lower impact, in the emergence of haemorrhagic viral disease in wild rabbits. The aim is to address a number of systems of associated causes and factors that are interdependent and may exert some influence. The main indications that this thesis addresses ecopathology are its extended time frame and the action of intermediate hosts, and in particular its treatment of a group pathology. The thesis therefore attempts to identify the factors that condition the appearance of this disease and determine their role. It focuses primarily on observational epidemiological studies in the field and includes an experimental study in laboratory conditions.

This is the first study in Extremadura to address little-studied issues associated with the persistence and distribution of the haemorrhagic disease virus in the wild and the effect of its presence on representative samples of wild rabbit populations in Extremadura. All previous

observational epidemiological studies have been of the "cross-sectional or prevalence" variety, conducted with information on the presence or absence of disease and whether or not there has been exposure to a factor obtained at a single moment of time in a representative population sample.

A series of extensive studies was conducted from 2002 to 2009, requiring an amount of fieldwork that is difficult to quantify. In 2002 and 2003, contacts were established with the main participants in both conservation and hunting of wild rabbits. Visits were made to the Hunting Federation of Extremadura and other hunting organisations, such as recreational clubs. Frequent contact was maintained with the public administration to produce as much information about wild rabbits as possible.

To prepare epidemiology charts and obtain samples from 2003 to 2009, visits were made to locations where samples were easy to obtain and local hunters provided assistance. Questionnaires were used to gather data and at times samples were collected by the people assisting us with our work, who had received specific training and written guidelines to help them (see appendix).

The fieldwork to assess densities, collect samples and monitor populations required many visits to natural areas of Extremadura. Much of the work published in this thesis is therefore based on work carried out exclusively in the field and was intended for eminently practical and specific purposes. Throughout the study, the doctorate student took part in awareness-raising activities during conferences, talks and courses (see annex). Some of the chapters of this thesis are based on these activities.

For the articles on RHDV sympatric hosts, many rodents were sampled with the corresponding permission from the public authorities and all efforts were made to ensure the most ethical conduct possible when handling samples. The experiment to produce an outbreak of haemorrhagic disease in controlled conditions was performed in biosecurity conditions with the necessary permission both from the donors of the virus strain and the authorities of the University of Extremadura.

In one of the chapters, data from rabbits in Valladolid are used to compare their morphometry with rabbits in Extremadura. In another chapter, rabbit management is compared, ranging from areas with no management at all to an area where rabbits are kept in semi-extensive facilities.

Lastly, chapter VII shows the results of a rabbit translocation carried out in 2009 using rabbits from an area in Toledo. This required initial visits to the area to sample the populations and analyse their characteristics from an epidemiological point of view and to determine their mitochondrial lineage. At the same time, background studies were conducted on the population of the release area

and the suitability of the location. On completion of the translocation, it was considered that the objectives of this doctoral thesis had been met. These were:

2. Objectives

1. To sample rodent species (*Apodemus sylvaticus* and *Mus spretus*) to verify the presence of RHD virus using RT-PCR and obtain a natural infection percentage. This involves taking liver samples from captured specimens living sympatrically with wild rabbit populations. Another objective is to sequence the virus strains of the rodents to determine whether or not the strains identified are species-specific.

2. To produce an outbreak of RHD (in *A. sylvaticus* and *M. spretus*) in controlled laboratory conditions, where rabbits are infected while in direct contact with rodents from the wild to determine whether the rodents show symptoms of RHD after the onset of infection in the rabbits. A further objective is to monitor the time RHDV can be harboured inside non-susceptible hosts (which mice are). The final objective in this section is to create an infection and/or immune response in a susceptible rabbit in contact with an infected mouse.

3. To determine the percentage of an apparently healthy wild rabbit population that could be hosting RHDV and the immunological status of these individuals; i.e., to consider the existence of persistently infected rabbits and the implications of this situation for rabbit population management.

4. To study the immunological status of some rabbit populations in Extremadura to myxomatosis and haemorrhagic viral disease in autumn and to relate the data on seropositivity between the two diseases in the areas studied, including variables such as age, sex and abundance.

5. To verify whether haemorrhagic viral disease and myxomatosis interact in natural conditions in dense wild rabbit populations in the province of Badajoz and to relate the titrations detected to the different types of management applied to the study populations.

6. To compare rabbits from the south of Extremadura with rabbits from the northern meseta (Valladolid) from the perspective of morphometry and body condition and to extend information on geographical suitability in rabbit restocking.

7. To put into practice knowledge that combines information on habitat control, genetics and health aspects through a wild rabbit translocation project and to study the results of translocation monitoring and obtain conclusions that can be used as management tools in other translocations.

ORIGINAL PUBLICATIONS

ORIGINAL PUBLICATIONS

This thesis is based on the following papers andmanuscripts and a scientific communication in the form of a poster. The original papers have been kept virtually unchanged (Chapters I and II) and the remaining material has been expanded, revised and discussed to develop the thesis. The chapters are referred to in the text by Roman numerals I-VII.

CHAPTER I

Detection of rabbit haemorrhagic disease virus (RHDV) in nonspecific vertebrate hosts sympatric to the European rabbit (*Oryctolagus cuniculus*)

Merchán, T.[a], Rocha, G.[a], Hidalgo, S. J.[b], Silva, E.[c], Thompson, G.[d], Alda, F.[e], Pagés, A.[f]

[a] Departamento de Ingeniería del Medio Agronómico y Forestal, University of Extremadura. Avda. Virgen del Puerto, 2. 10600 Plasencia, Cáceres, Spain. tmerchan@unex.es, gregorio@unex.es

[b] Grupo en Recursos Cinegéticos y Biodiversidad (RCB). Veterinary School. University of Extremadura. 10071 Caceres, Spain. shidalgo@unex.es

[c] Multidisciplinary Unit of Biomedical Investigation (UMIB), University of Porto, P-4099-003 Porto, Portugal. eli@mail.icav.up.pt

[d] Department of Veterinary Clinics, Abel Salazar Institute of Biomedical Sciences (ICBAS), University of Porto, P-4099-003 Porto, Portugal gat1@mail.icav.up.pt

[e] Instituto de Investigación en Recursos Cinegéticos (CSIC-UCLM-JCCM). Ronda de Toledo s/n, 13005 Ciudad Real, Spain. alda.fernando@gmail.com

[f] Laboratorios Hipra S.A., La Selva, 135. 17170 Amer, Girona, Spain, apmante@gmail.com

Merchán, T., Rocha, G., Alda, F., Silva, E., Thompson, G., Hidalgo, S., Pagés, A., 2011. Detection of rabbit haemorrhagic disease virus (RHDV) in nonspecific vertebrate hosts sympatric to the European wild rabbit (Oryctolagus cuniculus). ***Infection, Genetics and Evolution 11(6): 1469-74.***

1. Resumen

Desde su identificación en China en 1984, la enfermedad hemorrágica vírica (RHD) ha sido objeto de numerosos estudios. Sin embargo, el origen evolutivo del virus de la enfermedad hemorrágica vírica (RHDV) sigue siendo discutido, al igual que siguen siendo desconocidos aspectos de la epidemiología de la enfermedad como los hospedadores donde se alberga el virus entre brotes de enfermedad.

Para detectar la presencia del RHDV en micromamíferos simpátricos con el conejo, se analizaron 51 roedores (29 *Mus spretus* y 22 *Apodemus sylvaticus*) y 31 conejos (*Oryctolagus cuniculus*) de una misma localidad del centro de España. En las muestras en las que se detectó el virus se secuenció un fragmento del gen de la proteína VP60 de la cápside del RHDV y se analizaron las relaciones filogenéticas entre ellas y otras cepas de RHDV de la Península Ibérica.

En total se identificaron tres cepas de virus en *Apodemus sylvaticus*, *Mus spretus* y *Oryctolagus cuniculus.* Todas las cepas se encontraron bien soportadas dentro del clado de RHDV aislados en conejos de la Península Ibérica. Además, una de las cepas se encontró en las tres especies estudiadas, lo cual sugiere la capacidad del RHDV de infectar otros mamíferos diferentes al conejo y aún no testados. Se discute la vía de entrada del virus así como sus implicaciones ecoepidemiológicas.

Palabras clave: Enfermedad hemorrágica vírica, RHDV, anfitrión, *Apodemus*, *Mus*, *Oryctolagus cuniculus*

2. Abstract

Since its detection in China in 1984, rabbit haemorrhagic disease (RHD) has been the subject of numerous studies. However, the evolutionary origin of rabbit haemorrhagic disease virus (RHDV) is still under debate, just as aspects of the epidemiology of the disease are still unknown, such as where the virus is hosted between outbreaks.

To detect the presence of RHDV in rabbit-sympatric mice, a study was made of 51 rodents (29 *Mus spretus* and 22 *Apodemus sylvaticus*) and 31 rabbits (*Oryctolagus cuniculus*) from the same location in central Spain. A fragment of the VP60 protein gene from the RHDV capsid was sequenced in the samples where the virus was detected and the phylogenetic relationships between them and other strains of RHDV in the Iberian Peninsula were analysed.

In total, three viral strains were identified in *A. sylvaticus*, *M. spretus* and *O. cuniculus*. All the strains were well supported within the clade of RHDV found in rabbits in the Iberian Peninsula. Moreover, one of the strains was found in all three species under study, which suggests the ability of RHDV to infect other mammals, apart from the rabbit, that have not been tested. The means of entry of the virus is discussed, as well as its eco-epidemiological implications.

Key words: *Rabbit Haemorrhagic Disease Virus, RHDV, host, Apodemus, Mus, Oryctolagus cuniculus*

3. Introduction

Rabbit haemorrhagic disease, first discovered in China in 1984 (Liu *et al*., 1984), has a high rate of morbidity (100%) and mortality (40-100%) in adult rabbits. RHD is caused by a positive-sense, single-stranded RNA, a member of the *Lagovirus* genus belonging to the Caliciviridae family (Parra & Prieto, 1990; Ohlinger & Thiel, 1991; Moussa *et al*., 1992).

This genus also includes the European brown hare syndrome virus (EBHSV), as well as the non-pathogenic rabbit calicivirus (RCV), which causes asymptomatic seroconversion in rabbits and is considered to be a potential non-pathogenic ancestor of RHDV (Capucci *et al*., 1996). Recently a new calicivirus has been described, named the Michigan rabbit calicivirus (MRCV), which causes subclinical infections and whose genome shows an average similarity of 79% with RHDV (Bergin *et al., 2*009).

Since its identification, RHDV has been the subject of numerous studies. However, some aspects, such as the evolutionary origin of the virus, are still unknown or under debate (Forrester *et al*., 2006; Kerr *et al*., 2009). Various hypotheses have been put forward to explain the origin of RHDV, such as (i) the transmission of EBHSV from hares to rabbits (Nowotny *et al.,* 1997), (ii) that changes were produced in a non-pathogenic virus, making it virulent (Moss *et al*., 2002), and (iii) the emergence of RHDV from a different virus infecting another species (Fenner & Fantini, 1999).

All Lagoviruses, with the exception of EBHSV, which affects only hares (*Lepus spp*.) (Löliger & Eskens, 1991), are specific to *Oryctolagus cuniculus*, both domestic and wild, although the latter are more sensitive to the virus (Argüello *et al*., 1988; Pagés, 1989). Even though genetically RHDV and EBHSV show a similarity of 71% and they are relatedantigenically, as shown by the presence of common internal epitopes (Wirblich *et al*., 1995), cross-infection does not occur (Lavazza *et al*., 1996). Similarly, experimental infection of various species of laboratory and wild animals with RHDV produced no symptoms of disease (Gavier-Widen *et al*., 1997; Nowotny *et al*., 1999; Pagés, 1989; Smid *et al*., 1991). Nor did other species of American leporids (eastern cottontail *Sylvilagus floridanus*, volcano rabbit *Romerolagus diazi*, and black-tailed jackrabbit *Lepus californicus*) prove clinically affected on experimental exposure to RHDV (Gregg *et al*., 1991). Likewise, non-target mammals during periods of viral activity in New Zealand were not affected (Parkes *et al*., 2004). Replication of RHDV in other hosts apart from the rabbit has not been demonstrated. In Australia, seroconversion has been detected as a response to the viral antigene in mice inoculated with the virus (Gould *et al*., 1997), and also in New Zealand, in kiwis (*Apteryx australis*) exposed to contaminated material (Buddle *et al*., 1997). Similarly, in red foxes (*Vulpes vulpes*) that ingested

rabbits infected with RHDV, post-infection antibody titres were detected, without the viral replication ever fully coming to light (Leighton *et al*., 1995).

Subclinically diseased or infected rabbits are the main sources of infection (Xu & Chen, 1989). Moreover, rabbits with persistent infection (Löliger & Eskens, 1991) and carriers (Cancellotti & Renzi, 1991; Cooke, 2002), as well as young rabbits under 20 days old that manage to survive the disease (Rosell *et al*., 1989), can also act as sources of the virus. However, possible hosts of the virus and their role in epidemiology have barely been studied. Some field and laboratory studies have shown how predatory mammals and birds play a role in the transmission of RHDV (Chasey, 1994; Gavier-Widen & Morner, 1993; Simon *et al*., 1994) by acting as mechanical reservoirs or vectors. Schirrmeier *et al*. (1990) carried out an experiment with rodents and insects, suggesting that these did not act as reservoirs, although they did act as possible passive transmitters of the virus. Other studies have proven the importance of the role played by insects in transmitting the disease (Asgari *et al*., 1998; Gehrmann & Kratzschmar, 1991; Lenghaus *et al., 1*994) and the role of decomposing remains of infected rabbits (McColl *et al*., 2002) or their warrens in housing RHDV (Calvete *et al*., 2002).

This paper analyses the possible presence of RHDV in two species of rodent (wood mouse *A. sylvaticus* and Algerian mouse *M. spretus*) and the European rabbit sharing the same habitat in a specific area in the centre of the Iberian Peninsula, as it is vital to extend knowledge of the role that other wild species which have not yet been tested may have in both the origin and the epidemiology of this disease.

4. Material and methods

4.1. Area of study and sampling

The area studied is one of high relative abundance of rabbits, with a 5 km radius approx., in the municipality of Plasenzuela, in Extremadura, Spain (39º22'N 6º02'W). The land is a rich thicket of mostly *Retama sphaerocarpa* shrubs interspersed with rocks and the occasional Holm oak (*Quercus ilex*). The climate is typically Mediterranean with mild winters and very hot summers and is neither thermally nor pluviometrically homogeneous (an average of <500 mm per annum).

From 05 to 25 July 2005, 51 rodents were caught: 29 Algerian mice (*M. spretus*, Ms1-Ms29) and 22 wood mice (*A. sylvaticus* As1-As22). The mice were captured live using "hypolithic" traps (Carro *et al*., 2007) measuring 40x10x10cm. Thirty one rabbits were also ensnared in the same area of study, where they were found in abundance. The live animal trapping was authorised by the

Regional Government of Extremadura (exp. nº CN04/2328) and the specimens of European rabbit were obtained by hunters during a legal hunting period.

The captured mice were euthanised *in situ* by cervical dislocation. Animals were placed in individual bags, labelled and refrigerated for transportation to the laboratory, where they were frozen at -20ºC.

Sampling consisted of extracting all or part of the liver from the animals under study. This task was carried out at different times using sterile laboratory material under strict biosecurity measures to avoid any cross-contamination. Similarly, the three species (*O. cuniculus, A. sylvaticus* and *M. spretus*) were treated in separate laboratories to ensure no contamination occurred. All the samples were processed at premises belonging to the Cáceres Veterinary School of the University of Extremadura (Spain).

4.2. Laboratory methods

Viral RNA was extracted from an organ homogenate of 0.1g in 1ml phosphate buffered saline (PBS) using proteinase K and TriReagent LS (Sigma) followed by phenol-chloroform extraction. cDNA was synthesised using random priming and M-MLV reverse transcriptase (Invitrogen). Nested PCR was used to partially amplify RHDV capsid protein VP60 (Moss *et al*. 2002). Primers RHDV1 and 4 were used for the first PCR and primers RHDV2 and 3 for the nested reaction. For both PCRs the cycling conditions were 95ºC for 9 min, followed by 30 cycles of 95ºC for 40s, 50ºC for 40s, 72ºC for 2 min and the final elongation of 72ºC for 10 min. The size of the PCR products obtained was determined by ethidium bromide staining of a 2% agarose gel electrophoresis. The expected size of the final PCR product was 527bp corresponding to the region covering positions 6151 to 6703 of the major capsid protein VP60 of RHDV of the Spanish RHDV strain AST89 (Z49271). All the sequences obtained were deposited in Genbank with the following access numbers: HQ198365-HQ198366, HQ198368-HQ198371 and HQ413340-HQ413341.

The molecular analyses were carried out in the Clinical Veterinary Practice Department laboratories at the Abel Salazar Biomedical Science Institute (ICBAS) of the University of Porto (Portugal).

4.3. Phylogenetic analysis of RHDV VP60

Chromatograms were checked by eye and translated into amino acids using MacClade 4.05 (Maddison & Maddison, 2000) and manually aligned with other homologous sequences available in

GenBank from the Iberian Peninsula (Müller *et al*., 2009; Alda *et al*., 2010) and representatives of RHDV Genogroups 1-6 previously described (Le Gall-Recoulé *et al*., 2003).

Phylogenetic relationships among all the RHDV strains were inferred using Bayesian Inference (BI) in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2008), simulating four simultaneous Markov chains (MCMC) for 4 x 10^6 generations each, with a sampling frequency of 100 generations. The first 250,000 generations were discarded as burn-in. Bayesian posterior probabilities were obtained to assess the robustness of the BI trees. As an outgroup, we used Rabbit Calicivirus (RCV) (X96868).

5. Results

5.1. Detection of RHDV in mice and rabbits

All the animals sampled appeared healthy. RHDV positivity was detected by molecular analysis in the liver of three mice (5.8%): two *A. sylvaticus* (As15 and As26) and one *M. spretus* (Ms8). The rabbits analysed similarly revealed no markers consistent with the disease and were apparently healthy. Of the 31 rabbits caught, the virus was detected in seven (23.5%), six of which provided analysable sequences (Oc1-Oc6).

In all the animals analysed, three strains of virus were found, according to the sequences obtained from the VP60 gene. All the viruses detected were found inside the clade formed by the sequences of RHDV previously detected in the Iberian Peninsula (Fig. 1). Three rabbits (Oc1, Oc3 and Oc6), one wood mouse (As15) and one Algerian mouse (Ms8) showed the same sequence of RHDV, identical to a strain identified in Portugal in 2004. This strain was very similar to those detected in two other rabbits (Oc4 and Oc5) and they grouped together to form a highly resistant clade, which was the sibling group of another series of strains located in Spain between 2004 and 2006 (Figure 1). The other two strains of RHDV identified, corresponding to one rabbit (Oc2) and one wood mouse (As26), grouped together to from a differentiated, resistant clade which contained much older strains, discovered in Spain and Portugal between 1994 and 2005 (Figure 1).

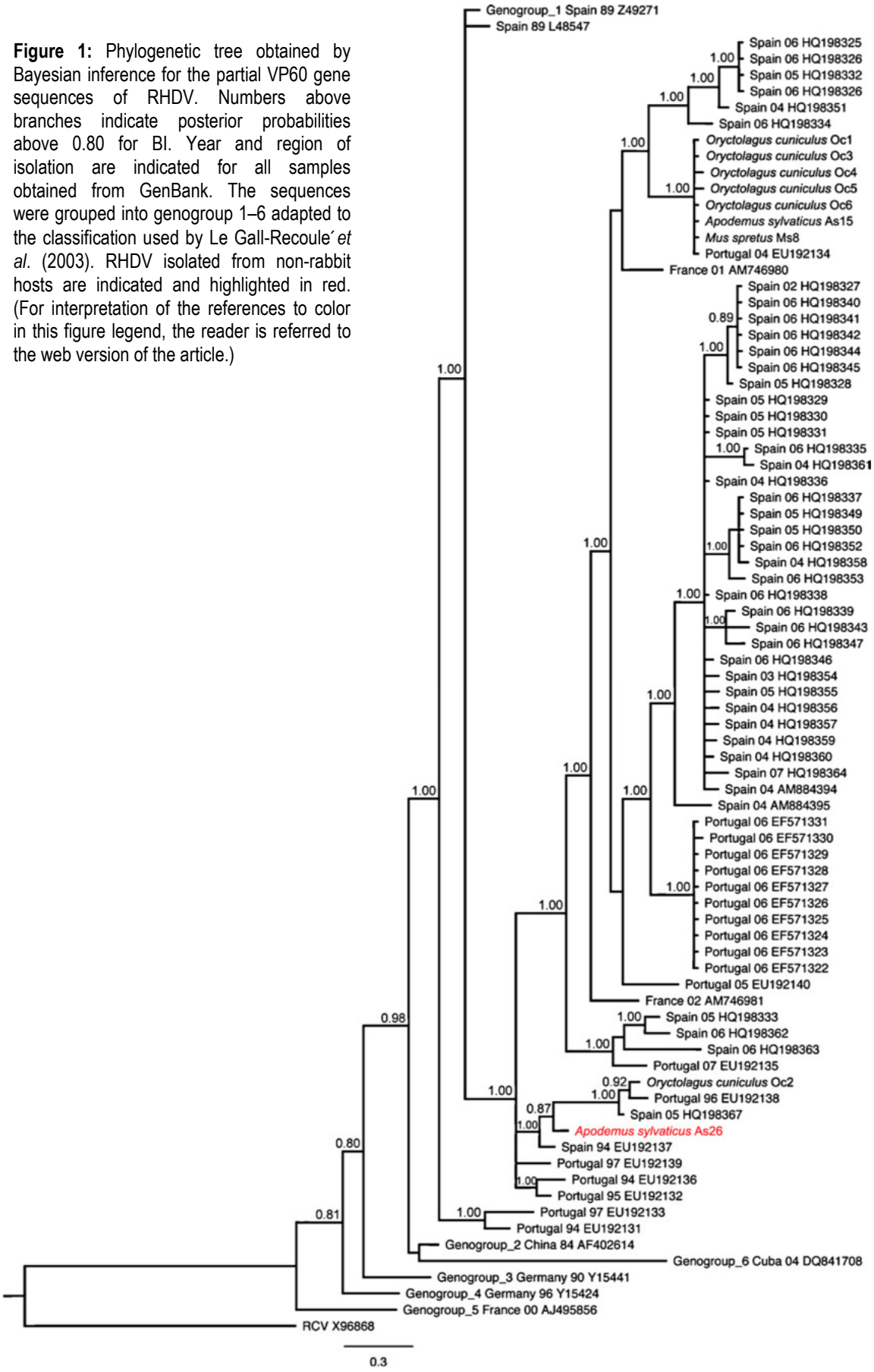


Figure 1: Phylogenetic tree obtained by Bayesian inference for the partial VP60 gene sequences of RHDV. Numbers above branches indicate posterior probabilities above 0.80 for BI. Year and region of isolation are indicated for all samples obtained from GenBank. The sequences were grouped into genogroup 1–6 adapted to the classification used by Le Gall-Recoule´ *et al*. (2003). RHDV isolated from non-rabbit hosts are indicated and highlighted in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

6. Discussion

For the first time, the natural presence of RHDV in wild mice of the species *A. sylvaticus* (9%) and *M. spretus* (3.4%) has been detected. These species were captured in the immediate vicinity of warrens in areas with a high density of rabbits. The study sample size of wood mice and Algerian mice does not permit differences with regards to RHDV infection rates to be established, although this value most likely depends not only on the abundance of each species in the area of study, but also on the use they make of the space.

In this case a greater appearance of RHDV in the wood mouse could be influenced by two factors: the wood mouse is the most abundant species and the most widely distributed in Mediterranean environments (Castién & Gosálbez, 2001), and it is found more closely linked to the European rabbit and its shelters in the Mediterranean, due to its dependence on stable warrens to shelter from the climate, light and predators (Diaz, 1992).

The fact that the viruses detected in mice were identical (Ms8 and As15) or very similar (As26) to the viruses isolated in rabbits in the same location and to the rest of the viruses that circulate in the Iberian Peninsula (Fig. 1) indicates that the strains of RHDV circulating in nature are capable of infecting different species of mammals. This finding is of great interest, given that if non-virulent circulation of RHDV were demonstrated in wild mice, this would substantiate the theory that the origin of RHDV may be in the transmission of this virus to rabbits from another species (Fenner & Fantini, 1999).

Similarly, the means of entry of the virus into mice is still unknown. In Mediterranean environments, warrens dug by rabbits are shelter structures used by a large variety of invertebrates and vertebrates such as *A. sylvaticus* (Delibes-Mateos *et al*., 2008), which may facilitate contact with the virus.

The presence of these species of mice during outbreaks of disease in rabbits could mean that they are infected passively by impregnation of viral particles in legs and hairs and subsequent ingestion of these particles during grooming. Another source of infection could be ingestion of partial remains of rabbits that have died of the disease, especially those that die inside their warrens, thus exposing their viruses in the warren (Cooke, 1996). Yet another possible means of infection is the faecal-oral route through excrement, as occurs in rabbits (Xu & Chen, 1989).

Rabbit excrement can carry the viruses both because the first viral replication occurs in crypts of Lieberkühn, in the intestine (Gregg *et al*., 1991), and because viruses come from intrahepatic

replication, through the bile duct, to the intestines (Marcato *et al*., 1991). In this way, species that ingest rabbit faeces, such as *A. sylvaticus* and *M. spretus* (Valverde, 1967), could be infected.

In this study, it was not possible to determine whether the murids with virus presented an immune response, as reported in predatory animals and scavengers after ingesting the remains of diseased or dead animals (Leighton *et al*., 1995; Parkes, 2004; Simon *et al*., 1994). Neither was it possible to prove whether they can contract the disease or if they have some sort of clinical manifestation due to the presence of RHDV, although at the time of their capture none showed any symptoms of disease.

Nevertheless, it would be important to determine the viability of RHDV and how long it can persist in natural murid populations. These data could provide information on intra- and interspecies transmission and spread of the virus (Merchán *et al*., in preparation) and on the cycles of occurrence of RHD, which could be influenced by the existence of associated rodent communities. It is not known whether these rodents have the ability to excrete and transmit the RHD virus, but were this the case, the persistence and spread of RHDV could be affected. Moreover, due to an effect of continuous contact with the virus, rodents could participate in inducing some immune response in individuals within sympatric rabbit populations if the rabbits are infected by carrier mice.

For this reason, the presence of rodent communities living sympatrically with the European rabbit could encourage the persistence and generalisation of these viral forms. If cross-species infection were proved conclusively, this could indicate the existence of an ecological relationship of passive collaborators in the increase of rabbit resistance to RHDV infections.

In view of these considerations, the preservation of communities of small herbivores in all their complexity and diversity could be a factor to keep in mind with regards to the delicate balance that European rabbit populations maintain with RHDV.

This report clearly shows the need for more studies to fully determine the potential of mice as harbourers of the disease in the environment or as spreaders of less fatal varieties of the virus, with the implications on the origin and the dynamics of the disease that this may have.

7. Acknowledgements

This project was funded by the DGIDTI of the Regional Government of Extremadura with file number: 2PR02A035. The authors are grateful to field work collaborators Begoña García Salazar, Francisco Cid, Francisco Ordiales and other students of the Cáceres Veterinary School, and would also like to thank Elizabeth Nestor for reviewing the English text.

8. References

- Alda, F., Gaitero, T., Alcaraz, L., Zardoya, R., Doadrio, I., Suárez, M., 2007. Coevolución de los virus de la Mixomatosis y de la Enfermedad Hemorrágica con el conejo (*Oryctolagus cuniculus* L., 1758) en la Península Ibérica. In: Ramírez, L., Asensio, B. (Eds). La investigación en Parques Nacionales: 2003-2006. Organismo Autónomo de Parques Nacionales-Ministerio de Medio Ambiente, Madrid, pp. 283-290.
- Alda, F., Gaitero, T., Suárez, M., Merchán, T., Rocha, G., Doadrio, I., 2010. Evolutionary history and molecular epidemiology of rabbit haemorrhagic disease virus in the Iberian Peninsula and Western Europe. BMC Evolutionary Biology 10: 347.
- Argüello, J.L., Llanos, A., Pérez, L.I., 1988. Enfermedad vírica hemorrágica del conejo en España. Medicina Veterinaria 5: 645-650.
- Asgari, S., Hardy, J.R., Sinclair, R.G., Cooke, B.D., 1998. Field evidence for mechanical transmission of Rabbit haemorrhagic disease virus (RHDV) by flies (Diptera: Calliphoridae) among wild rabbits in Australia. Virus Research 54: 123- 32.
- Bergin, I.L., Wise, A.G., Bolin, S.R., Mullaney, T.P., Kiupel, M., Maes, R.K., 2009. Novel calicivirus identified in rabbits, Michigan, USA. Emerging Infectious Diseases 15: 1955-1962.
- Buddle, B.M., de Lisle, G.W., McColl, K., Collins, B.J., Morrissy, C., Westbury, H. A., 1997. Response of the North Island brown kiwi, *Apteryx australis mantelli* and the lesser short-tailed bat, *Mystacina tuberculata* to a measured dose of Rabbit haemorrhagic disease virus. New Zealand Veterinary Journal 45: 109-113.
- Calvete, C., Estrada, R., Villafuerte, R., Osácar, J.J., Lucientes, J., 2002. Epidemiology of viral haemorrhagic disease and myxomatosis in a free-living population of wild rabbits. Veterinary Record 150: 776-782.
- Camacho, M., Moreno, M., 1989. Datos sobre la distribución espacial de los micromamíferos en el Parque Nacional de Doñana. Doñana Acta Vertebrata 16: 239-245.
- Cancellotti, F. M., Renzi, M., 1991. Epidemiology and current situation of viral haemorrhagic disease of rabbits and the European Brown Hare Syndrome. Revue scientifique et technique (International Office of Epizootics) 10: 409-421.
- Capucci, L., Fusi, P., Lavazza, A., Lodovica, M., Rossi, C., 1996. Detection and preliminary characterization of a new rabbit calicivirus related to rabbit haemorrhagic disease virus but nonpathogenic. Journal of Virology 70: 8614-8623.
- Capucci, L., Fallacara, F., Graziolis, S., Lavazza, A., Pacciarini, M., Brocchi, E., 1998. A further step in the evolution of rabbit haemorrhagic disease virus: the appearance of the first consistent antigenic variant. Virus Research 58: 115-126.
- Carro, F., Pérez Aranda, D., Lamosa, A., Schmalenberger, H.P., Pardavila, X., Gegundez, M.I., Soriguer, R.C., 2007. Eficiencia de tres tipos de trampas para la captura de micromamíferos. Galemys 19: 73-81.

- Castién, E., Gosálbez, J., 2001. Pequeños mamíferos forestales: influencia de las actividades forestales sobre las comunidades de Insectívoros y Roedores. In: Camprodon, J., Plana, E. (Eds). Conservación de la biodiversidad y gestión forestal: su aplicación en la fauna vertebrada. Edicions de la Universitat de Barcelona, Barcelona, pp. 353-364.
- Chasey, D., 1994. Possible origin of rabbit haemorrhagic disease in the United Kingdom. Veterinary Record 135: 496-499.
- Cooke, B. D., 1996. Field epidemiology of rabbit Calicivirus Disease in Australia. ESVV Symposium on caliciviruses, 15-17 September 1996. University of Reading.
- Cooke, B. D., 2002. Rabbit haemorrhagic disease: field epidemiology and the management of wild rabbit populations. Revue scientifique et technique (International Office of Epizootics) 2: 347-358.
- Delibes-Mateos, M., Delibes, M., Ferreras, P., Villafuerte, R., 2008. Key role of European rabbits in the conservation of the Western Mediterranean basin hotspot. Conservation Biological 22: 1106-1117.
- Díaz, M., 1992. Rodent seed predation in cereal crop areas of Central Spain: effects of physiognomy, food availability, and predation risk. Ecography 15: 77-85.
- Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783- 791.
- Fenner, F., Fantini, B., 1999. Biological control of vertebrate pests: the history of myxomatosis, an experiment in evolution. CABI Publishing, Wallingford.
- Forrester, N.L., Trout, R.C., Turner, S.L., Kelly, D., Boag, B., Moss, S., Gould, E.A., 2006. Unravelling the paradox of rabbit haemorrhagic disease virus emergence, using phylogenetic analysis; possible implications for rabbit conservation strategies. Biological Conservation 131: 296- 306.
- Gavier-Widen, D., Morner, T., 1993. Descriptive epizootiological study of European brown hare syndrome in Sweden. Journal of Wildlife Diseases 29: 15-20.
- Gavier-Widen, D., Berndtsson-Treiberg, L., Mejerland, T., Rivera, E., Morner, T., 1997. Experimental infection of silver foxes (*Vulpes vulpes*) and blue foxes (*Alopex lagopus*) with calicivirus of leporids. In: Chasey, D., Gaskell, R. M., Clarke, I.N. (Eds). Proceedings of the 1st International Symposium on Caliciviruses, UK, pp. 172-175.
- Gehrmann, B., Kretzschmar, C., 1991. Ein experimenteller Beitrag zur Epizootiologie der Viralen Hämorrhagischen Septikämie der Kaninchen (Rabbit haemorrhagic disease, RHD) - Ubertragung durch Fliegen. Berl Munch Tierarztl 104: 192-194.
- Green, K.Y., Ando, T., Balayan, M.S., Berke, T., Clarke, I.N., Estes, M.K., Matson, D.O., Nakata, S., Neill, J.D., Studdert, M.J., Thiel, H.J., 2000. Taxonomy of the caliciviruses. Journal of Infectious Diseases 181: 322-330.

- Gregg, D.A., House, C., Meyer, R. Berninger, M., 1991. Viral Haemorrhagic Disease of rabbits in Mexico: epidemiology and viral characterization. Revue scientifique et technique (International Office of Epizootics) 10: 435-451.
- Gould, A.R., Kattenbelt, J.A., Lenghaus, C., Morrissy, C., Chamberlain, T., Collins, B.J., Westbury, H.A., 1997. The complete nucleotide sequence of Rabbit haemorrhagic disease virus (Czech strain V351): use of the polymerase chain reaction to detect replication in Australian vertebrates and analysis of viral population sequence variation. Virus Research 47: 7-17.
- Kerr, P.J., Kitchen, A., Holmes, E.C., 2009. Origin and phylodynamics of rabbit hemorrhagic disease virus. Journal of Virology 83: 12129-12138.
- Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinformatics 5: 150-163.
- Lavazza, A., Scicluna, M.T., Capucci, L., 1996. Susceptibility of hares and rabbits to the European brown hare syndrome virus (EBHSV) and rabbit haemorrhagic disease virus (RHDV) under experimental conditions. Zentralblatt für Veterinärmedizin, Reihe B. Journal of Veterinary Medicine Series B. 43: 401-410.
- Le Gall-Recoulé, G., Zwingelstein, F., Laurent, S., de Boisséson, C., Portejoie, I., Rasschaert, D., 2003. Phylogenetic analysis of rabbit haemorrhagic disease virus in France between 1993 and 2000, and the characterisation of RDHV antigenic variants. Archives of Virology 148: 65-81.
- Leighton, F., Artois, M., Capucci, L., Gavier-Widen, D., Morisse, J., 1995. Antibody response to rabbit viral hemorrhagic disease virus in red foxes (*Vulpes vulpes*) consuming livers of infected rabbits (*Oryctolagus cuniculus*). Journal of Wildlife Diseases 31: 541-544.
- Lenghaus, C., Westbury, H., Collins, B., Ratnamohan, N., Morrissy, C., 1994. Overview of the RHD project in Australia. In: Munro, R. K., Williams, R.T. (Eds). Rabbit Haemorrhagic Disease: Issues in Assessment for Biological Control, Bureau of Resource Sciences, Australian Government Publishing Service, Canberra, pp. 104-129.
- Liu, S.J., Xue, H.P., Pu, B.Q., Qian, N.H., 1984. A new viral disease of rabbits. Animal Husband Veterinary medicine16: 253–255.
- Löliger, H. C., Eskens, U., 1991. Incidence, epizootiology and control of viral haemorrhagic disease of rabbits and the European brown hare syndrome in Germany. Revue scientifique et technique (International Office of Epizootics) 10: 423-430.
- Maddison, D.R., Maddison, W.P., 2000. MacClade 4: Analysis of phylogeny and character evolution. Sinauer Associates, Sunderland, Massachusetts.
- Marcato, P.S., Benazzi, C., Vecchi, G., Galeotti, M., Della Salda, L., Sarli, G., Lucidi, P., 1991. Clinical and pathological features of viral haemorrhagic disease of rabbits and the European brown hare syndrome. Revue Scientifique et Technique (International Office of Epizootics) 10: 371-392.
- Marchandeau, S., Le Gall-Reculé, G., Bertagnoli, S., Aubineau, J., Botti, G. Lavazza, A., 2005. Serological evidence for a non-protective RHDV-like virus. Veterinary Research 36: 53-62.

- McColl, K. A., Morrissy, C.J., Collins, B.J., Westbury, H.A., 2002. Persistence of rabbit haemorrhagic disease virus in decomposing rabbit carcases. Australian Veterinary Journal 80: 298-299.
- Moss, S. R., Turner, S.L., Trout, R.C., White, P.J., Hudson, P.J., Desai, A., Armesto, M., Forrester, N.L., Gould, E.A., 2002. Molecular epidemiology of rabbit haemorrhagic disease virus. Journal of General Virology 83: 2461-2467.
- Moussa, A., Chasey, D., Lavazza, A., Capucci, L., Smid, B., Meyers, G., Rossi, C., Thiel, H.J., Yasak, R., Ronsholt, L., 1992. Haemorrhagic disease of lagomorphs: evidence for a calicivirus. Veterinary Microbiology 33: 375- 381.
- Müller, A., Freitas, J., Silva, E., Le Gall-Reculé, G., Zwingelstein, F., Abrantes, J., Esteves, P.J., Alves, P.C., van der Loo, W., Kolodziejek, J., 2009. Evolution of rabbit haemorrhagic disease virus (RHDV) in the European rabbit (*Oryctolagus cuniculus*) from the Iberian Peninsula. Veterinary Microbiology 135: 368-373.
- Nowotny, N., Bascunana, C. R., Ballagi-Pordany, A., Gavier-Widen, D., Uhlen, M., Belak, S., 1997. Phylogenetic analysis of rabbit haemorrhagic disease and European brown hare syndrome viruses by comparison of sequences from the capsid protein gene. Archives of Virology 142: 657-673.
- Nowotny, N., Ros Bascunana, C., Ballagi-Pordany, A., Belak, S., Gavier-Widen, D., Uhlen, M., 1999. Phylogeny and variability of rabbit haemorrhagic disease virus, and the present situation of rabbit haemorrhagic disease in Europe. Rabbit control: RCD dilemmas and implications. Journal of the Royal Society of New Zealand Miscellaneous 55: 47–51.
- Ohlinger, V.F., Thiel, H.J., 1991. Identification of the viral haemorrhagic disease virus of rabbits as a calicivirus. Revue scientifique et technique (International Office of Epizootics) 10: 311-323.
- Pagés, A., 1989. Aspectos epidemiológicos y laboratoriales de la enfermedad hemorrágica de los conejos (RHD) en España. Medicina Veterinaria 6: 153-158.
- Parkes, J., Heyward, R. P., Henning, J., Motha, M.X.J., 2004. Antibody responses to rabbit haemorrhagic disease virus in predators, scavengers, and hares in New Zealand during epidemics in sympatric rabbit populations. New Zealand Veterinary Journal 52: 85-89.
- Parra, F., Prieto, M., 1990. Purification and characterization of a *Calicivirus* as the causative agent of a lethal haemorrhagic disease in rabbits. Journal of Virology 64: 4013-4015.
- Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3 Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572-1574.
- Rosell, J.M., Badiola, J.I., Pérez, A., Badiola, J.J., García, J.A., Vargas, M.A., 1989. Enfermedad vírica hemorrágica del conejo. I. Epizootiológica y clínica, Medicina Veterinaria 6: 275-284.
- Schirrmeier, H., Granzow, H., Bergmann, H., Schlüter, H., 1990. Experimentelle Untersuchungen zur Hämorrhagischen Septikämie der Kaninchen. Monatsh Veterinarmed 45: 193-197.

- Schirrmeier, H., Reimann, I., Koellner, B., Granzow, H., 1999. Pathogenic, antigenic and molecular properties of rabbit haemorrhagic disease virus (RHDV) isolated from vaccinated rabbits: detection and characterisation of antigenic variants. Archives of Virology 144: 719-735.
- Simon M.C., Muguruza R., Alonso J.L., Muzquiz C., Girones O., Haffar, A., 1994. Recherche du virus de la maladie hémorragique virale du lapin (RHD) chez le renard et rôle des canidés domestiques dans la transmission de la maladie. Recueil de Médecine Vétérinaire 170: 841-845.
- Smid, B., Valicek, L., Rodak, L., Stepanek, J., Jurak, E., 1991. Rabbit haemorrhagic disease: an investigation of some properties of the virus and evaluation of an inactivated vaccine. Veterinary Microbiology 26: 77-85.
- Takahashi, K., Nei, M., 2000. Efficiencies of fast algorithms of phylogenetic inference under the criteria of maximum parsimony, minimum evolution, and maximum likelihood when a large number of sequences are used. Molecular Biology and Evolution 17: 1251-1258.
- Valverde, J. A. 1967. Estructura de una Comunidad de Vertebrados Terrestres. C.S.I.C., Madrid.
- Wirblich, C., Sibilia, M., Boniotti, M.B., Rossi, C., Thiel, H.J., Meyers, G., 1995. 3C-like protease of rabbit hemorrhagic disease virus: identification of cleavage sites in the ORF1 polyprotein and analysis of cleavage specificity. Journal of Virology 69: 7159-7168.
- Xu, Z.J., Chen, W.X., 1989. Viral Hemorrhagic Disease in rabbits: a review. Veterinary Research Communications 13: 205-212.
- Zheng, T., Napier, A.M., Parkes, J.P., O'Keefe, J.S., Atkinson, P.H., 2002. Detection of RNA of rabbit haemorrhagic disease virus from New Zealand wild rabbits. Wildlife Research 29: 683-688.

CHAPTER II

Experimental transmission of rabbit haemorrhagic disease virus from rabbit to wild mice (*Mus spretus* and *Apodemus sylvaticus*) under laboratory conditions.

Merchán, T.[a], Rocha, G.[a], Alda, F.[b,c], Pagés, A.[d,e]

[a] Departamento de Ingeniería del Medio Agronómico y Forestal, University of Extremadura. Avda. Virgen del Puerto, 2. 10600 Plasencia, Cáceres, Spain. tmerchan@unex.es, gregorio@unex.es

[b] Instituto de Investigación en Recursos Cinegéticos (IREC, CSIC-UCLM-JCCM), Ronda de Toledo s/n, 13005 Ciudad Real, Spain

[c] Current address: Tulane University, 6823 St. Charles Ave. New Orleans, LA 70118, USA. alda.fernando@gmail.com

[d] Laboratorios Hipra S.A., La Selva, 135. 17170 Amer, Girona, Spain,

[e] Plaza del Mercado, 10. 17853 Tortella, Girona, Spain. apmante@gmail.com

Merchán, T., Rocha, G., Alda, F., Pagés, A., 2013.*Experimental transmission of rabbit haemorrhagic disease virus (RHDV) from rabbits to wild mice (Algerian mouse Mus spretus and wood mouse Apodemus sylvaticus)under laboratory conditions.* ***Preliminary short manuscript.***

1. Resumen

La enfermedad hemorrágica vírica está causada por un virus del género *Lagovirus* que provoca lesiones hemorrágicas en hígado y pulmones de conejos. La enfermedad hemorrágica vírica provoca graves pérdidas económicas y de biodiversidad en la Península Ibérica, sin embargo varios aspectos de su evolución, transmisión y epidemiología permanecen aún desconocidos. Para conocer mejor el papel que los roedores pueden tener como fuente de infección, cómo influyen sobre la persistencia del virus o su potencial capacidad como reservorios, nosotros llevamos a cabo un desafío experimental con el virus de la enfermedad hemorrágica del conejo (RHDV) en nueve conejos (*Oryctolagus cuniculus*) en contacto íntimo con 16 ratones salvajes (*Mus spretus*, n=12 y *Apodemus sylvaticus*, n=4) para estudiar el tiempo de permanencia y la viabilidad de estos virus en el interior de los roedores, así como su capacidad potencial para la transmisión posterior a los conejos. Mientras que el 100% de los conejos murió en un plazo de 96 horas post-desafío, ninguno de los roedores murió durante el experimento o presentó lesiones macroscópicas hemorrágicas en sus órganos internos.

Ocho ratones fueron sacrificados el día 7 post-contacto con conejos desafiados. En el 87.5% (n=7) de los ratones fue detectado RHDV en sus excrementos, y en el 50% (n=4) en los hígados. A los 14 días post-contacto, cuatro roedores eutanasiados no presentaron evidencias del RHDV, y a 68 días post-contacto, RHDV fue detectado en heces de uno de los cuatro ratones restantes.

Cuando uno ratón muerto infectado y sus excrementos fueron puestos en contacto con un conejo susceptible, este adquirió inmunidad (1/32). Sin embargo, los títulos no parecieron ser protectores, ya que el conejo murió a las 21 horas tras su desafío vírico.

Nuestros resultados permiten comprobar el trasiego interespecífico de RHDV y ampliar nuestro conocimiento acerca de la circulación del virus en micromamíferos simpátricos a los conejos, así como su papel potencial como fuentes de infección y en la transmisión de RHDV en la naturaleza.

Palabras clave: RHDV, enfermedad hemorrágica vírica, RHD, anfitriones, *Apodemus sylvaticus*, *Mus spretus*, *Oryctolagus cuniculus*

2. Abstract

Rabbit haemorrhagic disease (RHD) is caused by a virus of the genus Lagovirus which produces haemorrhagic lesions in rabbit liver and lungs. RHD causes severe economic and biodiversity losses in the Iberian Peninsula, although several aspects of its evolution, transmission and epidemiology are still unknown. To further understand the role that rodents may have in relation to sources of infection, virus persistence and potential reservoirs, we experimentally challenged nine rabbits with RHDV and put them in close contact with 16 wild mice (*Mus spretus*, n=12 and *Apodemus sylvaticus*, n=4) to study time to infection and time required for carrying the virus in their internal organs, as well as their potential capacity for subsequent transmission to rabbits. While 100 % of the rabbits died within 96 hours post-challenge, none of the rodents died from the experiment or presented macroscopic haemorrhagic lesions.

Eight mice were euthanised on day 7 post-contact with challenged rabbits. In 87.5% (n=7) RHDV was detected in faeces by RT-PCR, and in 50% (n=4) in the liver. By day 14 post-contact the four rodents euthanised showed no evidence of RHDV in faeces or liver, and 68 days post-contact RHDV was detected in the faeces of only one of the four remaining mice.

When one infected dead mouse and its faeces were brought into contact with a susceptible rabbit, the rabbit acquired immunity (1/32). However, the titres did not appear to be protective, as the rabbit died 21 hours after viral challenge.

Our results demonstrate rabbit to rodent cross-species transmission of RHDV and extend our knowledge about virus circulation in rodents sympatric to rabbits, as well as their potential role as sources of infection and in RHDV transmission in the wild.

Key words: RHDV, haemorrhagic disease virus, RHD, hosts, *Apodemus sylvaticus, Mus spretus, Oryctolagus cuniculus*

3. Introduction

Rabbit haemorrhagic disease (RHD) is caused by rabbit haemorrhagic disease virus (RHDV), a calicivirus of the genus *Lagovirus* (Ohlinger *et al*., 1990). It is characterised as extremely lethal and highly contagious, and affects both domestic and wild rabbits (*Oryctolagus cuniculus*). RHD was first reported in China (Liu *et al*., 1984) and affected rabbit populationsin Europefor the first time in the late 1980s. Since its detection in Spain in 1988 (Argüello *et al*., 1988), RHD has caused a reduction in rabbit population densities and significant changes in their spatial distribution throughout the country (Calvete *et al*., 2006; Delibes-Mateos *et al*., 2009). This population decline has directly affected both global biodiversity and economy, as rabbits are a multifunctional keystone species of the Mediterranean ecosystem (Delibes-Mateos *et al*., 2008) and one of the main hunting species in Spain (Alves & Ferreira 2004; Calvete *et al*., 2005).

The exact origin and evolution of the virus remain unknown. Although the first record comes from China, several studies have suggested that RHDV originated in Europe, where the virus had been circulating long before the first epidemic was reported in 1984 (Kerr *et al*., 2009; Alda *et al*., 2010; Kinnear & Linde, 2010). Some hypotheses on the origin of RHDV pathogenicity indicate a possible viral cross infection from hares to rabbits (Nowotny *et al.*, 1997). Others have attempted to explain its origin in possible changes occurring in non-pathogenic forms (Moss *et al*., 2002). Similarly, little is known about the key elements of RHDV epidemiology, such as aspects associated with its method of propagation and sources of infection. Documentation on the spread of the disease includes the activity of arthropods (Gould *et al*., 1997) and predatory mammals and birds (Mitro & Krauss, 1993), and the role of warrens in harbouring the virus after epizootic outbreaks (Cooke, 1996; Cooke *et al*., 2000; Calvete *et al*., 2002). The main sources of infection are known to be direct transmission from infected rabbits (Xu & Chen, 1989), young rabbits less than 20 days old that manage to survive the disease (Rosell *et al*., 1989) and rabbits with persistent infection (Löliger & Esken, 1991). Additionally, other variables that affect natural transmission mechanisms include environmental factors (Cooke, 2002), the presence of carcasses of animals that have died from the virus (McColl *et al*., 2002) and interaction with outbreaks of myxoma virus (Mutze *et al*., 2002).

So far it has not been reported that RHD affects any wild or domestic species other than the rabbit (Nowotny *et al*., 1997), although a long list of vertebrate species does show an antibody response after inoculation (Buddle *et al.*, 1997) and to RHDV oral challenge (Leighton *et al.,* 1995) in the laboratory. RHDV was recently detected in natural populations of two mice species (*Mus spretus* and *Apodemus sylvaticus*) that are commonly sympatric with wild rabbits in Mediterranean

environments of the Iberian Peninsula (Merchán *et al*. 2011). These mice were carriers of the same strain of virus as rabbits co-occurring with them, suggesting they may have an important role in the epidemiology of the virus. Nothing is known of the role these rodents could have in relation to sources of infection, persistence of the virus in the natural environment, or even as potential reservoirs in this epidemiological structure. Some authors have suggested that rather than acting as a reservoir, rodents are merely mechanical vectors (Schirrmeier *et al*., 1990), although no experimental studies have been conducted to explain the role of these sympatric mice in RHD epidemiology.

This study aimed to confirm whether RHDV-infected rabbits can infect wild Algerian mice (*M. spretus*) and wood mice (*A. sylvaticus*) after coexisting under controlled laboratory conditions. A severe RHD outbreak was produced in laboratory rabbits experimentally inoculated with a known strain of RHDV and rodents were tested to study time to infection and time required for carrying the virus in their internal organs, as well as their potential capacity for subsequent transmission to rabbits.

4. Material and methods

4.1. Experimental design

The study was conducted at a biosecurity facility in a 6x3x4 m sealed experimentation room prepared for the coexistence of the rabbits and mice. The room (coexistence room 1) was equipped with a one-way mirror to allow undetected observation from the outside. The experimentation room and all items contained in it were given a disinfecting and virucide treatment with a 1% Neozital® solution 24 hours before the animals were placed inside. The items treated comprised a feeder, a water spout and 16 plastic tubes for the mice to shelter in. Sterile cotton wool was also used as nesting for the mice. Rabbit food pellets were provided and water was available *ad libitum*.

The control mice were housed in cages in two separate rooms (negative control room and positive control room) isolated from the experimentation room. A decontaminated rabbit cage housed in a fourth, separate experimentation room (coexistence room 2) was used for the third phase of the experiment (Figure 1).

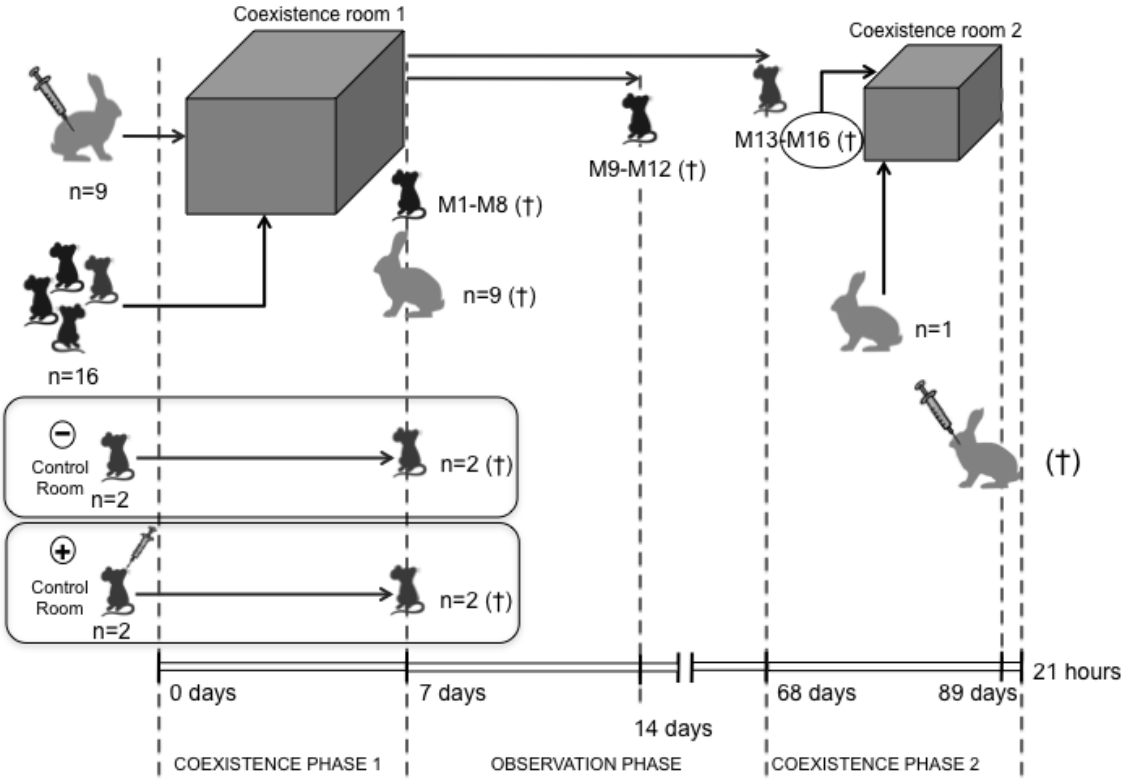


Figure 1: Schematic representation of the experimental design and timeline.

The experimental protocol was approved by the Ethical Committee for Animal Research of the Jesús Usón Minimally Invasive Surgery Centre, in Cáceres (Spain) as fully compliant with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and Spanish regulations on the use of experimental animals (Royal Decree 1201/2005, of 10th October, BOE 21st October 2005).

4.2. Study animals

The animals used in the study were nine New Zealand breed domestic rabbits and 20 wild mice (*M. spretus,* n=16and *A. sylvaticus,* n=4). The rabbits were disease free and had no history of RHD and no antibodies to RHDV, in accordance with Federation for Laboratory Animal Science Associations (FELASA) recommendations. All the rodents came from the municipality of Cáceres, in Extremadura (Spain), from an area that was free of rabbits. This circumstance, confirmed by extensive field analysis, minimised the probability of previous contact with RHDV. Mice were captured from 01-15 September 2007 using wire mesh traps ("hypolithic" traps) (Carro *et al.*, 2007) and kept in laboratory mouse cages until the experiment began on 09 October 2007, preventing further contact with the outside environment. Animal trapping was authorised by the Regional Government of Extremadura.

4.3. Timing

The experiment lasted 89 days and 21 hours (Figure 1). It was divided into three phases: the first phase, "coexistence phase 1", lasting 7 days, was designed to confirm transmission from infected and diseased rabbits to healthy mice; the second phase, "observation phase", from day 8 to 68 (61 days), was designed to confirm the duration of the presence of the virus in the rodents and its ability to cause infection in them; and the third phase, "coexistence phase 2", from day 69 to 89 (21 days and 21 hours), was designed to confirm transmission from mice infected during the experiment to healthy rabbits.

Before starting the first phase (coexistence phase 1), nine rabbits were inoculated with a viral resuspension obtained from the virulent RHDV reference strain RHD-4764-183-ET (23/09/97) supplied by Laboratorios Hipra S.A., with an infective dose of 1000 DLC/50 at 0.6 ml per rabbit. The nine rabbits and 16 mice (4 *A. sylvaticus* and 12 *M. spretus*) were immediately placed in experimentation room 1. At the same time, two positive control mice were inoculated with 0.1 ml of the same virus resuspension and placed in the positive control room, as well as the two negative control mice which were placed in the negative control room (Figure 1).

During coexistence phase 1, the experimentation room was closed off and no one entered it at any stage. Every morning and afternoon, 30-minute observations were made to check for the appearance of clinical signs and monitor the progress of the experiment. After seven days the room was entered and all the mice were placed in separate sterile containers and labelled. Immediately after this, eight mice were euthanised (M1 to M8). The negative and positive control group mice were also killed *in situ* by cervical dislocation seven days after the start of the experiment. Rabbit corpses were collected and placed in separate sterile containers and labelled. To detect the presence of RHDV infection, liver samples and dung pellets found in the intestinal tract were taken from all rodents and rabbits, as the first viral replication occurs in the crypts of Lieberkühn, in the intestine (Gregg *et al*., 1991), and because viruses come from intrahepatic replication, through the bile duct, to the intestines (Marcato *et al*., 1991. Sterile laboratory material was used under strict biosecurity measures to avoid cross-contamination. All samples were stored at -20 °C until analysis.

During the second phase (observation), the remaining mice (M9 to M16) were divided into two groups. Mice M9 to M12 were euthanised after seven days (14 days after the start of the experiment) and mice M13 to M16 were killed 68 days after the start of the experiment. Liver and faeces samples were taken from all individuals.

During coexistence phase 2, the carcass and faeces of mouse M16 were placed inside a cage with an RHD negative rabbit in coexistence room 2, where they remained for 21 days. After this, 1.5 ml rabbit blood was extracted by inserting a needle into the marginal ear vein to detect antibodies to RHD. A serum sample was obtained after the blood was centrifuged at 4500 rpm for 10 minutes and frozen at -20 ºC until screening. The rabbit used in the experiment in coexistence phase 2 was challenged on day 21 post contact with 0.6 ml virus resuspension.

4.4. Molecular laboratory methods

Viral RNA was extracted from all the liver and faecal samples collected. Approximately 125 mg of tissue were homogenised in 1.25 ml sterile PBS and centrifuged at 2500 x *g* for 15 min. One hundred microlitres of the homogenate was extracted using the TriPure reagent (Roche), following the manufacturer's instructions. A fragment of the major capsid protein gene VP60 was amplified and sequenced in all samples using primers RHDV1, RHDV2, RHDV3 and RHDV4 (Moss *et al.,* 2002), following the same conditions as in Alda *et al*., (2010). RHDV reference strain RHD-4764-183-ET was also amplified directly from the inoculum provided.

All the chromatograms obtained were checked, aligned and compared to the RHDV reference strain using Sequencer 4.6 (Gene Codes Corporation). The sequence obtained was deposited in GenBank under accession number KJ841711.

The serum sample from the rabbit in coexistence phase 2 was screened to detect antibodies and their titration through indirect ELISA using a commercial kit (INgezim RHDV 17.RHD.K.1, Ingenasa, Madrid, Spain). Absorbance of the sample was measured at a wavelength of 405 nm with shake duration (inside Normal) of 10 seconds. Screening results with Optical Density (OD) values higher than 0.300 were regarded as positive to RHDV antibodies and the titration of the sample was the maximum dilution with an OD value higher than 0.300.

5. Results

5.1. Coexistence phase 1

The first clinical signs associated with RHD appeared in a lethargic rabbit 48 hours post-inoculation. This rabbit and four others were found dead within 72 hours. The remaining four rabbits all died within 96 hours. Thus 100% of the rabbits had died by the end of the first phase and presented lesions consistent with RHD. In all the liver and faecal samples RHDV was positively detected by RT-PCR, and all the sequences matched that of the strain inoculated.

None of the rodents, including the control group, showed either behavioural changes or clinical signs of disease during the first phase of the experiment. Although no lesions or macroscopic alterations were observed in the samples extracted after the first phase, RHDV was detected in the faeces of 87.5% (n=7) of the mice, and in 50% (n=4) of the liver (Table 1). Whenever the virus was detected in the liver, it was also detected in the faeces. In three individuals (37.5%) the virus was detected only in faecal samples, and in only one mouse (12.5%) the virus was not detected in either liver or faeces. Positive control mice presented RHDV in liver and faeces, whereas negative control mice did not show RHDV in any sample (Table 1).

Table 1: Results from the RT-PCR detection of RHDV in mice liver and faeces during coexistence phase 1 and the observation phase of the experiment. As: *Apodemus sylvaticus*, Ms: *Mus spretus*,C(-): negative control C(+): positive control, (+): RHDV detected positively, (-) RHDV not detected by RT-PCR.

Individual	Species	RT-PCR Faeces	RT-PCR Liver	Experiment Phase	Day euthanised
M1	As	+	-	Coexistence 1	7
M2	As	+	+	Coexistence 1	7
M3	Ms	-	-	Coexistence 1	7
M4	Ms	+	+	Coexistence 1	7
M5	As	+	-	Coexistence 1	7
M6	As	+	+	Coexistence 1	7
M7	Ms	+	-	Coexistence 1	7
M8	Ms	+	+	Coexistence 1	7
M9	Ms	-	-	Observation	14
M10	Ms	-	-	Observation	14
M11	Ms	-	-	Observation	14
M12	Ms	-	-	Observation	14
M13	Ms	-	-	Observation	68
M14	Ms	-	-	Observation	68
M15	Ms	-	-	Observation	68
M16	Ms	+	-	Observation	68
C(-)1	Ms	-	-	Coexistence 1	7
C(-)2	Ms	-	-	Coexistence 1	7
C(+)1	Ms	+	+	Coexistence 1	7
C(+)2	Ms	+	+	Coexistence 1	7

All the virus samples amplified from mice showed sequences identical to the virus strain inoculated into the rabbits.

5.2. Observation phase

In the second phase, the four mice (M9-M12) killed 14 days after contact with the infected rabbits did not show evidence of RHDV infection, after RT-PCR of liver and faecal sample. Similarly, RT-PCR failed to detect RHDV in all the samples from the four mice analysed 68 days post-contact (M13-M16), except in the faeces of mouse M16 (Table 1). In this case, the VP60 sequence obtained also matched the sequence of the virus strain inoculated into the rabbits.

5.3. Coexistence phase 2

At the end of coexistence phase 2, the rabbit that was in contact with the carcass and infected faeces of mouse M16, showed an RHD ELISA OD of 0.302 with a 1/32 titration, but RHDV was not detected in its faeces. The rabbit looked healthy and had no symptoms consistent with RHD. However, after it was inoculated with RHDV, the rabbit died with RHD symptoms 21 hours later and the virus was detected in liver and faeces.

6. Discussion

This study shows, for the first time, experimental evidence of RHDV transmission by direct contact between infected rabbits and two wild mouse species, *A. sylvaticus* and *M. spretus*. Although much has been documented about the main transmission mechanisms of the disease among rabbits and the importance of rabbit carcasses as a source of infection in natural conditions (Ohlinger *et al*., 1993; McColl *et al*., 2002), cross-species infection and seroconversion had not been demonstrated before. Experimental inoculation of RHDV in 31 domestic, feral (including *Mus musculus*) and Australian native mammal and bird species failed to show any evidence of RHDV infection, or its genome (Lenghaus *et al*., 1994; Collins *et al*., 1995; Gould *et al*., 1997).

Although RHDV is considered highly species-specific (Lenghaus *et al*., 2000; Cooke & Fenner, 2002), this study shows that when infected rabbits are brought into contact with wild mice under controlled laboratory conditions, virus transmission can occur from rabbits to rodents and persist in the latter. The virus was detected in the faeces of 87.5% of the rodents tested seven days post-contact and in 25% of those tested 68 days post-contact. This may indicate that *M. spretus* (and possibly *A. sylvaticus*) is capable of hosting the virus for at least 68 days. These results strengthen the hypothesis of the important participation of these two species of mice in RHDV epidemiology when they co-occur with wild rabbits (Merchán *et al.*, 2011).

The presence of RHDV, or its genome, in mice seven days post-contact with infected rabbits, and in one mouse 68 days post-contact, with no clinical symptoms, suggests that although there may

be barriers against infection (e.g., macrophages, membrane specificity, mucus and ciliated epithelium), RHDV is capable of viral replication in a non-target species and therefore is capable of remaining in this host for a long time. However, little is known about many aspects of the effect the virus has on these rodents, such as the type of immune response (cellular or humoral), the existence of immunopathology or immune adherence phenomena, and the existence of specific cell receptors or other components required for replication of this virus, which are necessary conditions for infection (Pastoret, 1990). Studies on human caliciviruses (Nyström *et al*., 2011) have indicated that RHDV attachment factors are also present on human cells, constituting potential points of entry for the virus, and suggesting that the molecular mechanisms for the entry of RHDV are not entirely limited to rabbits, as our study also indicates.

The more frequent presence of RHDV in mice faeces than in livers could suggest that the intestine is the organ where the virus is located after entry through the oral route, as in the case of RCV (Marchandeau *et al*., 2005), or that the action of the antiviral response may be compromised at the intestinal epithelial cell level. These results open up new lines of study to shed light on RHDV pathogenesis in wild mice and its possible cross-species origin (Fenner & Fantini, 1999). In fact, some researchers have alluded to the potential for mutation of RHDV, which could enable it to cross species barriers and cause disease (Smith, 1998), and to how colonisation of new but related host species may represent the principle mode of macroevolution in RNA viruses (Kitchen *et al.,* 2011).

In mice where RHDV was detected after coexistence phase 1, the virus may have been at an early stage of infection, when viral particles would not yet have passed through the intestinal mucous membrane on their way to the liver cells. Similarly, the virus may have invaded the digestive tract naturally after the rodent feeding process in a virus-filled environment. The absence of signs of RHDV genetic material in faeces and liver after day 7could also be explained by RHDV infection in mice having a specific duration, either because the mice end up fighting the virus or because the infection reaches a phase of latency with such a low number of viruses in the organs that they are no longer detectable by RT-PCR.

The non-susceptibility of mice to RHD could indicate a case of weak viral attachment, or even suggest an adaptation to the host genetic diversity (Nyström *et al*., 2011). Greater knowledge of possible resistance mechanisms in these rodent species could also be useful to extend information about the origin of the virus (which could be associated with the rodents themselves, thus explaining the apparent non-virulent coexistence in these species). The higher resistance in rabbits less than two months old compared to adult rabbits could also provide valuable information, as in these younger individuals it has been shown that attachment factors are weakly expressed in respiratory

and digestive cells, which is where the first virus replication occurs in rabbits (Ruvoen-Clouet *et al*., 1995; Ruvoen-Clouet *et al*., 2000). This could partially explain resistance to RHD infection in rodents as well.In any case, it would be interesting to conduct this type of research on sympatric rodents

The oral route is considered the preferred means for RHDV transmission in the field (Parkes *et al*., 2001), so it is likely that in this experiment, where an environment with a high viral load contaminates water and food, the route of entry in the rodents might be the same (Cooke, 2002). Furthermore, considering that virus particles in rabbit faeces can remain infectious for 1-2 weeks (Henning *et al*., 2005), the ingestion of contaminated rabbit faeces (Ohlinger *et al*., 1993) may greatly enhance transmission in the wild. In consequence, rodent consumption of rabbit faeces when sharing the same habitat (Valverde, 1967), combined with the ubiquitous nature of mice, which commonly use rabbit warrens for shelter (Delibes-Mateos *et al*., 2008), could support their role as sources of infection and also aid in RHDV transmission.

The detection of antibodies in the rabbit in coexistence phase 2 indicates a serological response after contact with the faeces and carcass of the infected mouse. The low titre seroconversion reaction without infection could have been caused by exposure to a low viral load. On the other hand, too much time may have elapsed between the initial contact and sample collection, thus leading to a decline in antibody level. Antibodies anti-VP60 appear approximately five to six days after the first contact and last for several weeks or even months if the dose is sufficient (Bertagnoli *et al*., 1996; Laurent *et al*., 1994). However, in the present study, the titres detected after the viral challenge were not protective. OD values should be at least 0.900 to provide protection, according to the kit used. However, cases are known where 1/20 antibody titres provide protection against RHDV (Laurent *et al*., 1994), and thus it appears that more in-depth knowledge of this aspect is required and the results of this study should be regarded as highly preliminary due to the sample size. It is also necessary to know whether seroconversion occurs in infected rodents, as demonstrated in other closely related species such as hares (*Lepus* spp.) by Lavazza *et al*., 1996 and in red fox (*Vulpes vulpes*) and the domestic house cat (*Felis catus*) fed with RHDV infected liver (Leighton *et al*., 1995; Zheng *et al*., 2003), although this procedure was not included in the present study.

The viability of viruses found in rodents is currently unknown, but all indications are that mice could contribute to the persistence and spread of RHDV until at least 68 days post-contact. This finding extends epidemiological information on where viruses are harboured between outbreaks, which was mostly attributed to rabbit kits carrying the virus or the inside of warrens (Cooke *et al*., 2000; Cooke, 2002). The fact that rodents may host the virus could somehow influence the density

dependent dynamics of the virus itself (Fa *et al*., 2001; Calvete, 2006) and affect its viral impact. The population dynamics of these hosts could therefore determine the introduction, survival or disappearance of the virus at a given site, as suggested by Mills *et al*., 1992 and Porcasi *et al*., 2005. However, more in-depth studies are needed about aspects such as the virus load per animal, the infectious capacity of the virus and the potential of these new hosts to spread the virus. Coexistence of rodents and rabbits can encourage cross-species transmission of the virus, with epidemiological consequences that have yet to be determined, both on the impact of RHD and on rabbit survival.

7. Acknowledgements

The authors are grateful to Laboratorios Hipra S.A. for providing the RHDV virus reference strain RHD-4764-183-ET (23/09/97) and to the Animal Research department at the Jesús Usón Minimally Invasive Surgery Centre, Cáceres (Spain), for providing experimental facilities.

8. References

- Alda F., Gaitero T., Suárez M., Merchán T., Rocha G., Doadrio, I., 2010.Evolutionary history and molecular epidemiology of rabbit haemorrhagic disease virus in the Iberian Peninsula and Western Europe. BMC Evolutionary Biology 10: 347.
- Alves, P.C., Ferreira, C., 2004. Revisão do Livro Vermelho dos Vertebrados de Portugal. Determinação da abundância relativa das populações de coelho-bravo (*Oryctolagus cuniculus*) em Portugal Continental. Relatório Final. CIBIO, ICETA, Universidade do Porto.
- Argüello, J.L., Llanos, A., Pérez, L.I., 1988. Enfermedad vírica hemorrágica del conejo en España. Medicina Veterinaria 5: 645-650.
- Bertagnoli, S., Gelfi, J., Le Gall, G., Boilletot, E., Vautherot, J. F., Rasschaert, D., Laurent, S., Petit, F., Boucraut-Baralon, C., Milon, A., 1996. Protection against myxomatosis and rabbit viral hemorrhagic disease with recombinant myxoma viruses expressing rabbit hemorrhagic disease virus capsid protein. Journal Virology 70: 5061-5066.
- Buddle, B.M., de Lisle, G.W., McColl, K., Collins, B.J., Morrissy, C., Westbury, H.A., 1997. Response of the North Island brown kiwi, *Apteryx australismantelli* and the lesser short-tailed bat, *Mystacina tuberculata* to a measured dose of rabbit haemorrhagic disease virus. New Zealand Veterinary Journal 45: 109-113.
- Calvete C., 2006. Modelling the effect of population dynamics on the impact of rabbit hemorrhagic disease. Conservation Biology 20: 1232–1241.
- Calvete, C., Estrada, R., Villafuerte, R., Osácar, J.J., Lucientes, J., 2002. Epidemiology of viral haemorrhagic disease and myxomatosis in a free-living population of wild rabbits. Veterinary Record 1 50: 776-782.
- Calvete, C., Angulo, E. Estrada, R., 2005. Conservation of European wild rabbit populations when hunting is age and sex selective. Biology Conservation 121: 623–634.

- Calvete, C., Pelayo, E., Sampietro, J., 2006. Habitat factors related to wild rabbit population trends after the initial impact of rabbit haemorrhagic disease. Wildlife Research 33: 467–474.
- Carro, F., Pérez Aranda, D., Lamosa, A., Schmalenberger, H.P., Pardavila, X.,Gegundez, M.I., Soriguer, R.C., 2007. Eficiencia de tres tipos de trampas para la captura de micromamíferos. Galemys 19 (nº extra): 73-81.
- Collins, B.J., White, J.R., Lenghaus, C., Boyd, V., Westbury, H.A., 1995. A competition ELISA for the detection of antibodies to rabbit haemorrhagic disease virus. Veterinary Microbiology 43: 85-96.
- Cooke, B.D., 1996. Field epidemiology of rabbit Calicivirus Disease in Australia. ESVV Symposium on caliciviruses, 15-17 Septiembre. Universidad de Reading. Reino Unido.
- Cooke, B.D., 2002. Rabbit hemorrhagic disease: field epidemiology and the management of wild rabbit populations. 2002. Revue Scientifique et Technique. Office Internationale des Epizooties 21: 347-358.
- Cooke, B.D., Robinson, A.J., Merchant, J.C., Nardin, A., Capucci, L., 2000. Use of ELISAs in field studies of rabbit haemorrhagic disease (RHD) in Australia. Epidemiology Infection 124: 563-76.
- Cooke, B.D., Fenner, F., 2002. Rabbit haemorrhagic disease and the biological control of wild rabbits, *Oryctolagus cuniculus*, in Australia and New Zealand. Wildlife Research 29: 689-706.
- Delibes-Mateos, M., Delibes, M., Ferreras, P., Villafuerte, R., 2008. Key role of European rabbits in the conservation of the Western Mediterranean basin hotspot. Conservation Biology 22: 1106–1117.
- Delibes-Mateos, M., Ferreras, P., Villafuerte, R., 2009. European rabbit population trends and associated factors: a review of the situation in the Iberian Peninsula. Mammal Review 39: 124–140.
- Fa, J.E., Sharples, C.M., Bell, D.J., De Angelis, D., 2001. An individual-based model of rabbit viral haemorrhagic disease in European wild rabbits (*Oryctolagus cuniculus*). Ecological Modelling 144: 121–138.
- Fenner, F., Fantini, B., 1999. Biological control of vertebrate pests: the history of myxomatosis; an experiment in evolution, 1 edn. CABI Publishing.
- Gould, A.R., Kattenbelt, J.A., Lenghaus, C., Morrissy, C., Chamberlain, T., Collins, B.J., Westbury, H.A., 1997. The complete nucleotide sequence of rabbit haemorrhagic disease virus (Czech strain V351): use of the polymerase chain reaction to detect replication in Australian vertebrates and analysis of viral population sequence variation. Virus Research 47: 7–17.
- Gregg, D.A., House, C., Meyer, R., Berninger, M., 1991. Viral haemorrhagic disease of rabbits in Mexico: epidemiology and viral characterisation. Revue Scientifique et Technique. Office Internationale des Epizooties 10(2): 435-448.
- Henning, J., Meers, P.R., Davies R., Morris, S., 2005. Survival of rabbit haemorrhagic disease virus (RHDV) in the environment. Epidemiology and Infection 133: 719-730.

- Kerr, P.J., Kitchen, A., Holmes, E.C., 2009. Origin and phylodynamics of rabbit hemorrhagic disease virus. Journal of Virology 83: 12129–12138
- Kinnear, M., Linde, C.C., 2010. Capsid gene divergence in rabbit hemorrhagic disease virus.Journal of General Virology 91(Pt 1): 174-81.
- Kitchen, A., Shackelton, L.A., Holmes, E.C., 2011. Family level phylogenies reveal modes of macroevolution in RNA viruses. Proceedings of the National Academy of Sciences of the United States of America PNAS, USA 108: 238-43.
- Laurent, S., Vautherot, J.F., Madelaine, M.F., Le Gall, G., Rasschaert, D., 1994. Recombinant rabbit hemorrhagic disease virus capsid protein expressed in baculovirus self-assembles into viruslike particles and induces protection. Journal Virology 68: 6794-6798.
- Lavazza, A., Scicluna, M.T., Capucci, L., 1996. Susceptibility of hares and rabbits to the European Brown Hare Syndrome Virus (EBHSV) and Rabbit Hemorrhagic Disease Virus (RHDV) under experimental conditions. Journal Veterinary Medicine [B] 43: 401-410.
- Leighton, F.A., Artois, M. Capucci, L. Gavier-Widen, D. Morisse, J.P., 1995. Antibody response to rabbit viral hemorrhagic disease virus in red foxes (*Vulpes vulpes*) consuming livers of infected rabbits (*Oryctolagus cuniculus*). Journal of Wildlife Diseases31: 541-544.
- Lenghaus, C., Westbury, H., Collins, B., Ratnamohan, N., Morrissy, C., 1994. Overview of the RHD project in Australia. In 'Rabbit Haemorrhagic Disease: Issues in Assessment for Biological Control'. (Eds Munro, R.K. & Williams, R.T.) pp. 104-129. (Bureau of Resource Sciences, Australian Government Publishing Service: Canberra.)
- Lenghaus, C., Studdert, M.J., Gavier-Widen, D., 2000. Calicivirus infections. In 'Infectious Diseases of Wild Mammals'. (Eds Williams, E.S. & Barker, I.K.) pp. 280-291. (Iowa State University Press, Ames, USA.)
- Liu, S.J., Xue, H.P., Pu, B.Q., Qian, N.H., 1984. A new viral disease of rabbits [in Chinese]. Animal Husband Veterinary Medicine 16: 253–5.
- Löliger, H.C., Eskens, U., 1991. Incidence, epizootiology and control of viral haemorrhagic disease of rabbits and the European brown hare syndrome in Germany. Revue Scientifique et Technique. Office Internationale des Epizooties 10: 423-430.
- Marcato, P.S., Benazzi, C., Vecchi, G., Galeotti, M., Della Salda, L., Sarli, G., Lucidi, P., 1991. Clinical and pathological features of viral haemorrhagic disease of rabbits and the European brown hare syndrome. Revue Scientifique et Technique (International Office of Epizootics) 10: 371-392.
- Marchandeau, S., Le Gall-Reculé, G., Bertagnoli, S., Aubineau, J., Botti, G., Lavazza, A., 2005. Serological evidence for a non-protective RHDV-like virus. Veterinary Research 36(1): 53-62.
- McColl, K.A., Morrissy, C.J., Collins, B.J., Westbury, H.A., 2002. Persistence of rabbit haemorrhagic disease virus in decomposing rabbit carcases. Australian Veterinary Journal 80(5): 298-299.

- Merchán, T., Rocha, G., Alda, F., Silva, E., Thompson, G., de Trucios, S.H., Pagés, A., 2011. Detection of rabbit haemorrhagic disease virus (RHDV) in nonspecific vertebrate hosts sympatric to the European wild rabbit (*Oryctolagus cuniculus)*. Infection, Genetics and Evolution 11(6): 1469-74.
- Mills, J.N., Ellis, B.A., McKee, K.T., Calderon, G.E, Maiztegui J.I., Nelson, G.O., Ksiazek, T.G., Peters, C.J., Childs, J.E., 1992. A longitudinal study of Junin virus activity in the rodent reservoir of Argentine hemorrhagic fever. American Journal of Tropical Medicine and Hygiene 47(6): 749-63.
- Mitro, S., Krauss, H., 1993. Rabbit haemorrhagic disease: a review with special reference to its epizootiology. European Journal of Epidemiology 9: 70-8.
- Moss, S.R., Turner, S.L., Trout, R.C., White, P.J., Hudson, P.J., Desai, A., Armesto, M., Forrester, N.L., Gould, E.A., 2002. Molecular epidemiology of rabbit haemorrhagic disease virus. Journal of General Virology 83: 2461-2467.
- Mutze, G., Bird, P., Kovaliski, J., Peacock, D., Jennings, S., Cooke, B., 2002. Emerging epidemiological patterns in rabbit haemorrhagic disease, its interaction with myxomatosis, and their effects on rabbit populations in South Australia. Wildlife Research 29: 577-590.
- Nowotny, N., Bascuñana, C.R., Ballagi-Pordány, A., Gavier-Widén, D., Uhlén, M., Belák, S.,1997. Phylogenetic analysis of rabbit haemorrhagic disease and European brown hare syndrome viruses by comparison of sequences from the capsid protein gene. Archives of Virology 142: 657-673.
- Nyström, K., Le Gall-Reculé, G., Grassi, P., Abrantes, J., Ruvoën-Clouet, N., Le Moullac-Vaidye, B., Lopes, A.M., Esteves, P.J., Strive, T., Marchandeau, S., Dell, A., Haslam, S.M., Le Pendu, J., 2011. Histo-blood group antigens act as attachment factors of rabbit hemorrhagic disease virus infection in a virus strain-dependent manner. PLoS Pathogens 7: e1002188.
- Ohlinger, V.F., Haas, B., Meyers, G., Weiland, F., Thiel, H.J., 1990. Identification and characterization of the virus causing rabbit hemorrhagic disease. Journal Virology 64: 3331–3336.
- Ohlinger, V.F., Haas, B., Thiel, H.J., 1993. Rabbit hemorrhagic disease (RHD): characterization of the causative calicivirus. Veterinary Research 24: 103-116.
- Parkes, J.P., Norbury, G.L., Heyward, R.P., Heath, A.C.G., 2001. Proceedings of the New Zealand Society of Animal Production 61: 68-70.
- Pastoret, P.P., Govaerts, A. Bazin, H., 1990. Immunologie Animale Flammarion Medicine Sciences, Paris, pp. 243-260.
- Porcasi, X., Calderon, G., Lamfri, M., Gardenal, N., Polop, J., Sabattini, M., Scavuzzo, C., 2005. The use of satellite data in modeling population dynamics and prevalence of infection in the rodent reservoir of Junin virus. Ecological Modelling 185 (2-4): 437-449.
- Royal Decree 1201/2005, de 10 de octubre, sobre protección de los animales utilizados para experimentación y otros fines científicos. Boletín Oficial del Estado núm 252, p 34367.

- Rosell, J.M., Badiola, J.I., Pérez, A., Badiola, J.J., García, J.A., Vargas, M.A., 1989. Enfermedad vírica hemorrágica del conejo. I. Epizootiológica y clínica. Medicina Veterinaria 6 (5): 275-284.
- Ruvoen-Clouet, N., Blanchard, D., André-Fontaine, G., Ganière, J.P., 1995. Partial characterization of the human erythrocyte receptor for rabbit haemorrhagic disease virus. Research in Virology 146: 33–41.
- Ruvoen-Clouet, N., Ganière, J.P., André-Fontaine, G., Blanchard, D., Le Pendu, J., 2000. Binding of rabbit hemorrhagic disease virus to antigens of the ABH histo-blood group family. Journal of Virology 74: 11950-11954.
- Schirrmeier, H., Granzow, H., Bergmann, H., Schlüter, H., 1990. Experimentelle Untersuchungen zur Hämorrhagischen Septikämie der Kaninchen [in German]. Monatshefte Veterinärmedizin 45: 193-197.
- Smith, A.W., 1998. Calicivirus models of emerging and zoonotic diseases. In Proceedings of the rabbit control, RCD: dilemmas and implications conference. Wellington, New Zealand, March 30. Vol. 31: 1998.
- Valverde, J.A., 1967. Estructura de una Comunidad de Vertebrados Terrestres. C.S.I.C., Madrid.
- www.ingenasa.es/pdf%5C17_RHD_K1_espan_protocolo.pdf
- Xu, Z.J., Chen, W.X., 1989. Viral Hemorrhagic Disease in rabbits: a review. Veterinary Research Communications 13: 205-212.
- Zheng, T., Lu, G., Napier, A.M., Lockyer, S.J., 2003. No virus replication in cats fed with RHDV-infected rabbit livers. Veterinary Microbiology 95: 61.

CHAPTER III

Immune and virological status of European wild rabbit *Oryctolagus cuniculus* to rabbit haemorrhagic disease (RHD) in two dense populations in Extremadura determined during culling

Merchán, T., Rocha, G.

Departamento de Ingeniería del Medio Agronómico y Forestal, University of Extremadura. Avda. Virgen del Puerto, 2. 10600 Plasencia, Cáceres, Spain. tmerchan@unex.es, gregorio@unex.es

Merchán Sánchez, T., Rocha Camarero, G., 2009. Situación inmunitaria y virológica frente a la enfermedad hemorrágica vírica (RHD) del conejo de monte Oryctolagus cuniculus en dos poblaciones densas de Extremadura durante el descaste. Caza, desarrollo rural y sostenibilidad ambiental en la Andalucía del siglo XXI. Consejería de Medio Ambiente. Dirección General de Gestión del Medio Natural. Octubre de 2009. Córdoba. ***Oral Communication Science Congress.***

1. Resumen

Durante una jornada de caza en descaste (julio de 2005) se recogieron muestras de sangre e hígado de conejos abatidos en dos terrenos cinegéticos de Extremadura. Las dos zonas presentan unas características ecológicas similares compuestas por un sistema de dehesas abierto donde domina la encina (*Quercusilex*) y la retama (*Retama sphaerocarpa*). En Ocadal-Las Golondrinas (O-LG) (Cáceres), la abundancia relativa de conejos en julio fue 83 letrinas/hora, muy similar a la detectada en Quintos del Viar (QV) (Badajoz) con 87 letrinas/hora, catalogándose ambas con la categoría de abundancia alta.

Con el fin de determinar los niveles de anticuerpos encontrados frente a la enfermedad hemorrágica vírica (RHD) se analizaron los sueros mediante técnicas de enzimoinmunoensayo (ELISA) en 31 conejos de monte de la zona O-LG y 24 de la zona QV. Por otra parte, para evidenciar la presencia de ARN vírico mediante análisis de RT-PCR se analizaron los hígados de 17 conejos de los 31 capturados en O-LG y de 29 de Quintos de Viar (incluyendo los 24 del análisis anterior).

La seropositividad (títulos positivos/total) en O-LG fue tan sólo del 10%, mientras que en QV alcanzó el 54%, existiendo diferencias significativas entre ambas zonas (test de Fisher, $p<0.01$). Atendiendo a estos datos, en O-LG las condiciones serían propicias para que la entrada de un virus provocase un brote epizoótico de alto impacto debido a la escasa protección inmunitaria detectada en la muestra. Tanto es así, que con la llegada de las adversas condiciones climatológicas del otoño, se constató una elevadísima mortalidad por RHD a finales de septiembre. Los datos de QV parecen sugerir o una mayor circulación del virus de RHD con una presentación más frecuente, o la aparición de un brote reciente de RHD lo cual permitiría explicar el elevado porcentaje de seropositivos encontrado. Este hecho confiere a esta población una mejor respuesta ante la llegada de la enfermedad en otoño, donde la mortalidad por RHD afectaría únicamente a la porción poblacional no inmunizada. De esta manera, los brotes pueden provocar escasa mortalidad e incluso no detectarse, como de hecho ocurrió en QV.

Por otro lado, los análisis de los hígados en ambas poblaciones revelaron un porcentaje de PCR positivos muy similar (23,5% en O-LG y 24,1% en QV). Estos resultados permiten ratificar como la presencia del virus en conejos vivos es una constante en poblaciones de alta densidad y nos alertan sobre la necesidad de comprobar en cualquier movimiento de conejos cuál es la cepa vírica que podamos introducir para evitar la translocación de variantes más patógenas. Al mismo tiempo, las casi idénticas proporciones de PCR positivos en ambas poblaciones, pero los desiguales resultados inmunológicos, podrían ser indicativos de la presencia en QV de formas víricas apatógenas o con

menor poder patógeno, pero con capacidad inmunógena que permitiría detectar mayores porcentajes de seropositivos en épocas alejadas de los brotes primaverales y otoñales.

2. Abstract

On a single day of culling in July 2005, blood and liver samples were collected from rabbits shot in two hunting grounds in different parts of Extremadura. These areas have similar ecological characteristics, comprising open woodland with a predominance of holmoak (*Quercus ilex*) and broom (*Retama sphaerocarpa*). In Ocadal-Las Golondrinas (O-LG) (province of Cáceres), rabbit relative abundance in July was 83 latrines/hour, very similar to the abundance detected in Quintos del Viar (QV) (province of Badajoz), with 87 latrines/hour. Both areas can be classified in the high abundance category.

Sera were analysed through enzyme immunoassay techniques (indirect ELISA) in 31 wild rabbits from O-LG and 24 rabbits from QV to determine the levels of antibodies to rabbit haemorrhagic disease (RHD). A further study was conducted to confirm the presence of viral RNA through Polymerase Chain Reaction (PCR) analysis using the livers of 17 of the 31 rabbits captured in O-LG and 29 rabbits from QV (including the 24 from the sera analysis).

Seropositivity (positive titres/total) in O-LG was only 10%, as opposed to 54% in QV, with significant differences between the two areas (Fisher's test, $p<0.01$). Based on these data, conditions in O-LG would be favourable for entry of the virus to cause a high impact epizootic outbreak due to the limited immune protection detected in the sample. A very high RHD mortality rate was reported at the end of September, with the arrival of adverse autumn weather. Data from QV seem to suggest either greater circulation of the RHD virus with more frequent presentation, or a recent outbreak of RHD, which would explain the high percentage of seropositive samples found. This circumstance gives the QV population a better response to the arrival of the disease in autumn, as RHD mortality would affect only the non-immunised portion of the population. Thus outbreaks could cause only slight mortality or may not even be detected, as happened in the QV area.

Liver analyses in both populations showed a very similar PCR positive percentage (23.5% in O-LG and 24.1% in QV). These results allow us to confirm that the presence of the virus in live rabbits is a constant feature of high density populations. They also warn us of the need to confirm which viral strain we could be introducing with any movement of rabbits, to avoid translocating more pathogenic variants. At the same time, the almost identical PCR positive proportions in the populations, but with different immunological results, may indicate the presence of non-pathogenic or less virulent forms in QV. These forms are likely to have an immunogenic capacity that would make it possible to detect higher percentages of seropositive individuals during periods outside spring and autumn outbreaks.

3. Introduction

Rabbit haemorrhagic disease (RHD) was first detected in 1984 in domestic rabbits in China (Liu *et al.*, 1984). It was introduced into Europe in 1988, occurring in Germany, Belgium, Italy, France and Spain, and was first described in Spain in Asturias and León (Argüello *et al.*, 1988). It is thought that the disease could have been introduced through live animals or frozen meat, leading to the appearance of geographically isolated focal points. It was later detected in Segovia, Murcia, the Canary Islands, Almería and Málaga (Villafuerte & Moreno, 1991).

The origin of the RHD virus has not been clearly identified. Authors of recent serological and phylogenetic studies refer to the existence of non-virulent forms in European rabbits several decades before the major epidemics at the end of the 1980s (Forrester *et al.*, 2006). Other authors maintain that rabbit caliciviruses could have come from possible changes occurring in non-pathogenic forms (Moss *et al., 2*002) and even refer to rabbit haemorrhagic disease virus (RHDV) emerging from a different virus that affects other species (Fenner & Fantini, 1999). The Caliciviridae family has seven different genogroups in Europe (Forrester *et al.*, 2006) that maintain high mutation rates.

Infection occurs from infected to healthy animals, through the nasal, conjunctive, and oral routes and also through skin wounds. It also occurs indirectly, through contaminated food and water, urine and faeces. Flies (*Hybopygia varia*, Sarcophagidae) can carry sublethal doses of RHDV. Similarly, scavengers and predators (hunting dogs, foxes) can spread the virus after feeding off diseased animals or their carcasses. It has recently been discovered that free-living rodents in sympatry with wild rabbits can host and spread the virus (Merchán *et al.*, 2011).

RHD can be highly acute, acute or subacute and disappears 8-10 days after the first cases. The virus is highly labile as soon as it leaves the host and loses its activity a few hours after affecting adults and sub-adults but not young individuals (less than 50-60 days). A new RHDV strain capable of infecting young farm-raised rabbits during their first 60 days of life has recently been discovered (Dalton *et al., 2*012).

The objectives of this study are, firstly, to determine antibody levels to rabbit haemorrhagic disease from samples obtained during culling (outside the period of spring and autumn disease outbreaks) of two high density wild rabbit populations using indirect ELISA techniques; secondly, to detect the presence of viral RNA in both populations through RT-PCR analysis of two wild rabbit populations and comparison of the populations; and thirdly, to study the immune status of rabbits in which the virus is present (PCR positive).

4. Methodology

The two study areas chosen in Extremadura (Figure 1) are characterised by their thermo-Mediterranean climate and habitat of woodland and grassland dominated by *Quercus* spp., *Retamasphaerocarpa*, *Cistus* spp. and *Ulici eriocladi-Cistetum ladaniferi* (Rivas-Martínez, 1983).

Ocadal-Las Golondrinas is in the municipality of Plasenzuela, in the province of Cáceres, and Quintos del Viar is in the municipality of Reina, in the province of Badajoz. A high density rabbit population was chosen in each location.

Rabbit relative abundance categories were estimated based on the number of latrines/hour during sampling (Guzmán *et al*., 2004) in each location. In Plasenzuela (Ocadal-Las Golondrinas hunting ground), counting conducted in July in a two-hectare area showed a rabbit relative abundance of 83 latrines/hour. In Reina (Quintos del Viar hunting ground), the result in an area of a similar size was 87 latrines/hour. The rabbits used for sampling were killed on one day's hunting in July 2005, during the period referred to as culling.

In Ocadal-Las Golondrinas, 31 rabbits were obtained, from which 31 blood and 17 liver samples were taken. In Quintos del Viar, 29 rabbits were obtained, from which 24 blood and 29 liver samples were taken.

For the laboratory study, parts of the rabbit liver samples were extracted to determine the presence of RHDV. This task was performed at different times using sterile laboratory material, under strict biosecurity conditions to avoid cross-contamination. All samples were stored at -20 ºC until analysis.

From the liver samples, 125 mg of tissue was homogenised in 1.25 mg sterile PBS (8mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.4) and centrifuged at 2500 rpm for 15 min. Supernatant was collected and stored at -20 ºC until RNA extraction. Viral RNA extraction was performed on 100 µl of the homogenate using TriPure reagent (Roche), following the manufacturer's instructions. The PCR programme consisted of an initial denaturation stage for 2 min at 95 ºC, 40 1-min cycles at 95 ºC, 1 min at 64 ºC and 1 min at 72 ºC, and one final extension step of 5 min at 72 ºC. All RNA extraction and amplification stages were performed in a dedicated laboratory and all reactions included both positive and negative controls.

The serum sample was obtained through blood centrifugation at 2500 rpm for 15 min and screened to detect antibodies and their titration using RHD CIVTest cuni (Laboratorios Hipra S.A., Gerona, Spain). We expressed antibody concentration as the index of Relative Immunity (RI), in

which the optical density (OD) of each sample was related to the OD of positive and negative controls. RI is used because the OD values in positive serum can have a high standard deviation and high standard error due to variation in the absorbance of the plate. For this reason the formula RI = (T-N)(P-N)x100 was applied and then validated on reading, as this is when the values P (Positive), N (Negative) and T (tested serum) are obtained. RI values ≥ 1.5 are considered positive.

5. Results and discussion

5.1. Immunology

Significant differences between seropositivity percentages were determined in both populations (Fisher's test, $p<0.01$). In the 31 tested rabbits in the O-LG area, prevalence was 10% seropositive and 90% seronegative. Prevalence was taken as the number of positive cases/total x 100. The low positive percentage may make the population vulnerable to the entry of a virulent strain or the activation of a local strain, with the possibility of causing high morbidity and mortality from RHD.

In the 24 rabbits analysed in the QV area, prevalence was 54% seropositive rabbits and 46% seronegative rabbits. These seropositivity results are lower than those detected in the first years following the initial outbreak of the disease (Simón *et al*., 1998), which was 80-100% of the population. The results of this study are very similar to findings in autumn in other parts of the south of Extremadura (Rocha & Merchán, 2008).

The percentages indicate that half the population could be better protected against the entry of a virulent strain or increased virulence of a local strain. However, they could also indicate a recent appearance of an outbreak or the existence of non-pathogenic forms in the population sampled; these forms have immunogenic capacity and can be detected with ELISA screening. It has not yet been possible to describe this situation in Spain, although it is known in other European populations (Le Gall-Reculé *et al*., 2011). In the positive cases we do not know the real protective capacity of the antibodies detected.

5.2. RHDV detection

Analysis of the 17 rabbits in the O-LG area showed that 23.5% were PCR positive. In the QC area 29 rabbits were analysed and 24.1% were PCR positive. No differences were found between populations in terms of the percentage of the virus presence even though the populations showed different seropositivity. This circumstance appears to indicate that the presence of the virus is habitual in high density populations and that resistance increases over time, as the rabbits were

healthy. Although fewer viruses would be expected to be circulating in the environment after adaptation of the virus to the host, the percentage of virus presence (24.1 and 23.5%) in the rabbits was the highest ever determined, as previous findings were just 7.2% of animals sampled with vestiges of the RHD virus (Simón *et al*., 1995). These differences are probably due to the different diagnostic techniques used by these authors (Dot ELISA). The technique we used in this study was RT-PCR, which is more specific and sensitive than Dot ELISA.

In the O-LG population (n=17 rabbits) no rabbits were both seropositive and PCR positive. However, 10.3% rabbits were seropositive and PCR positive to RHDV in the QV sample (n=29). This can be interpreted in a variety of ways and requires knowledge of recent RHD epidemiology and subsequent monitoring of its evolution. With this information, we know that the rabbits were not in the final phase of infection and therefore had not seroconverted in response and neither were they in the acute phase.

This finding shows that carrier animals may exist. The percentage of animals with carrier status is higher than the figure of 1.6% reported by Simón *et al*. (1995), and allows us to discuss the existence of individuals in rabbit populations that are persistently infected and could host non-pathogenic forms or other uncommon strains different from the virulent strains, but which are capable of producing an immune response.

In addition, 13.8% of samples were both seronegative and PCR negative. Information on the background and evolution of the population sampled allows us to rule out the possibility that this was a recently infected population which had not yet been seroconverted, as no disease outbreaks occurred.

Therefore, it could be a case of immunotolerant rabbits or persistently infected rabbits that are seronegative to the specific strain detected, but which could develop resistance antibodies to other strains. Further studies are needed, not only to determine the virulence of these types of strains, but also to use sequencing techniques to determine the forms of *Calicivirus*, their variants and the presence of antigenically similar viruses, as addressed in studies in other geographical areas (Capucci *et al*., 1996; Bergin *et al*., 2009; Kerr *et al*., 2009). From the result of this study and using information about the Iberian Peninsula already published (Alda *et al*., 2010), we can infer that greater caution is required when translocating individuals from high density populations, as the viruses associated with these populations are transferred at the same time, regardless of the immune status and immunity type (natural or induced). The effects of translocations, involving the

introduction and spread of strains different from local strains, are stochastic processes that have a highly negative impact on the recovery of wild rabbit populations.

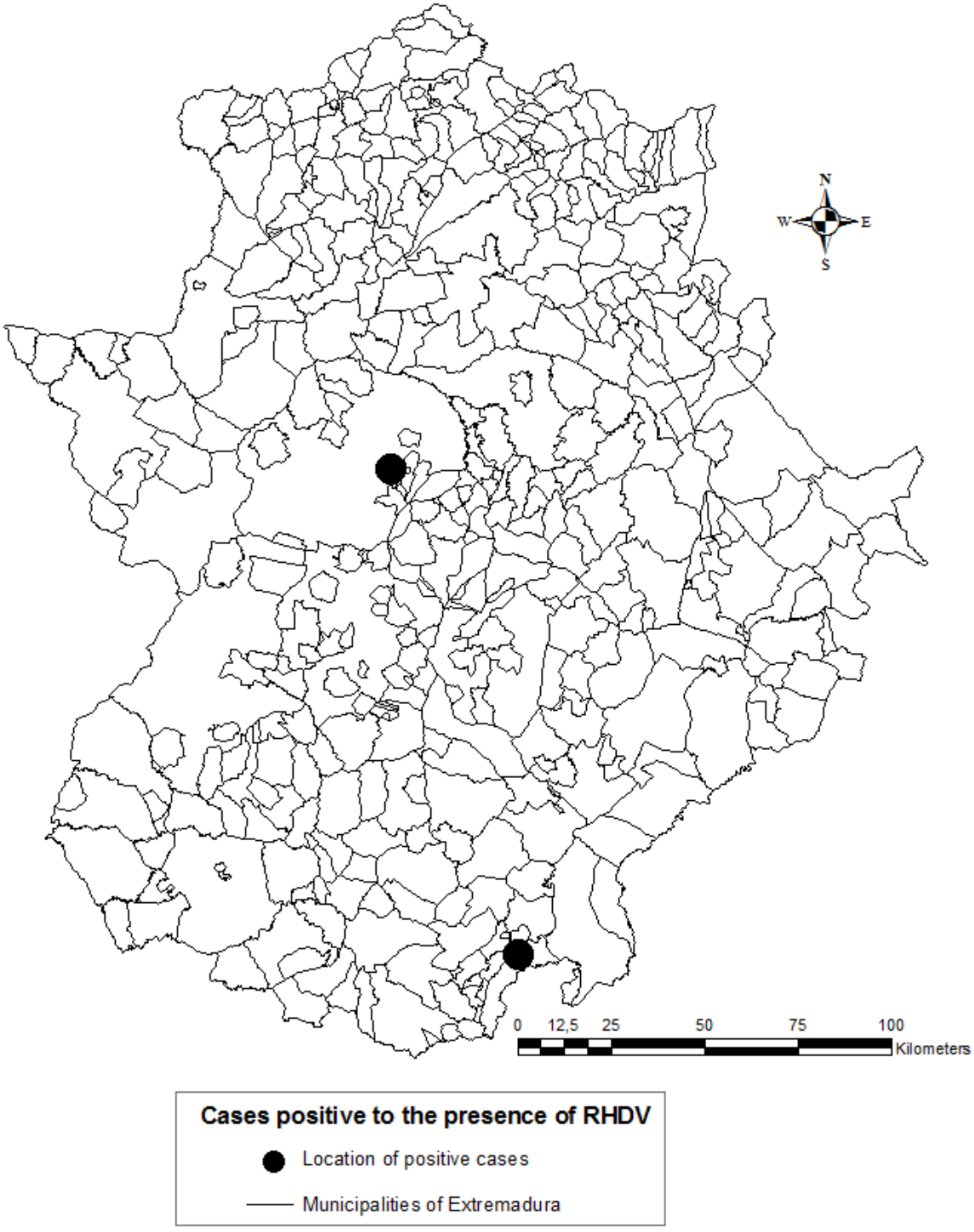


Figure 1: Study areas and location of positive cases

6. Conclusions

The following conclusions can be drawn from the results of this study:

1. Serological testing provides very useful information to determine the immune status of a population at a given time and allows us to predict a situation of risk that may require implementation of a particular control. A context of low immune status in a population commonly hunted during culling can indicate whether continued hunting is advisable during this period.

2. When the antibody level of a population is known, its suitability as a donor for translocations can be determined. Although density appears to be a factor that favours appropriate immune status, the two dense populations in this study showed different levels of seropositivity and therefore checks should be performed at different times of year to determine what actions can be taken.

3. Virus presence in the population (24.1% and 23.5%) and the high rate of RHDV mutation highlight the risk involved in transferring rabbits from different geographical areas. The existence of rabbits that are both seropositive and PCR positive (10.3%) and seronegative and PCR positive (13.8%) can provide information on the presence of low virulence forms in carrier rabbits.

7. References

- Alda, F., Gaitero, T., Suárez, M., Merchán, T., Rocha, G., Doadrio, I., 2010. Evolutionary history and molecular epidemiology of rabbit hemorrhagic disease virus in the Iberian Peninsula and Western Europe. BMC Evolutionary Biology 10: 347.
- Argüello, J.L., Llanos, A., Pérez, L.I., 1988. Enfermedad vírica hemorrágica del conejo en España. Medicina Veterinaria 5: 645-650.
- Bergin, I.L., Wise, A.G., Bolin, S.R., Mullaney, T.P., Kiupel, M., Maes, R.K., 2009. Novel calicivirus identified in rabbits, Michigan, USA. Emerging Infectious Diseases 15: 1955-1962.
- Capucci, L., Fusi, P., Lavazza, A., Pacciarini, M.L., Rossi, C., 1996. Detection and preliminary characterization of a new rabbit calicivirus related to rabbit hemorrhagic disease virus but nonpathogenic. Journal of Virology 70: 8614–8623.

- Dalton, K.P., Nicieza, I., Balseiro, A., Muguerza, M., Rosell, J.M., Casais, R., 2012. Variant rabbit hemorrhagic disease virus in young rabbits, Spain. Emerging Infectious Diseases. Vol. 18, Num. 12, Dec. 2012. [Internet]. 2012 Nov [date cited]. http://dx.doi.org/10.3201/eid1812.120341
- Forrester, N.L., Trout, R.C., Turner, S.L., Kelly, D., Boag, B., Moss, S., Gould, E.A., 2006. Unravelling the paradox of rabbit haemorrhagic disease virus emergence, using phylogenetic analysis; possible implications for rabbit conservation strategies. Biological Conservation 131: 296- 306.
- Fenner, F., Fantini, B., 1999. Biological control of vertebrate pests: the history of myxomatosis; an experiment in evolution, 1 edn. CABI Publishing, Wallingford.
- Guzmán, J.N., García, F.J., Garrote, G., Pérez de Ayala, R., 2004. El lince ibérico (*Lynx pardinus*) en España y Portugal. Censo-diagnóstico de sus poblaciones. Organismo Autónomo de Parques Nacionales. Madrid. 174 pp.
- Kerr, P.J., Kitchen, A., Holmes, E.C., 2009. Origin and phylodynamics of rabbit hemorrhagic disease virus. Journal of Virology 83: 12129-12138.
- Liu, S.J, Xue, H.P., Pu, B.Q., Qian, N.H., 1984. A new viral disease of rabbits. Animal Husband Veterinary Medicine 16: 253–255.
- Le Gall-Reculé, G., Zwingelstein, F., Fages, M.P., Bertagnoli, S., Gelfi, J., Aubineau, J., Roobrouck, A., Botti, G., Lavazza, A., Marchandeau, S., 2011. Characterisation of a non-pathogenic and non-protective infectious rabbit lagovirus related to RHDV. Virology 410: 395–402.
- Merchán, T., Rocha, G., Alda, F., Silva, E., Thompson, G., de Trucios, S.H., Pagés, A., 2011. Detection of rabbit haemorrhagic disease virus (RHDV) in nonspecific vertebrate hosts sympatric to the European wild rabbit (*Oryctolagus cuniculus*). Infection, Genetics and Evolution 11(6): 1469-74.
- Moss, S.R., Turner, S.L., Trout, R.C., White, P.J., Hudson, P.J., Desai, A., Armesto, M., Forrester, N.L., Gould, E.A. 2002. Molecular epidemiology of rabbit haemorrhagic disease virus. Journal of General Virology 83: 2461-2467.
- Rivas Martínez, S., 1983. Pisos bioclimáticos de España, Lazaroa 5: 33-43.
- Rocha, G., Merchán, T., 2008. Seropositividad otoñal frente a Mixomatosis y a Enfermedad Hemorrágica Vírica en 8 poblaciones de conejo silvestre de Extremadura. Actas III Congreso Andaluz de Caza. Consejería de Medio Ambiente de la Junta de Andalucía y Federación Andaluza de Caza. Córdoba (España).
- Simón, M.C., Gironés, O., Muguruza, R., Alonso, J.L., Muzquiz, J.L., Ortega, C., 1995. Diagnostic survey of viral haemorrhagic disease in wild rabbits (*Oryctolagus cuniculus*) in four regions of Spain. Revue Scientifique et Technique/Office International des Epizooties 14 : 801-810.
- Simón, M.C., Ortega, C., Maynar, P., Muzquiz, J.L., De Blas, I., Muguruza, R., Gironés, O., Alonso, J.L., Sánchez, J., 1998. Studies in wild rabbit (*Oryctolagus cuniculus*) populations in

Navarra, Spain. I. Epidemiology of rabbit viral haemorrhagic disease. Gibier Faune Sauvage, Game and Wildlife 15: 47-64.

- Villafuerte, R., Moreno, S., 1991. Viral haemorrhagic disease in Doñana National Park. XXth International Theriological Congress. Rome, August 1991.

CHAPTER IV

Autumn seropositivity to myxomatosis and rabbit haemorrhagic disease of eight wild rabbit populations in Extremadura, Spain

Rocha, G., Merchán, T.

Departamento de Ingeniería del Medio Agronómico y Forestal, University of Extremadura. Avda. Virgen del Puerto, 2. 10600 Plasencia, Cáceres, Spain. gregorio@unex.es tmerchan@unex.es,

Rocha Camarero, G., Merchán Sánchez, T., 2009. Seropositividad Otoñal frente a la mixomatosis y a la enfermedad hemorrágica vírica en ocho poblaciones de conejo silvestre de Extremadura. Caza, desarrollo rural y sostenibilidad ambiental en la Andalucía del siglo XXI. Consejería de Medio Ambiente. Dirección General de Gestión del Medio Natural. Octubre de 2009. Córdoba. ***Oral Communication Science Congress.***

1. Resumen

El presente trabajo pretende realizar una aproximación a la situación inmunitaria frente a mixomatosis y la enfermedad hemorrágica vírica de varias poblaciones naturales de conejo silvestre. Se seleccionaron 8 zonas repartidas a lo largo y ancho de Extremadura (5 en Badajoz y 3 en Cáceres), eligiendo el periodo otoñal (de octubre a diciembre) de 2004 por la facilidad en la obtención de muestras a partir de cacerías. Se recopiló información de dichas zonas relativa a la presencia de algún brote de cualquiera de ambas patologías en campo en esas fechas, sin que se apreciaran indicios de enfermedad en ninguna de ellas. Los individuos abatidos eran pesados y sexados en el momento de su captura o antes de las dos horas siguientes. En ese momento, a cada animal se le extraían dos muestras se sangre de la cavidad torácica y posteriormente eran eviscerados. La sangre, tras su centrifugación y obtención del suero se analizaba para la determinación de la titulación de anticuerpos frente a mixomatosis y a enfermedad hemorrágica vírica mediante técnicas ELISA. En total se analizaron muestras de 107 animales.

La inmensa mayoría de las poblaciones extremeñas estudiadas (88%) presentaron anticuerpos frente a mixomatosis con porcentajes de seropositividad superiores al 80%, lo que indica que dichos animales habían tenido contacto previo con el virus y habían sobrevivido, encontrándose en ese periodo del año inmunizados frente a dicha enfermedad.

En cuanto a la enfermedad hemorrágica, la situación es bien distinta; tan solo 2 de las 8 poblaciones muestreadas (25%) presentaron porcentajes de seropositividad elevados (>80%). El 37,5% del total, presentaron porcentajes de seropositividad que se situaban entre el 53-60% de la población, mientras que la proporción restante (3 de cada 8) poseía una seropositividad muy baja (menor del 27%). No existen diferencias significativas en la seropositividad frente a ambas enfermedades en cuanto al sexo pero si encontramos diferencias atendiendo a las dos clases de edad consideradas (joven y adulto), siendo mayor el porcentaje de individuos adultos seropositivos que el de jóvenes (mixomatosis: adultos 96%, jóvenes 62,5%; hemorrágica: adultos 58%, jóvenes 12%).

Los datos parecen indicar que los animales que tienen contacto con el virus de la mixomatosis sobreviven en mayor medida que los que contactan con el virus de la enfermedad hemorrágica, aunque también podría ocurrir que la aparición del último brote de esta última enfermedad en las poblaciones tuvo lugar más tiempo atrás que el de la primera. Por las fechas de muestreo, sin brotes en campo y tras el paso del verano, podemos deducir que los elevados porcentajes de seropositividad frente a mixomatosis son la respuesta al hipotético brote epizoótico que ocurrió pocos meses antes (en verano). Finalmente, la poca seropositividad detectada frente a la

enfermedad hemorrágica también nos alerta de la susceptibilidad de la mayoría de las poblaciones a la aparición de esta patología.

2. Abstract

This paper examines the immune status of several natural wild rabbit populations to myxomatosis and rabbit haemorrhagic disease (RHD). Eight areas were chosen throughout Extremadura (five in Badajoz and three in Cáceres). The study was conducted in autumn 2004 (October to December). This season was chosen because samples are easy to obtain from hunting at this time of year. Information was gathered on the presence of any outbreak of either pathology in the field during the study. No evidence of disease was found in any of the areas. The individuals killed were weighed and sexed at capture or within two hours. Two blood samples were taken from the thoracic cavity of each rabbit before evisceration. Blood was centrifuged and the serum obtained was analysed to determine antibody titration to myxomatosis and RHD through ELISA techniques. Samples from 107 rabbits were analysed.

Most of the Extremadura rabbit populations studied (88%) had antibodies to myxomatosis, showing percentages of seropositivity higher than 80%. This indicates that these rabbits had been in previous contact with the virus and had survived. At the time of year of the study, they were immunised against the disease. For RHD, the situation was quite different. Only two of the eight populations sampled (25%) had high percentages of seropositivity (>80%). Of the total rabbits analysed, 37.5% showed percentages of seropositivity of 53-60%. The remaining populations (three out of eight) had very low seropositivity (<27%). No significant differences in seropositivity by sex to either disease were observed, although differences were found in the age classes considered (young/adult). Adults had a higher percentage of seropositivity than young rabbits (myxomatosis: adults 96%, young 62.5%; RHD: adults 58%, young 12%).

The data suggest that rabbits in contact with the myxoma virus have a higher survival rate than rabbits in contact with RHD. However, the latest outbreak of RHD in the populations sampled may have occurred earlier than the most recent myxomatosis outbreak. From the sampling dates, after summer and with no outbreaks in the field, it can be deduced that the high percentages of seropositivity to myxomatosis are a response to an epizootic outbreak a few months earlier (in summer). The low seropositivity to RHD detected warns us of the susceptibility of most populations to the emergence of this pathology.

3. Introduction

Myxomatosis is a viral disease (Poxviridae) introduced as a biological control method in Australia in 1950 and in France in 1952. From France the virus immediately spread throughout Western Europe (Fenner & Ross, 1994). RHD is similarly caused by a virus (Caliciviridae). It first appeared in China in 1984 (Liu *et al*., 1984) and in Europe (Italy) in 1986 (Cancellotti, 1990). Some authors, however, claim that the virus was already present in Europe in a non-virulent form decades before the great epidemic of the late 1980s (Forrester *et al*., 2006).

Both pathologies have caused, and continue to cause, high mortalities in rabbit populations, changing both their density and distribution (Argüello *et al*., 1988). In the Iberian Peninsula, both pathologies are now enzootic, with epizootic outbreaks (Calvete & Estrada, 2000), and they seriously harm the situation of rabbits and other species that depend on them (Virgós & Travaini, 2005). One very important aspect for understanding the development and incidence of these pathological processes is the study of the immunity of rabbit populations (Calvete & Estrada, 2000).

This study examines the immune status of several wild rabbit populations in Extremadura to myxomatosis and RHD at a specific time of year (autumn) to find possible differences in immune protection between sexes and between adult and young rabbits. We will also attempt to relate the seropositivity found to the rabbit relative abundance using a hunting abundance index.

4. Methodology

Eight areas were selected throughout Extremadura: five in Badajoz and three in Cáceres (Table 1). Autumn (October to December) was chosen because samples can easily be obtained from hunting at this time of year. The study was conducted in 2004.

Table 1: Name of each sampling point indicating the area (estate or hunting ground) and the municipality and province in Extremadura of each location.

Area	Municipality	Province
Los Baldíos	Botija	Cáceres
El Guijo	La Cumbre	Cáceres
Ocadal	Plasenzuela	Cáceres
Las Calderonas	Reina	Badajoz
El Calaverón	Mérida	Badajoz
Los Rincones	Talarrubias	Badajoz
La Rusal	Valencia de las Torres	Badajoz
La Coronada	Villafranca de los Barros	Badajoz

Information was gathered from these areas on the presence of any outbreak of either pathology in the field at the time. This was done by continually checking with the owners and administrators of the hunting grounds. No evidence of disease was observed in any of the areas.

At the end of the hunting season, annual catches were tallied for each population (hunting ground) and the hunting activity (number of hunters x number of days). These data were used to compile a Hunting Abundance Index (ICA, from the Spanish) which shows the ratio between catch and activity: ICA= catch/activity. The ICA was calculated for each area studied.

After the rabbits had been killed, they were weighed and sexed. In all cases this was done within two hours of capture to avoid deterioration. Two age groups were defined (young and adults) by palpating the cartilage or studying the lower epiphysis of the forelimb (Watson & Tyndale, 1953). At the same time, blood samples were taken from the thoracic cavity of all individuals and then the rabbits were eviscerated for macroscopic examination to rule out lesions consistent with either disease.

Serum obtained after blood centrifugation was analysed to determine antibody titration to myxomatosis and RHD using indirect ELISA techniques (CIVTest cuni, Laboratorios Hipra S.A., Gerona, Spain, for myxomatosis, and CIVTest cuni, Laboratorios Hipra S.A., Gerona, Spain, for RHD). We expressed antibody concentration as the index of Relative Immunity (RI), in which the optical density (OD) of each sample was related to the OD of positive and negative controls. RI is used because the OD values in positive serum can have a high standard deviation and high standard error due to variation in the absorption of the plate. For this reason the formula RI = (T-N)(P-N)x100 was applied and then validated on reading, as this is when the values P (Positive), N (Negative) and T (tested serum) are obtained. In the case of myxomatosis, the optical density (OD) of each sample was related to the OD of positive and negative controls. RI values ranged from 1 to 10, and sera with RI≥2 were considered positive. For RHD, RI values ≥1.5 were considered positive.

The statistical tests used were Fisher's exact test and Spearman's rank correlation. Samples from 107 rabbits were analysed.

5. Results and discussion

Most of the Extremadura populations studied (88%) showed antibodies to myxomatosis with percentages of seropositivity higher than 80% (Figure 1). This indicates that the rabbits had been in previous contact with the virus and had survived. At the time of year of the study, they were immunised against the disease.

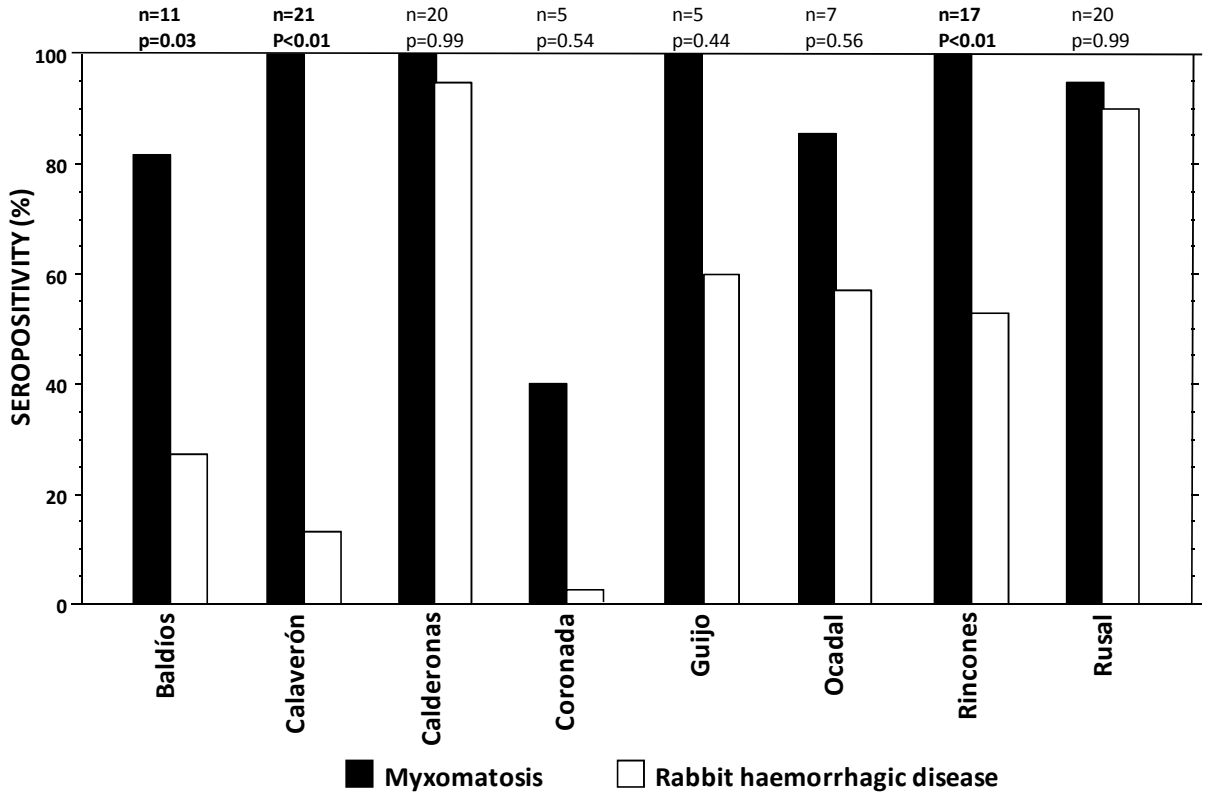


Figure 1: Percentage of rabbits seropositive to myxomatosis and RHD from the total population sampled in each area studied. The number of samples (n) and the level of statistical significance (p) are shown at the top of the graph.

For RHD (Figure 1), the situation is quite different. Only two of the eight populations sampled (25%) had high percentages of seropositivity (>80%). Of the total rabbits analysed, 37.5% showed percentages of seropositivity of 53-60%. The remaining populations (three out of eight) had very low seropositivity (<27%). The finding of a higher prevalence of myxomatosis than RHD concurs with reports by other authors for the Iberian Peninsula (Alda *et al., 2*006; Calvete & Estrada, 2000).

No significant differences in seropositivity by sex to either disease were observed (Figure 2). This concurs with the findings of other studies (Liu *et al*., 1984; Villafuerte *et al., 1*994; Calvete & Estrada, 2000).

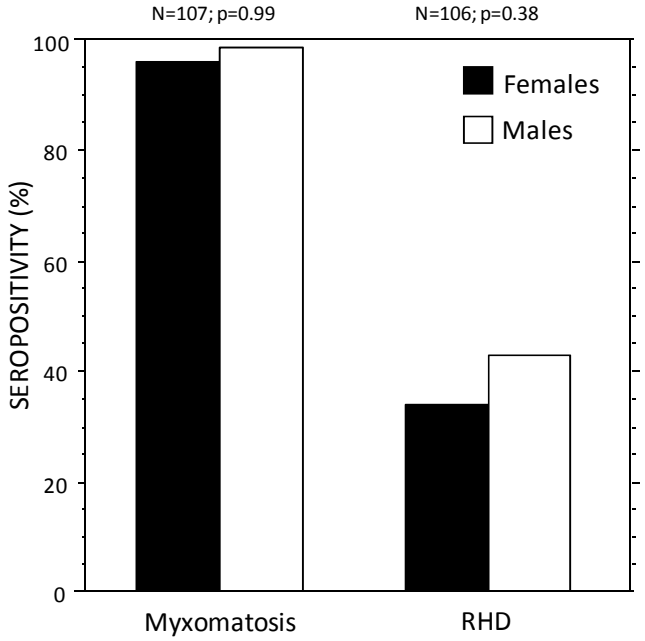


Figure 2: Percentage of rabbits seropositive to myxomatosis and RHD from the total population sampled in all areas studied, by sex. The number of samples (n) and the level of statistical significance (p) are shown at the top of the graph.

In contrast, we found significant differences in the two age classes considered (young and adult). The percentage of seropositivity was higher in adults than in young rabbits (Figure 3).

Overall, the data suggest that survival is higher in rabbits in contact with the myxoma virus than in rabbits in contact with the RHD virus. However, the most recent outbreak of RHD in the populations analysed may have occurred before the latest myxomatosis outbreak.

In view of the sampling dates, following summer and with no outbreaks in the field, we can deduce that the high percentages of seropositivity to myxomatosis are in response to the habitual epizootic outbreaks that occur a few months earlier (in summer). Similarly, the low seropositivity to RHD warns us of the susceptibility of most of the populations studied to the emergence of this pathology.

In Baldíos, Calaverón and Calderona, the few positive cases may be due to the isolation of these populations, as they are far from other rabbit areas. This prevents them from coming into contact with potentially diseased individuals and/or carriers from other populations.

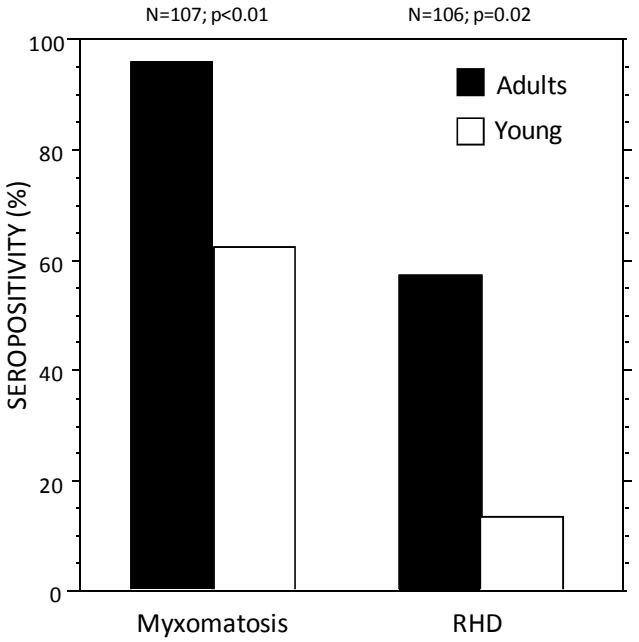


Figure 3: Percentage of rabbits seropositive to myxomatosis and RHD from the total population sampled in all areas studied, by age (adult or young). The number of samples (n) and the level of statistical significance (p) are shown at the top of the graph.

In recent years, there appears to be an overall higher resistance to myxomatosis. This can be explained as a result of more than 50 years of coexistence with the virus (Calvete, 2005). RHD has not yet reached the same situation, although this disease can be expected to gradually achieve a virus-host balance.

Seropositivity to myxomatosis is not related to the hunting abundance index. We believe this may be due to the involvement of arthropod vectors in the epidemiology of this disease. Thus higher or lower rabbit abundance will be a less important determining factor in seropositivity than vector abundance.

Seropositivity to RHD is, however, related to the hunting abundance index. Following the same working hypothesis, high rabbit abundance is a factor that encourages immunogenicity; i.e., better status to the emergence of RHD. This can lead to populations that are more resistant to this disease.

These results can help in planning recovery actions for wild rabbits in our hunting grounds, especially when we know that when rabbit abundance is higher (higher ICA) in a hunting ground, protection from RHD will be higher, at least in autumn. For example, it could be advisable to maintain populations at a high density or abundance when epizootic outbreaks are likely to occur. When translocations are necessary, the rabbits to be translocated should come from areas with high abundance and should be moved at this time of year (autumn).

6. Conclusions

The following conclusions can be drawn from the results of this study:

1. In autumn, the adult rabbit population presents higher rates of protection from both diseases than the young population.

2. At this time of year in these areas, wild rabbit populations have higher percentages of seropositivity to myxomatosis than to RHD.

3. No significant differences by sex were found in the proportion of seropositive rabbits to both pathologies.

4. Autumn seropositivity to RHD is positively and significantly correlated to rabbit relative abundance.

5. Knowledge of the immune status of a population at certain times of the annual cycle can shed light on specific management actions.

7. References

- Alda, F., Doadrio, I., Hernández, M., Muñoz, J., Silvestre, F., 2006. Genetic and immunological management for managing translocations and reintroductions of rabbits (*Oryctolagus cuniculus* L., 1758) in Spain. In: San Miguel, A. (Coord.). Handbook habitat management Iberian lynx (*Lynx pardinus*) and their main prey, the rabbit (*Oryctolagus cuniculus*). CBD-Habitat Foundation. Madrid.
- Argüello, J.L., Llanos, A., Pérez, L.I., 1988. Rabbit haemorrhagic disease virus in Spain. Veterinary Medicine 5: 645-650.
- Calvete, C., 2005. Modeling the effect of population dynamics on the impact of rabbit haemorrhagic disease. Conservation Biology 20: 1232-1241.
- Calvete, C., Estrada, R., 2000. Epidemiología de enfermedad hemorrágica (VHD) y mixomatosis en el conejo silvestre en el valle medio del Ebro -Herramientas de gestión-. Publicaciones del Consejo de Protección de la Naturaleza de Aragón, Zaragoza, Spain.
- Fenner, F., Ross, J., 1994. Myxomatosis: The European rabbit. The history and biology of a successful colonizer. Edited by: Thompson, H.V. & King, C.M., pp. 205-239.
- Forrester, N.L., Trout, R.C., Turner, S.L., Nelly, D., Boag, B., Moss, S., Gould, E.A., 2006. Unravelling the paradox of rabbit haemorrhagic disease virus emergence, using phylogenetic

analysis; possible implications for rabbit conservation strategies. Conservations Biology 131: 296-306.

- Liu, S.J., Xue, H.P., Pu, B.Q., Qian, N.H., 1984. A new viral disease in rabbits. Animal Husbandry and Veterinary Medicine 16: 253-255.
- Virgós, E., Travaini, A., 2005. Relationship between small-hunting and carnivore diversity in Central Spain. Biodiversity and Conversation 14: 3475-3486.
- Villafuerte, R., Calvete, C., Gortázar, C., Moreno, S., 1994. First epizootic of rabbit haemorrhagic disease in free living populations of *Oryctolagus cuniculus* at Doñana national park, Spain. Journal Wildlife Diseases 30(2): 176-179.
- Watson, J.S., Tyndale-Biscoe, C.H., 1953. The apophyseal line as an age indicator for the wild rabbit. New Zealand Journal of Science and Technology 34(6) B: 427-435.

CHAPTER V

Study of antibody prevalence to myxoma virus and rabbit haemorrhagic disease virus in populations of the European wild rabbit (*Oryctolagus cuniculus*) under different types of management in southern Extremadura, Spain

Merchán, T., Rocha, G., Gómez, F., Cortázar, G., Hidalgo, S., Ordiales, F., Serrano, S.

Grupo de Investigación en Recursos Cinegéticos y Biodiversidad (RCB). Veterinary School. University of Extremadura (Spain)

Merchán, T., Rocha, G., Gómez, F., Cortázar, G., Hidalgo, S., Ordiales, F., Serrano, S., 2004. Antibody prevalence to myxoma virus and RHD analysis in wild rabbit (Oryctolagus cuniculus) populations under different type of management in south Extremadura (Spain). 2º World Lagomorph Conference, Vairao, Portugal, from 26 to 31 July 2004. ***Communication (poster) at the International Congress.***

1. Resumen

Durante los meses de junio y julio de 2003, cuatro poblaciones de conejo de monte del sur de Badajoz (Extremadura) fueron muestreadas para detectar anticuerpos frente al virus dela mixomatosis (MV) y al virus de la enfermedad hemorrágica vírica (RHDV). Estas poblaciones estaban sometidas a un manejo diferente, desde nulo, o sin intervención alguna, a un manejo exhaustivo. Tres de las poblaciones estudiadas (Valencia de las Torres, Reina y Talarrubias) mostraron niveles altos de anticuerpos frente a MV y RHDV en junio y julio, y este hecho podría determinar una mayor supervivencia en los conejos en los meses siguientes, ya que, en principio, las condiciones inmunes frente a futuros brotes de enfermedades víricas serían adecuadas. Los niveles de prevalencia fueron superiores en la RHDV con respecto a MV, lo cual puede ser debido a la irrupción posterior de RHD en la Península Ibérica. También podría deberse al patrón epidemiológico de los brotes endémicos de la enfermedad o a la persistencia de RHDV en el medio por efecto de reservorios o formas víricas parcialmente no identificados. También se observa un gradiente decreciente en el número de animales seropositivos con un control sanitario intenso. Las poblaciones confinadas bajo intensas medidas sanitarias, como la vacunación periódica y la desparasitación, mostraron diferencias significativas en los niveles de seropositividad independientemente del control sanitario empleado. La utilidad de las medidas sanitarias directas se cuestiona frente a las técnicas de gestión del hábitat.

Palabras clave: prevalencia, MV, RHDV, rabbit, manejo poblacional, control sanitario

2. Abstract

In June and July 2003, four populations of European wild rabbits from the south of Badajoz (Extremadura) were screened to detect antibodies to myxoma virus (MV) and rabbit haemorrhagic disease virus (RHDV). The populations received a variety of control measures, ranging from no intervention to full control. Three of the populations studied (Valencia de las Torres, Reina and Talarrubias) showed high antibody levels to MV and RHDV in June and July. This circumstance may have determined a higher rate of survival among the rabbits in the following months as, in theory, their immune status to future outbreaks of viral diseases would have been adequate. The levels of prevalence were higher for RHDV than for MV. This may be due to the later appearance of RHD than myxomatosis in the Iberian Peninsula as a result of the epidemiological pattern of the endemic outbreaks of this disease, or to the persistence of RHDV in the environment caused by viral reservoirs or viral forms that are only partially identified. A declining trend was also observed in the number of seropositive rabbits under intense sanitary control. Populations enclosed under intense health measures such as regular vaccination and anti-parasite treatment showed significant differences in the levels of seropositivity regardless of the sanitary control used. The utility of direct health measures compared to habitat management is questioned.

Key words: prevalence, MV, RHDV, rabbit, population management, sanitary control

3. Introduction

The appearance of myxomatosis in the 1950s, followed by rabbit haemorrhagic disease in the 1980s (Argüello *et al*., 1988), caused a majorreduction in both the density and the distribution of the European wild rabbit (*Oryctolagus cuniculus*, L. 1758) in the Iberian Peninsula.

Despite the enormous influence of both pathologies on the ecology of this species, data on the epidemiology for these diseases in wild populations are limited for Spain in general and non-existent in the case of Extremadura. In view of this situation, we decided to study the prevalence of positive antibodies to the myxoma virus (MV) and the rabbit haemorrhagic disease virus (RHDV) in four wild rabbit populations in the province of Badajoz.

4. Material and methods

The study areas have high population densities and similar ecological characteristics and are not used for hunting. Both free-living and controlled populations were chosen. As the health management of the populations ranges from no control to full intervention, it was possible to assess the effect of the type of sanitary control on antibody levels. The populations are identified by the name of the municipality where they are found: Valencia de las Torres (VT), Reina (RE), Talarrubias (TA) and Jerez de los Caballeros (JC).

In 2003, 93 wild rabbits of all the age classes found during the mating season (June-July) were captured live, using ferrets, nets and manual extraction from artificial warrens. All rabbits were clinically assessed on capture to determine whether they had disease symptoms. Blood samples were obtained from the marginal ear vein and analysed to determine antibody titration through a commercial laboratory kit: indirect ELISA (INgezim RHDV 17.RHD.K.1 and INgezim Mixomatosis 17.MIX.K.1, Ingenasa, Madrid, Spain). Absorbance was measured at 405 nm with a shake duration of 10 seconds. Titres were considered positive when values were > 2 for MV and > 1.5 for RHDV.

5. Results and discussion

5.1. RHDV

External symptoms of Rabbit Haemorrhagic Disease (RHD) were not found in any of the rabbits studied at random. Prevalence rates (No. positive cases/population studied) were higher for RHDV than for MV (Figure 1) in all the study areas except JC. The prevalence rates detected follow a similar pattern to that described by Simón *et al*. (1998) in Navarra, of 80-100%. This could be explained by the more recent emergence of RHDV and therefore the persistence of a greater virus pool in the

environment. Another reason is that RHDV presents an epidemiological pattern with epizootic outbreaks, partly due to the presence of persistent epidemiological reservoirs in the environment (as yet not fully identified), which would keep the virus in specific areas. In JC, it appears that the absence of positive cases may be caused by the isolation of these rabbits, which prevents them from coming into contact with diseased and/or carrier rabbits from other areas.

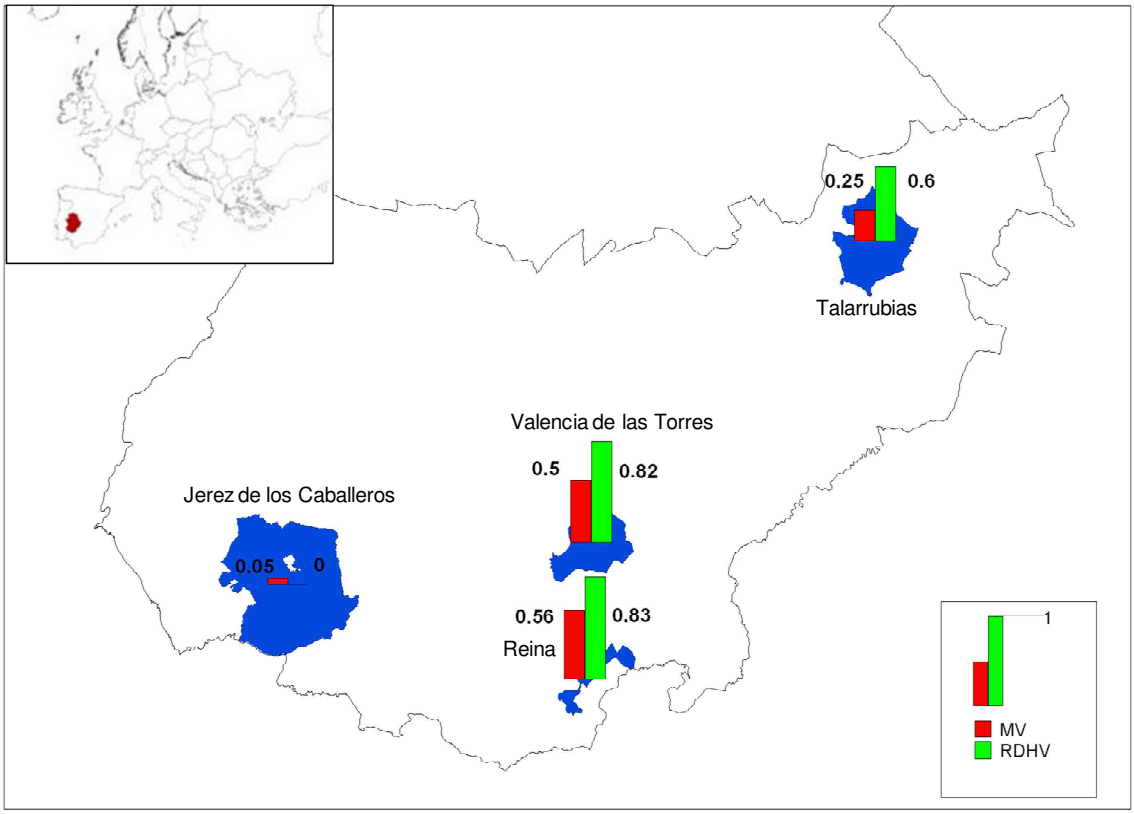


Figure 1: Antibody prevalence to MV and RHDV in the study areas.

A survey was conducted through 754 questionnaires completed by people who were in contact with the natural environment, in relation to an annual cycle from 2002-2003, in the province of Badajoz (Figure 2).

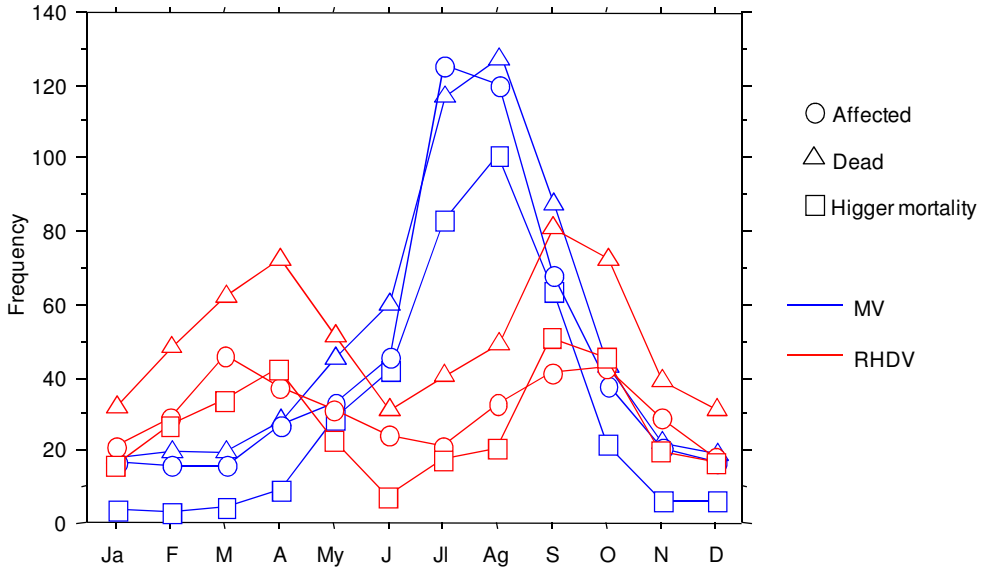


Figure 2: Phenology of MV and RHDV in the province of Badajoz.

The results were compared with the positive titration results obtained in June and July, when the incidence of RHDV is lower. It can be seen that the percentage of positive rabbits is higher. This is because the rabbits sampled had survived the outbreak in March and had time to acquire a sufficient immunisation level. This level of immunity also suggests recent contact with the virus and thus all these rabbits would, in theory, be immune to RHDV. Examination of the mean immunity levels shows statistically significant differences (Kruskal-Wallis test, p = 0.0001, n = 93) in these levels to RHDV in the four metapopulations. The differences are due to the effect of management in the JC population (Figure 3), as a pair analysis for RHDV conducted in the other municipalities shows no significant differences (Mann-Whitney U = 290 RE *vs* VT: p = 0.7582, n = 52; M-W U = 160 RE *vs* TA: p = 0.5583, n = 38; M-W U = 316 TA *vs* VT: p = 0.6670, n = 54).

Thus the various sanitary treatments the populations are given do not significantly affect immunity levels, except in JC, suggesting that the populations studied may be reaching some level of natural immunity. This circumstance appears to be enhanced by high density (Calvete & Estrada, 2000) or a change in the epizootiology of the disease (Cooke, 1994).

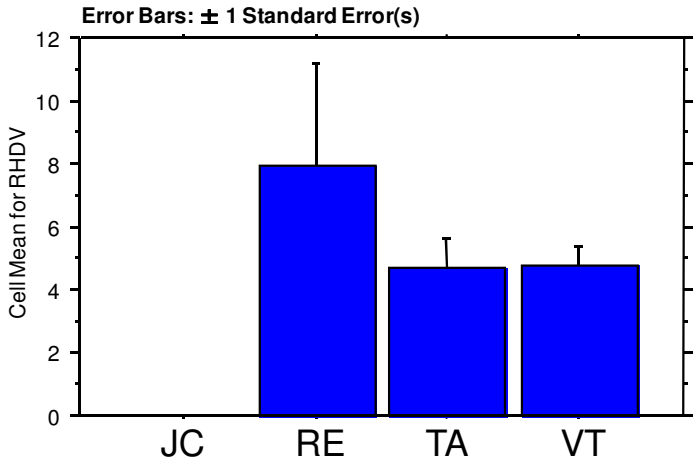


Figure 3: Mean immunity levels to RHDV in the study areas. JC: Jerez de los Caballeros. RE: Reina. TA: Talarrubias. VT: Valencia de las Torres.

In VT and RE, rabbits receive no health intervention (vaccinations or treatment of warrens with disinfectants and anti-parasite products). In TA and JC, preventive measures are regularly taken. In both areas the most recent vaccination for MV and RHDV was one year before the study. In TA the results for mean antibody level and prevalence indicate the limited effectiveness of the sanitary intervention, although this is difficult to measure.

Immunity and prevalence levels are slightly lower in TA than in VT and RE. In JC a response to sanitary intervention is observed, as no positive cases were found. This is undoubtedly due to the perimeter fence that isolates this population and stops individual rabbits coming and going. However, this situation could be highly dangerous, as confined rabbits that are not appropriately vaccinated could be susceptible to infection if diseased or carrier rabbits enter the enclosure.

5.2. MV

Some authors consider myxomatosis responsible for the direct or indirect mortality of at least 50% of young rabbits from the time they leave the warren (Calvete *et al*., 1995). This circumstance aids understanding of the phenology of this disease in the province of Badajoz (Figure 2). The highest incidence of affected and/or dead rabbits is in June and July (although according to Soriguer (1980), the usual period is from July to October in southeast Spain), just when the last cohort of young rabbits appears before reproduction ceases during summer. Many young rabbits die once they have been infected (Ross *et al*., 1989; Rogers *et al*., 1994). Thus it can be expected that the rabbits sampled, mostly adults, will have high prevalences of seropositivity.

However, due to the epidemiology of myxomatosis, which includes the intervention of vectors, and the time this disease has been in existence, prevalence rates are lower than for RHD. A new

outbreak of myxomatosis is likely to have occurred at the time of the study. This situation, shown in Figure 2, concurs with the findings of other authors when the prevalence of positive antibodies in a population decreases from 50-20 % (Arthur & Louzis, 1988; Rogers *et al*. 1994; Calvete & Estrada, 2000).

Analysis of the mean immunity levels reveals statistically significant differences (Kruskal-Wallis Test, p = 0.0001, n = 93) in immunity levels to MV in the four metapopulations. These differences are once again due to the JC population (Figure 4). A pair analysis for MV in the remaining municipalities shows no significant differences (Mann-Whitney U = 300 RE *vs* VT: p = 0.90, n = 52; M-W U = 137 RE *vs* TA: p = 0.178, n = 38), except VT compared to TA (M-W U = 236 TA *vs* VT: p = 0.05, n = 54), where statistical differences are observed. Thus it can be inferred that the sanitary intervention in the case of myxomatosis appears to affect positive antibody levels to MV.

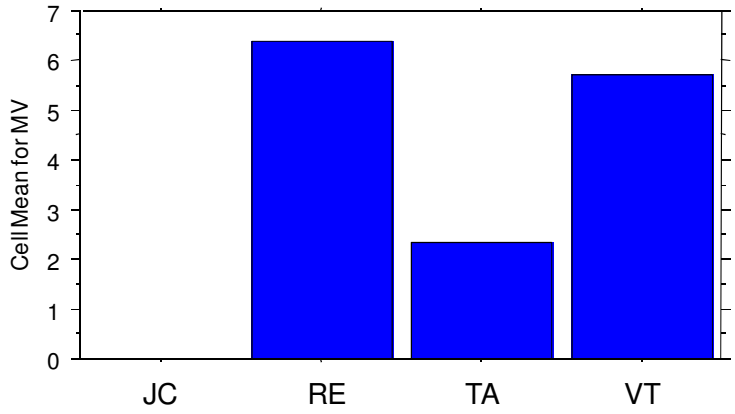


Figure 4: Mean immunity levels to MV in the study areas.JC: Jerez de los Caballeros. RE: Reina. TA: Talarrubias. VT: Valencia de las Torres.

5.3. RHDV *versus* MV

On comparing only positive cases to both RHDV and MV appearing simultaneously in individual rabbits, we find a positive correlation between the two diseases (as suggested by Calvete *et al*., (1995) who described complementarity rather than additivity between diseases). Therefore, when an individual rabbit presents higher values for MV, it also presents higher values for RHDV (Spearman's R = 0.081 p = 0.0004, n = 30) (Figure 5).

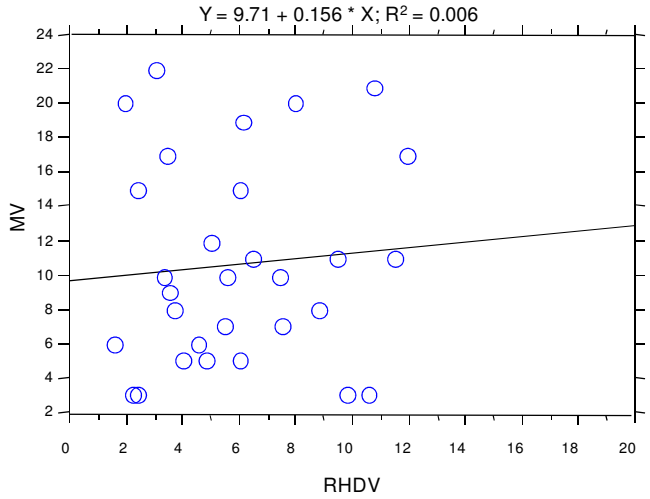


Figure 5: Simple linear regression between antibody prevalences to MV and RHDV.

If we consider each municipality separately, however, there is no significant correlation between any of them, except for marginal statistical significance in RE and VT (RE: Spearman's R = 0.4 p = 0.06, n = 18; VT: Spearman's R = 0.255 p = 0.06, n = 34) (Figures 6 and 7).

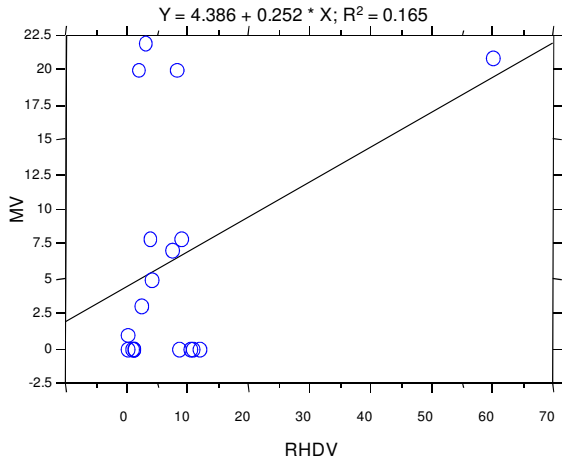


Figure 6: Simple linear regression in Reina.

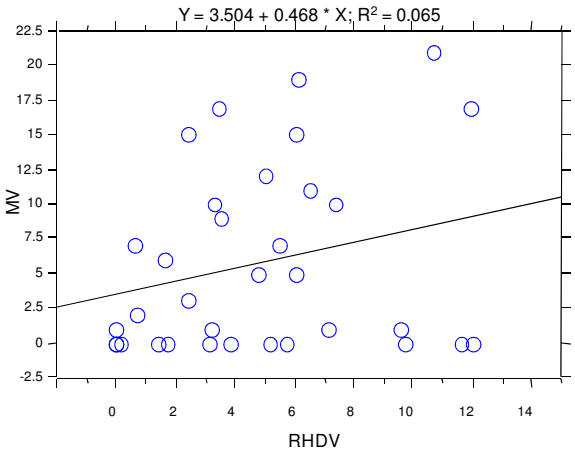


Figure 7: Simple linear regression in Valencia de las Torres.

An overall comparison of the means of the antibody levels of positive cases to MV and RHDV showed significant differences (t Test = 4.04, p = 0.0001, n = 84) (Figure 8), as the mean values of positive cases to MV are higher than those for RHDV.

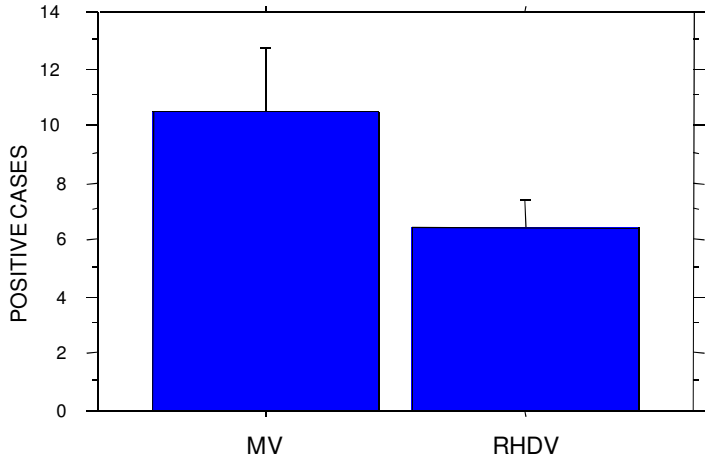


Figure 8: Means of positive cases for MV and RHDV, with a 95% confidence interval.

This circumstance could be explained by the longer existence of myxomatosis in rabbits and recent, reiterated contact with the virus. The effect of this could be similar to revaccination, as antibody levels to MV would increase after the disease is caught for the first time. This situation could be amplified due to the intervention of arthropod vectors, which are very abundant during the time of year the study was conducted. A further influence is that most of the individuals sampled were adults and therefore the negative effect of young rabbits on mean antibody levels is removed. This accounts for the high antibody levels detected in the rabbits sampled.

6. Conclusions

1. The populations studied in Valencia de las Torres, Reina and Talarrubias, in the province of Badajoz, present high antibody levels to MV and RHDV in June and July. This could increase rabbit survival in the following months and allow rabbits to reach a probable second epizootic outbreak of RHD in optimal immune conditions.

2. At the time of the study, the rabbits in Jerez de los Caballeros showed no immunity. This situation calls for caution with this type of management, as immunity is acquired primarily through appropriate, controlled vaccination. This population is therefore highly exposed to an epizootic outbreak. After vaccination, these rabbits would have better immune status from the effect of the higher protection provided by artificial induction of immunity (Calvete & Estrada, 2000). This would also make them suitable candidates, at this time of year, to reinforce populations near their enclosure.

3. In the areas of free-ranging rabbits studied, the naturally acquired high immunity levels make it advisable to adopt measures designed to keep population density high, and to assess actions such as vaccination. In some cases, apart from the financial and human resources required, vaccination could be counterproductive for rabbits (Calvete & Estrada, 2003). It is essential to know the level of naturally acquired immunity to MV and RHDV in any rabbit population that is to be vaccinated to determine whether or not vaccination is advisable.

4. The prevalences of seropositivity detected in the study areas with free-ranging rabbits are higher to RHDV (around 80%) than to MV (around 50%). This reduces the importance of RHD as a mortality factor of a pathological nature during the time of year of the study. Thus myxomatosis is the disease that could act as a destabilising factor in the populations due to the time of its appearance (June and July) and because it affects younger age classes.

5. The presence of RHDV positive antibody levels shows complementarity to MV antibody levels. The mean positive antibody level is higher to MV than to RHDV.

4. Acknowledgements

The authors are very grateful to local property owners and administrators for their invaluable help in the fieldwork.This study was funded by the Regional Government of Extremadura through a collaboration agreement between the University of Extremadura and the Extremadura Regional Ministry of Agriculture and the Environment (Ref. No. 379/01) and also by the Extremadura Regional Ministry of Education, Science and Technology (ERDF Funds: II PRI+DT+I - 2nd Regional Plan for Investigation, Technical Development and Innovation - File No. 2PR02A035).

5. References

- Arthur, C.P., Louzis, C., 1988. Myxomatose du lapin en France: une revue. Revue Scientifique et Technique. Office International des Epizooties, 7: 939-957.
- Calvete, C., Estrada, R., Villafuerte, R., Osácar, J.J., Lucientes, J., 1995. Primeros resultados en campo sobre la enfermedad hemorrágica del conejo. Trofeo, 304: 22-28.
- Calvete, C., Estrada, R., 2000. Epidemiología de enfermedad hemorrágica (VHD) y mixomatosis en el conejo silvestre en el valle medio del Ebro -Herramientas de gestión-. Publicaciones del Consejo de Protección de la Naturaleza de Aragón, Zaragoza, Spain.

- Calvete, C., Estrada, R., 2003. Las Campañas de vacunación contra la Mixomatosis y Enfermedad Hemorrágica (RHD) tienen un impacto negativo sobre la supervivencia a corto plazo de los conejos silvestres. Resúmenes VI Jornadas SECEM, Ciudad Real, p. 30.
- Cooke, B.D., 1994. Rabbit haemorrhagic disease in wild rabbits. Animal and plant control commission. Unpublished report, 16 pp.
- Rogers, P.M., Arthur, C.P., Soriguer, R.C., 1994. The rabbit in continental Europe. In: Thompson, H.V. & King, C.M. (Eds). The European rabbit: the history and biology of a successful colonizer, pp. 22-63. Oxford University Press, Oxford.
- Ross, J., Tittensor, A.M., Fox, A.P., Sanders, M.F., 1989. Myxomatosis in farmland rabbit populations in England and Wales. Epidemiology and Infection 103: 333-357.
- Simón, M.C., Ortega, C., Maynar, P., Muzquiz, J.L., De Blas, I., Gironés, O., Alonso, J.L., Sánchez, J., 1998. Studies in wild rabbit (*Oryctolagus cuniculus*) populations in Navarra (Spain). I. Epidemiology of rabbit viral haemorrhagic disease. Gibier Faune Sauvage, Game Wildlife, 15(1): 47-64.
- Soriguer, R.C., 1980. Mixomatosis en una población de conejos de Andalucía occidental. Evolución temporal, epidemia invernal y resistencia genética. I Reunión iberoamericana de Zoología y Conservación de Vertebrados. La Rábida, 241- 250.

CHAPTER VI

Differences in weight, length and body condition between two wild populations of European wild rabbit (*Oryctolagus cuniculus*, L. 1758) ingeographically distant areas in western Spain

Díez, C.[a], Merchán, T.[b], Pérez, J.A.[a], Cortázar, G.[b], Bartolomé, D.[a], Gómez, P.[b], Alonso, M. E.[a], Hidalgo, S.[b]

[a] Dpto. Producción Animal II. Veterinary School. University of León. (España)

[b] Grupo de Investigación en Recursos Cinegéticos y Biodiversidad (RCB). Veterinary School. University of Extremadura. 10071 Cáceres, Spain

Díez, C., Merchán, T., Pérez, J.A., Cortázar, G., Bartolomé, D., Gómez, P., Alonso, M.E., Hidalgo, S., 2004. Weight, length and body condition differences between two wild rabbit (Oryctolagus cuniculus, L. 1758) poulations from two far western Spain areas. 2º World Lagomorph Conference, Vairao, Portugal, from 26 to 31 July 2004. ***Communication (poster) at the International Congress.***

1. Resumen

En octubre de 2003, dos poblaciones de conejo de monte (*Oryctolagus cuniculus*, L. 1758) fueron estudiadas para determinar diferencias en el peso y en la condición corporal. Las poblaciones provenían del norte de la provincia de Valladolid (n=40, 10 machos y 30 hembras) y del sur de la provincia de Badajoz (n=40, 10 machos y 30 hembras). Se encontró que los animales procedentes de Valladolid fueron de mayor peso y longitud que los procedentes de Badajoz, siendo común esta diferencia para ambos sexos. Si bien estas diferencias no fueron significativas, sí eran esperables por ser el conejo de monte una especie caracterizada por una variación gradual de tamaño en función de la latitud ocupada.

Por otro lado, se estableció un índice de condición física (ICF= (P/LTx3)x106), que indicó que las variaciones relativas al peso corporal están relacionadas con el estado nutricional de los individuos. De este modo se pudo comprobar que la condición corporal resultó ser significativamente mayor para los animales procedentes de Badajoz. Este resultado puede ser debido a las condiciones meteorológicas y de hábitat que caracterizan ambas zonas de estudio, donde la disponibilidad de una mayor abundancia de alimento es anterior en el sur (Badajoz), alcanzando los conejos más temprano un estado corporal óptimo para iniciar la temporada reproductiva.

Palabras clave: índice de condición física, longitud, peso, este español, conejo de monte

2. Abstract

In October 2003 two populations of European wild rabbit (*Oryctolagus cuniculus*, L. 1758) were studied to determine differences in weight and body length. The populations were from the north of the province of Valladolid (n=40, 10 males and 30 females) and the south of the province of Badajoz (n=40, 10 males and 30 females). The Valladolid rabbits were larger than the Badajoz rabbits and the size difference was similar but not significant when rabbits were compared by sex. Differences of this type are common because wild rabbits, like many species, are characterised by a gradual variation in size in relation to the latitude of their habitat. A body condition index (BCI= (W/TLx3)x106) was also established. This index shows that variations in body weight are related to the nutritional state of individual rabbits. The body condition index was found to be significantly higher for the Badajoz rabbits. This result may be due to the different weather and habitat conditions in the study areas, as the availability of greater food abundance occurs earlier in the south (Badajoz) and rabbits in this location reach optimum body condition earlier for the start of the reproductive season.

Key words: body condition index, length, weight, western Spain, wild rabbit

3. Introduction and objectives

Taxonomic differences among subspecies of European rabbit (*Oryctolagus cuniculus*, L. 1758) have been widely studied and discussed in recent years (Gibb, 1990; Flux & Fullager, 1992). Differences in coat colour have been questioned as indications of taxonomic variations (Corbet, 1994). In terms of size variations between rabbits in northern and southern Europe, analyses of cranial data correlate positively between latitude and body size, showing a decrease in size southward (Sharples *et al*. 1996).

However, this study found no evidence on the basis of skull size for separation into the subspecies described to date in Europe. Later studies based on genetic analysis of mitochondrial DNA revealed the existence of two lineages, one in the north of Europe, France and northern Spain, and another in the south of Spain. Molecular biology studies have clearly established two lineages of rabbits in the Iberian Peninsula (*O.c. cuniculus* and *O.c. algirus*) and their geographical distribution (Branco *et al*. 2000).

In this study, two populations (Figure 1) of wild rabbits from the subspecies described in the Iberian Peninsula were examined to determine differences in weight and body length. The body condition index was also calculated. The populations were from the north of the province of Valladolid (*O.c. cuniculus*) and the south of the province of Badajoz (*O.c. algirus*).

Figure 1: Study areas.

4. Material and methods

In October 2003, 40 adult wild rabbits were captured from each area (10 males and 30 females), all older than nine months. The capture methods used were hunting with ferrets and manual extraction from artificial warrens.

On capture, rabbits were sexed and their weights (W) and total body lengths (TL) were recorded. Total length was measured from the tip of the nose to the start of the tail, with rabbits standing, following the dorsal midline. Body condition index was also calculated [BCI = (W/TLx3)x106] (Alves, 1994; Alves, 1996). The BCI showed that the relative variations in body weight were associated with the nutritional state of the rabbits.

Statistical analysis was performed using SPSS v.11.0 for Windows.

5. Results and discussion

Valladolid rabbits were found to have higher weight and TL values than Badajoz rabbits, both when the entire population of the two groups was analysed and when males were compared as a group and females were compared as a group. In each population males and females showed no differences to each other, although on analysing both groups together, Valladolid rabbits were larger than Badajoz rabbits. Despite this difference, no statistically significant difference was found except in body condition, where the values were higher for Badajoz rabbits. Although Iberian rabbits are known to be the smallest of their species in Europe (Anderson *et al.*, 1979; Sothern, 1940), few studies have addressed differences in size (weight and total length) between the *algirus* lineage (Badajoz) and the *cuniculus* lineage (Valladolid) as this study does. Similar results for morphological differences have recently been described for the two subspecies (Castro *et al.*, 2011).

Differences of this type are common, because the wild rabbit, like many species, is characterised by a gradual variation in size associated with the latitude of their habitat (Bergmann, 1847). Soriguer (1980) found an average weight of 1092 grams in a sample of 520 rabbits in Huelva (southern Spain). This value is also consistent with the general pattern of decreased size with decreasing latitude and the smaller body size observed in the Badajoz rabbits than in the Valladolid rabbits. The larger size of the Valladolid rabbits appears to be associated with the subspecies *cuniculus*, which predominates in the area. Another explanation is that these populations grow faster, in line with the hypothesis of Geist (1987), who proposed that body size follows the duration of the annual productivity pulse and is therefore a function of the availability of nutrients and energy during growth periods and is not related to thermoregulation. This hypothesis considers not only the

annual productivity pulse, but alsothe influenceof climate conditionson there production of small mammals (lagomorphs). These factors suggest that northern rabbits grow faster and are larger than southern animals living in less productive, arid climates (Williams & Moore, 1989; Bell & Webb, 1991).

A study of the BCI revealed significantly higher values for Badajoz rabbits. This result may be due to the different weather and habitat conditions in the study areas. The study was conducted in October, when rabbits in southern Spain were closer to the reproductive period, had improved quality and abundance of food and had attained good body condition.

Table 1: Results in each area (Medium values ± standard deviation).

Populations	Weight (g)	Total length (mm)	Body condition index
Badajoz rabbits	1107.22+/-98.68	381.5+/-16.81	20.05+/-2.47
Valladolid rabbits	1267.65+/-19.68	408.03+/-24.72	18.81+/-2.69

6. References

- Alves, P.C., 1994. Estudo da reproduçao e do estado da condiçao física de duas populaçoes portuguesas de coelho-bravo, *Oryctolagus cuniculus*. Dissertaçao de mestrado em ecologia aplicada. F.C.U.P. Porto 86 pp.
- Alves, M.G., 1996. Incidência da doença hemorrágica viral do coelho-bravo, Oryctolagus cuniculus, L. 1758, na Beira Interior. Relatorio Final de Estágio. Vila Real.
- Bell, D.J., Webb, N.J., 1991. Effects of climate on reproduction in the European wild rabbit (*Oryctolugus cuniculus*). Journal of Zoology 224: 639–648.
- Bergmann, C., 1847. Über die Verhältnisse der Wärmeökonomie der Thiere zu ihrer Grösse. Göttinger Studien Pt. 1: 595-708.
- Branco, M., Ferrand, N., Monnerot, M., 2000. Phylogeography of the European rabbit (*Oryctolagus cuniculus*) in the Iberian Peninsula inferred from RFLP analysis of the cytochrome b gene. Heredity 85: 307-317.
- Castro, F., Ramírez, E., Ferreira, C., Aparicio, F., Álvaro, PJ., Manners, RE., Redpath, S., Villafuerte, R., 2011. ¿*Algirus* o *cuniculus*? Pequeñas y grandes diferencias. En Díaz-Portero, M.A., Sánchez, J.F. & Robles, M. (Eds). II Congreso internacional sobre el conejo de monte. Ponencias y comunicaciones. Proyecto LIFE+ 07 NAT/E/000742 “Priorimancha”. Toledo 2011.

- Corbet, G.B., 1994. Taxonomy and origins. The European Rabbit: the History and Biology of a Successful Coloniser (Eds Thompson, H.V. & King, C.M.), 1-7. Oxford Science Publications, Oxford, UK.
- Flux, J.E.C., Fullagar, P.J., 1992. World distribution of the Rabbit *Oryctolagus cuniculus* on islands. Mammal Review 22: 151–205.
- Geist, V., 1987. Bergmann's rule is invalid. Canadian Journal of Zoology 65: 1035-1038
- Gibb, J.A., 1990. The European rabbit *Oryctolagus cuniculus*. Rabbit, hares and pikas: status survey and conservation action plan. (Eds Chapman, J.A. & Flux, J.E.C.), 116–120 IUCN/SCC Lagomorph Specialist Group, Information Press, Oxford, United Kingdom.
- Sharples, C.M., Fa, J.E., Bell, D.J., 1996. Geographical variation in size in the European rabbit *Oryctolagus cuniculus* (Lagomorpha: Leporidae) in Western Europe and North Africa. Zoological Journal of the Linnean Society 117: 141–158.
- Soriguer, R.C., 1980. El conejo, *Oryctolagus cuniculus* (L), en Andalucía Occidental: Parámetros corporales y curva de crecimiento. Doñana, Acta Vertebrata 7(1): 83-90, 1980.
- Williams, C.K., Moore, R.J., 1989. Environmental and Genetic Influences on Growth of the Wild Rabbit, *Oryctolagus cuniculus* (L) in Australia. Australian Journal of Zoology 37(5): 591-59.

CHAPTER VII

Experimental introduction of the European wild rabbit (*Oryctolagus cuniculus*) inside an enclosurein the Monte de Granadilla restricted hunting area (Cáceres, Spain)

Merchán, T., Rocha, G.

Departamento de Ingeniería del Medio Agronómico y Forestal, University of Extremadura. Avda. Virgen del Puerto, 2. 10600 Plasencia, Cáceres, Spain. tmerchan@unex.es, gregorio@unex.es

Merchán, T., Rocha, G., 2011. Experiencia de implantación de conejo de monte (Oryctolagus cuniculus) en un área cercada en la zona de caza controlada de Granadilla (Cáceres). II Congreso Internacional sobre el conejo de monte. Universidad de Castilla la Mancha. 24 a 25 de noviembre de 2011. Toledo. ***Communication (poster) at the International Congress.***

1. Resumen

Con el objeto de recuperar poblaciones de conejo de monte en el área de Granadilla, de cara a una posible futura reintroducción del lince ibérico (*Lynx pardinus* L.), se procedió a implantar de forma experimental en el año 2009, una población fundadora de 95 conejos de monte (58 hembras y 37 machos) en dos fases: 52 individuos en mayo y 43 en diciembre. Los animales fueron soltados en un cercón vallado de 0,54 hectáreas, impermeable para la especie hasta el 16/12/09, instalado en una zona de monte mediterráneo, previamente elegida como óptima y dotada de refugio, alimento y agua. En el exterior del vallado se preparó un área del tamaño y de características similares a las del interior.

Varios meses antes de la suelta, se estimó la abundancia de la población nativa mediante conteos nocturnos obteniendo un resultado de 0,93 conejos/km. En las mismas fechas, a partir de individuos cazados (n=11), se analizaron muestras de sangre que arrojaron valores de seropositividad del 45,4% frente al virus de la enfermedad hemorrágica vírica (RHDV) y al de la mixomatosis (MV). Se determinó la subespecie de conejo en la zona a partir del análisis de ADN mitocondrial de 10 animales, dando un resultado del 50% para cada uno de los 2 linajes: A (*O. c. algirus*) y B (*O. c. cuniculus*).

La población donante fue elegida por su proximidad dentro de la misma cuenca hidrográfica; además se partió de la premisa de que los muestreos serológicos previos que se realizaron en esas fechas debían aportar cifras elevadas de inmunidad natural frente a ambas enfermedades. Los resultados obtenidos fueron del 100% de seropositividad frente al RHDV y MV (n=10). Las proporciones de linajes fueron similares a las descritas de la población nativa.

En la población implantada se realizaron chequeos serológicos (tras su vacunación frente a enfermedades víricas) al mes y a los cinco meses, obteniendo un 100% de seropositividad en los animales monitoreados.

Las tasas de supervivencia en el interior del vallado, transcurridos 5 meses desde la primera suelta, se situaron entre el 45 y el 49%.

La abundancia relativa en el exterior del vallado, basada en conteos de cagarruteros/hora a los 5 meses tras la apertura de pasos al exterior (abril 2010), fue un 193% superior a la obtenida un año antes.

2. Abstract

European wild rabbits were experimentally introduced in the Monte de Granadilla area in 2009 as preparation for reintroduction of the Iberian lynx (*Lynx pardinus*). The founder population comprised 95 rabbits (58 females and 37 males) released in two phases: 52 in May and 43 in December. The rabbits were released into a 0.54 ha enclosure they could not break through until openings were made in the fencing on 16 December 2009. The enclosure was established in an area of Mediterranean forest chosen for its optimum conditions and was equipped with shelter, food and water. The area immediately outside the enclosure was prepared in a similar way.

Several months before release, the abundance of the native population was estimated by night counting, giving a result of 0.93 rabbits/km. During these same months, blood samples taken from hunted rabbits (n=11) showed seropositivity values of 45.4% to rabbit haemorrhagic disease virus (RHDV) and myxoma virus (MV). The rabbit subspecies in the area was determined by mitochondrial DNA analysis of 10 rabbits, giving a result of 50% for each lineage: A (*O. c. algirus*) and B (*O. c. cuniculus*).

The donor population was chosen because of its proximity within the same hydrographic basin. It was also considered that the serological screening before release would have provided high levels of natural immunity to both diseases. The results were 100% seropositivity to RHDV and MV (n=10). The lineage proportions were similar to those in the native population.

The introduced population was vaccinated against viral diseases, and serological tests were performed at two intervals: one month and five months post introduction. The rabbits monitored showed 100% seropositivity. Five months after the first release, survival rates inside the enclosure were estimated at 45-49%. In April 2010, five months after openings had been made in the enclosure, relative abundance in the area immediately outside was 193% higher than the previous year according to the number of latrines/hour.

3. Introduction

The emergence of the myxoma virus (MV) in the 1950s, followed by the appearance of the rabbit haemorrhagic disease virus (RHDV) in the 1980s (Argüello *et al*., 1988), caused a significant reduction in the density and distribution of the wild rabbit (*Oryctolagus cuniculus*, L. 1758) in the Iberian Peninsula. This situation of decline had a major impact on rabbit predators, particularly in the case of specialist predators such as the Iberian imperial eagle (*Aquila adalberti* B.) and the Iberian lynx (*Lynx pardinus*) (Moreno *et al*., 2007). The importance of the rabbit is magnified because it is a multifunctional keystone species within the Mediterranean ecosystem that positively affects the maintenance of biodiversity (Delibes-Mateos *et al*., 2008).

To enhance the ecological value of the areas where wild rabbit populations have declined, intervention is sometimes necessary in both the habitat and the populations, following technical criteria at all times. In recent years, due to the scarcity of wild rabbits in many areas and as a way to recover prey populations for endangered apex predators, much work has been done to recover this species (Guerrero-Casado *et al*., 2011). Strict sanitary aspects have been included in action protocols only recently (Calvete *et al*., 2005; Narváez *et al*., 2011).

In this study we describe actions to reintroduce wild rabbits in a specific area. These actions combine ecological, ethological, health, genetic and management criteria obtained exclusively from the study of wild rabbit populations in the region of Extremadura.

4. Study area

The Monte de Granadilla restricted hunting area, in the municipality of Zarza de la Granadilla, is in the north of the province of Cáceres and covers 5,188 ha. Its location is approximately 40º 13’ 46’’ north and 6º 3’ 19’’ west, in the central western area of the Iberian Peninsula (Figure 1). The study area has potential vegetation typical of the Mediterranean and is dominated by *Quercus* spp., *Retama sphaerocarpa*, *Cistus* spp. and *Ulici eriocladi-Cistetum ladaniferi* according to Rivas Martínez (1983), although forest policy in the 1970s and 1980s led to a major transformation of the area into pine stands and low forest of heath and rockrose. The landscape is characterised by slopes dotted with small valleys and flat hilltops with pastureland that encourages the presence of wild rabbits.

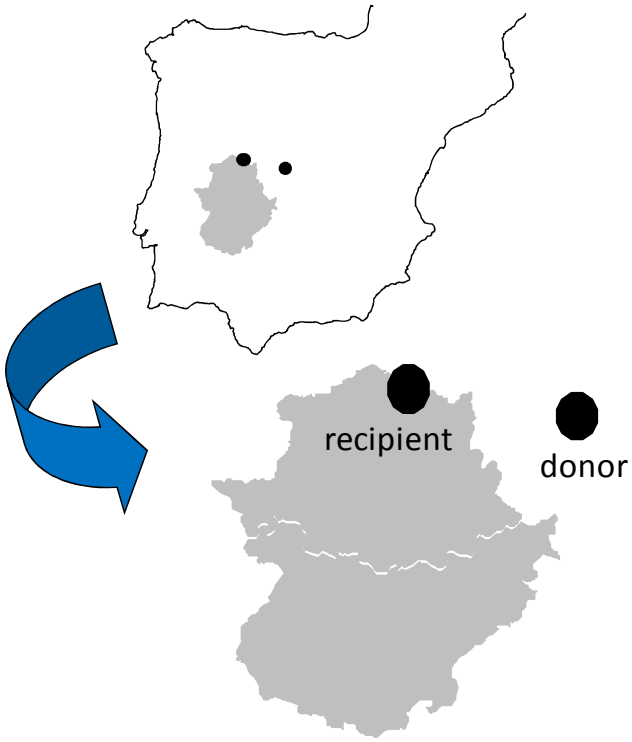


Figure 1: Close-up of the study area showing the locations of the donor and recipient populations.

5. Objectives

The objectives of this study are:

1. To describe the action areas and analyse the suitability of the habitat.
2. To study the genetic and serological status of the recipient and donor populations.
3. To establish a high density settlement of rabbits in a location where action had been taken to create an appropriate environment.
4. To monitor the health of the rabbits, including serological analysis of the introduced (founder) population.

6. Material and methods

A plot of land prepared in 1999 was used for this study. The plot is in an area known as Cuatro Caminos and comprises pine stands and Mediterranean forest. Small subpopulations of wild rabbits can be found nearby, with a kilometric abundance index (KAI) of 0.93 rabbits/km, allowing individuals to move between populations in the area described (Figure 2).

Actions taken to recover the rabbit population in this particular area were: making the 0.54 ha enclosure impenetrable to terrestrial predators; removing 50% of the scrub; building rabbit warrens and creating mounds and ridges of soil to aid warren building; and preparing an area for sowing plants and installing feeders and drinking spouts.

In the area immediately outside the enclosure, netting was installed as protection from ungulates and the habitat was modified in the same way as inside the enclosure. The aim was to create an initial dispersal area and enhance the capacity of the exterior to receive rabbits after release. All introduced rabbits were sexed, vaccinated, treated for parasites and identified with ear tags and had come from a region in the same hydrographic basin, in the province of Toledo.

The serum sample was screened to identify antibodies and their titration by indirect ELISA (INgezim Mixomatosis 17.MIX.K.1, Ingenasa, Madrid, Spain, for MV and INgezim RHDV 17.RHD.K.1 for RHDV). Absorbance was measured at 405 nm with a shake duration of 10 seconds. All samples with Optical Density (OD) higher than 0.289 were considered positive to RHDV and the titre was the maximum dilution with an OD value higher than 0.289. For MV, samples were considered positive when OD values were higher than 0.265.

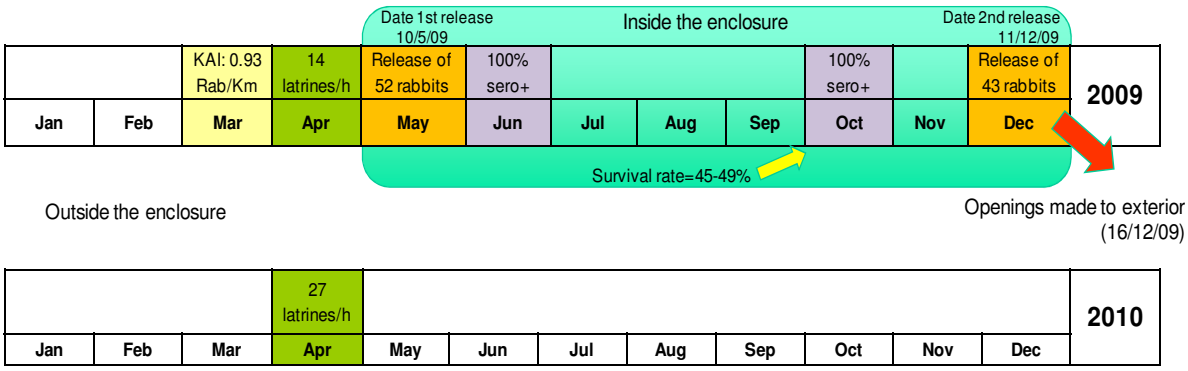


Figure 2: Schedule of actions; abundance results inside and outside the enclosure; seropositivity; and survival rate.

3. Results and discussion

3.1. Genetic aspects

No significant differences were observed in genetic structure at nuclear level between mitochondrial lineages A and B (Tables 1 and 2), either in the donor or the recipient sample. This may indicate homogenisation of the nuclear genetic characteristics, as the two types of rabbit intermingle in the central area of the Peninsula (Alda *et al.*, 2006; Branco *et al.*, 2000).

3.2. Aspects of immunity to RHDV and MV before introduction

Donor area: 100% of the rabbits were seropositive to both diseases (Table 1), indicating that they had been exposed to the viruses. All animals were healthy on capture and came from a specific high density area. Rabbits with antibodies to MV may have been in the phases prior to seasonal outbreaks, or could have had the disease at a subclinical level.

In the case of RHD, the seropositivity found to the virus could have been due to recent outbreaks or even to the existence of only slightly virulent viruses in the environment which would have led to crossed immunity. This possibility has not yet been confirmed in the Iberian Peninsula (Lavazza & Capucci, 2008; Alda *et al*., 2010), although the hypothesis could be supported by reiterated findings of RHDV in healthy rabbits in the area (24.1% PCR positive) (Merchán & Rocha, 2008).

Table 1: Antibody titration, genetics and other data from the donor population (4/4/2008).

Sample	Sex	Age	RHDV	Titration	MV	Titration	Restriction pattern	mtDNA ancestor
1	F	>9 m	**POSITIVE**	(1/256)	**POSITIVE**	(1/512)	A	**B**
2	M	<9 m	**POSITIVE**	(1/512)	**POSITIVE**	(1/512)	D	**B**
3	M	<9 m	**POSITIVE**	(1/256)	**POSITIVE**	(1/256)	D	**A**
4	F	<9 m	**POSITIVE**	(1/256)	**POSITIVE**	(1/512)	A	**B**
5	F	>9 m	**POSITIVE**	(1/256)	**POSITIVE**	(1/512)	D	**A**
6	M	>9 m	**POSITIVE**	(1/512)	**POSITIVE**	(1/512)	D	**B**
7	F	<9 m	**POSITIVE**	(1/256)	**POSITIVE**	(1/256)	A	**A**
8	F	>9 m	**POSITIVE**	(1/512)	**POSITIVE**	(1/256)	B	**B**
9	F	<9 m	**POSITIVE**	(1/256)	**POSITIVE**	(1/512)	D	**A**
10	M	<9 m	**POSITIVE**	(1/512)	**POSITIVE**	(1/512)	D	**A**

Recipient area: 45.5% of the rabbits screened were seropositive to both diseases and asymptomatic (Table 2). This could indicate that the native subpopulation was also (or had been) in contact with the circulating viruses. The lower proportion of seropositive rabbits could be explained by the spatial isolation between these low density subpopulations, which would give them less contact with the viruses. It could also be explained by the existence of more virulent viral forms that would act as a limiting factor in population growth.

Table 2: Antibody titration, genetics and other data from the recipient population (11/8/2008).

Sample	Sex	Age	RHDV	Titration	MV	Titration	Restriction pattern	mtDNA ancestor
1	M	>9 m	**POSITIVE**	1/1024	NEGATIVE	-	A	**B**
2	M	>9 m	**POSITIVE**	1/512	NEGATIVE	-	D	**A**
3	F	>9 m	NEGATIVE	-	**POSITIVE**	1/128	D	**A**
4	F	<9 m	NEGATIVE	-	NEGATIVE	-	D	**A**
5	M	>9 m	**POSITIVE**	1/256	**POSITIVE**	1/64	D	**A**
6	M	>9 m	NEGATIVE	-	NEGATIVE	-	B	**B**
7	F	>9 m	**POSITIVE**	1/128	NEGATIVE	-	A	**B**
8	M	>9 m	NEGATIVE	-	**POSITIVE**	1/128	A	**B**
9	F	>9 m	**POSITIVE**	1/512	NEGATIVE	-	D	**A**
10	F	<9 m	NEGATIVE	-	**POSITIVE**	1/512	B	**B**
11	M	<9 m	NEGATIVE	-	**POSITIVE**	1/128	-	-

3.3. Aspects of immunity to RHDV and MV after introduction

As expected, when tested at one month and five months after introduction inside the enclosure, 100% of the rabbits had positive titres to both diseases (Figure 2), as they had been vaccinated before release. Although they had a high percentage of natural immunity, we decided to vaccinate all donor rabbits to create longer-lasting hyperimmunisation. This was eventually demonstrated by monitoring five sentinel rabbits for eight months (Figure 3).

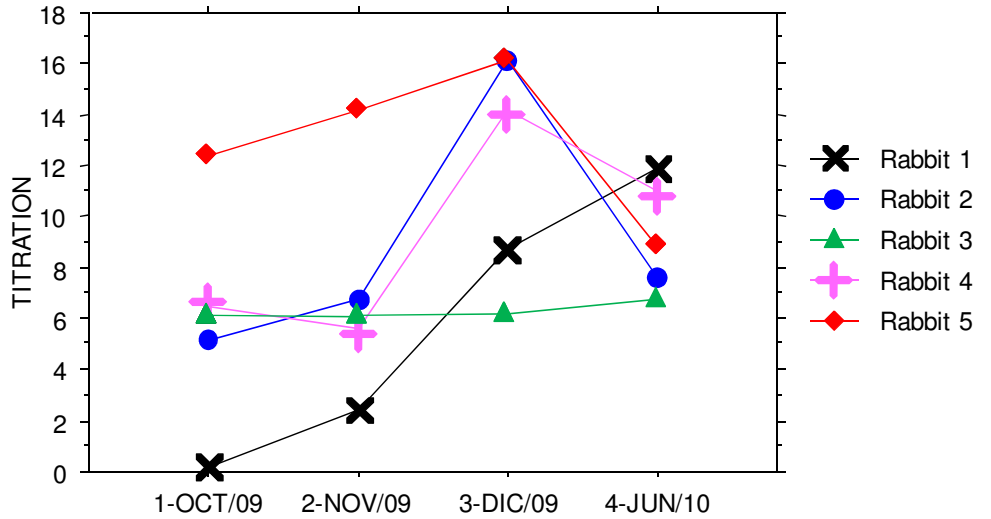


Figure 3: Antibody levels to RHDV over 8 months in 5 vaccinated rabbits with natural immunity. Technique: ELISA (CIVTEST RHD).

The high natural immune status, combined with the suitable habitat conditions both inside and outside the enclosure, positively affected rabbit survival, as Cabezas *et al*. (2011) recently reported.

3.4. Survival inside the enclosure

The survival rate five months after the first release, obtained using various scientific methods, was 45%-49% based on the faeces count (Figure 4) and 48.1% from the capture/re-capture method using marks, following the Lincoln-Petersen method (Begon, 1979), whose basic model is (M/N = m/n).

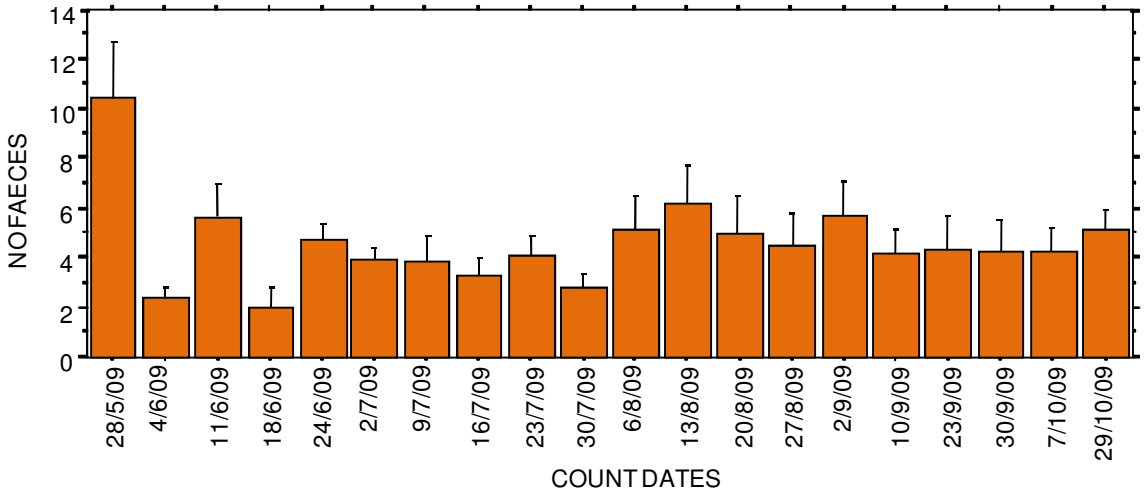


Figure 4: Mean and standard error of fresh faeces/day recorded using fixed linear transects (25 cm long x 1 m wide)

We considered the survival rate or success level high compared to levels in free releases, where survival is around 9% just in the first month (Calvete & Estrada, 2004). The highest mortality detected was caused by aerial predators (19.2% in the first five months).

No viral diseases were observed at any stage of the study.

3.5. Rabbit densities outside the enclosure

The dispersal phase began with the opening of exits in the enclosure. Rabbits from outside the enclosure showed a natural inclination to stay in the prepared area, where they found crops and shelter. In April 2010, five months after openings had been made in the fencing (December 2009), relative abundance outside the enclosure was 27 latrines/hour according to the faeces counts. This was 193% higher than the count obtained in April 2009 (14 latrines/hour).

Based on the categorisation of rabbit relative abundance accepted by the scientific community (Guzmán *et al*., 2005), the change was from low to medium relative abundance outside the enclosure.

4. Conclusions

The following conclusions can be drawn from this study:

1. In translocations, rabbits should come from a neighbouring area, their genetic structures should be known and they should have high natural immunity; they should also be hyperimmunised by vaccination.

2. Raising rabbit density, in both time and space, has a positive effect; after possible seasonal outbreaks or other events, a balance can be achieved between rabbits and their viruses.

3. Creating translocation units by forcing situations of high rabbit density based on the experiment in this study can be effective and will help to interconnect increasingly larger areas.

4. It may be necessary to regularly reinforce populations and carry out other indirect habitat controls to ensure a minimum density threshold that will facilitate the viability of reintroductions.

5. References

- Alda, F., Doadrio, I., Hernández, M., Muñoz, J., Silvestre, F., 2006. Gestión genética e inmunológica para el manejo de las translocaciones y reintroducciones de conejo *Oryctolagus cuniculus* en España. In: San Miguel (Ed.) 2006. 1ª reimp. Manual para la gestión del habitat del lince ibérico (*Lynx pardinus*, Temminck) y de su presa principal, el conejo de monte (*Oryctolagus cuniculus* L). Fundación CBD-Hábitat. Madrid. Pp: 217-241.
- Alda, F., Gaitero, T., Suárez, M., Merchán, T., Rocha, G., Doadrio, I., 2010. Evolutionary history and molecular epidemiology of rabbit haemorrhagic disease virus in the Iberian Peninsula and Western Europe. BMC Evolutionary Biology 10: 347.
- Begon, M., 1979. Investigating Animal Abundance: Capture-Recapture for Biologists. Edward Arnold, London.
- Argüello, J.L., Llanos, A., Pérez, L.I., 1988. Enfermedad vírica hemorrágica del conejo en España. Medicina Veterinaria 5: 645–650.
- Branco, M., Ferrand, N., Monnerot, M., 2000. Phylogeography of the European rabbit (*Oryctolagus cuniculus*) in the Iberian Peninsula inferred from RFLP analysis of the cytochrome b gene. Heredity 85: 307-317.

- Cabezas, S., Calvete, C., Moreno, S., 2011. Survival of translocated wild rabbits: importance of habitat, physiological and immune condition. Animal Conservation 14: 665-675.
- Calvete, C., Estrada, R., 2004. Short-term survival and dispersal of translocated European wild rabbits. Improving the release protocol. Biological Conservation 120(4): 507-516.
- Calvete, C., Angulo, E., Estrada, R., Moreno, S., Villafuerte, R., 2005. Quarantine length and survival of translocated European wild rabbits. Journal of Wildlife Management 69(3): 1063-1072.
- Delibes-Mateos, M., Delibes, M., Ferreras, P., Villafuerte, R., 2008. Key role of European rabbits in the conservation of the Western Mediterranean basin hotspot. Conservation Biological 22: 1106–1117.
- Guerrero-Casado, J.M., Ruiz-Aizpurua, L., Carpio, A., Arenas, R., Sánchez-Tortosa, F., 2011. Eficacia de los cercados y del manejo del hábitat como método para recuperar las poblaciones de conejo a gran escala. Díaz-Portero, M.A., Sánchez, J.F. & Robles, M. (Eds) 2011. II Congreso internacional sobre el conejo de monte. Ponencias y comunicaciones. Toledo 2011. Proyecto LIFE+ 07 NAT/E/000742 "Priorimancha".
- Guzmán, J.N., García, F.J., Garrote, G., Pérez de Ayala, R., 2005. El lince ibérico (*Lynx pardinus*) en España y Portugal. Censo-diagnóstico de sus poblaciones. Organismo Autónomo de Parques Nacionales. Madrid. 174 pp.
- Lavazza, A., Capucci, L., 2008. How many caliciviruses are there in rabbits? A review on RHDV and correlated viruses. In: Alves, P.C., Ferrand, N., Hackländer, K., (Eds) Lagomorph biology: evolution, ecology and conservation. Springer, Berlin, pp. 263–278.
- Merchán T., Rocha, G., 2008. Situación inmunitaria y virológica frente a la enfermedad hemorrágica vírica (RHD) del conejo de monte *Oryctolagus cuniculus* en dos poblaciones densas de Extremadura durante el descaste. III Congreso Andaluz de Caza, 3, 4 & 5 October 2007. Córdoba.
- Moreno, S., Beltrán, J.F., Cotilla, I., Kuffner, B., Laffite, R. Jordán, G., Ayala, J., Quintero, C., Jiménez, A., Castro, F., Cabezas, S., Villafuerte, R., 2007. Long-term decline of the European wild rabbit (*Oryctolagus cuniculus*) in south-western Spain. Wildlife Research 34: 652–658.
- Narváez, E., Bertos, E., Fernández, P., 2011. Manejo sanitario en las translocaciones de conejo realizadas en los montes lugar nuevo y selladores contadero. Díaz-Portero, M.A., Sánchez, J.F. & Robles, M. (Eds) 2011. II Congreso internacional sobre el conejo de monte. Ponencias y comunicaciones. Toledo 2011. Proyecto LIFE+ 07 NAT/E/000742 "Priorimancha".
- Rivas-Martínez, S., 1983. Pisos bioclimáticos de España, Lazaroa 5: 33-43.

GENERAL DISCUSSION AND EPILOGUE

GENERAL DISCUSSION AND EPILOGUE

1. General discussion

The situation of wild rabbits in the Extremadura region and the southern borders of Castile and León is generally disparate. In some zones rabbits are very abundant, occupying smaller or larger areas with interconnecting, stable subpopulations. In other zones, a high percentage of areas are found where the species has disappeared or occurs in very low densities. This situation requires the study of aspects that can be addressed from applied research, allowing practical conclusions to be drawn from the results to aid the recovery of wild rabbit populations in Extremadura. This thesis examines previously unstudied aspects of the epidemiology of rabbit haemorrhagic disease virus (RHDV).

The RHDV strains circulating in the Iberian Peninsula also infect mice sympatric to wild rabbit populations. The virus has been detected in two of the most abundant and widely distributed rodent species in Mediterranean environments, *Apodemus sylvaticus* and *Mus spretus*, which commonly share a habitat with rabbits (Delibes-Mateos *et al*, 2008; Aparicio *et al*., 2011). This finding not only opens up the possibility of these species being involved in the epidemiology of RHDV, but also suggests that other species not yet screened may similarly be involved.

It has been shown that rabbits are the only species susceptible to RHDV, regardless of the subspecies (Muller *et al*., 2009), even though Caliciviridae appear in a wide range of hosts, including humans (Van Regenmortel, 2000). Studies on human caliciviruses (Nyström *et al*., 2012) have indicated that RHDV attachment factors are present in human cells, constituting possible points of entry for RHDV. This suggests that the range of RHDV hosts is not as limited as previously thought. Recent phylogenetic analysis has also shown that caliciviruses exhibit high levels of host switching (Kitchen *et al*., 2011). In the chapter on the sensitivity of rodents brought into contact with the virus, it was shown for the first time that *A. sylvaticus* and *M. spretus* have no apparent sensitivity to RHDV. No clinical symptoms were observed, but the lack of serological and microscope information is not sufficient to determine the absence of sensitivity with any certainty. Neither is it possible to state whether or not viral replication occurred in the rodents, although the virus persisted inside them for up to 68 days. This virus may have remained in a lysogenic phase, although what is particularly interesting is that it was excreted through the faeces and was not found in the liver. Because of this, and with the results of this study, in which the virus was found more often in the intestine than in the liver, we can consider that RHDV may have a special tropism in rodents for the intestinal cells. RHDV is thought to be intestinal in origin and to have evolved from non-virulent to virulent (Park *et al*.,

1995). This circumstance could therefore give RHDV the innate capacity to remain in enteric cells of rodents as a prophage in the lysogenic phase. Although this is merely speculation, the results can shed light on the origin of RNA viruses. Some researchers concluded that the colonisation of new but related host species may represent the principle mode of macroevolution in RNA viruses (Kitchen *et al.,* 2011).

Regardless of how RHD viruses remain inside rodents, they are actually eliminated, at least through the faeces. This enables the spread of virus particles that cause an immunological response when they come into contact with susceptible rabbits. The scope and epidemiological repercussions of this spread of viruses opens up a new line of study and research. If non-virulent circulation of RHDV in wild mice is demonstrated, it would be consistent with the possibility of RHDV originating by the virus "jumping" species (Fenner & Fantini, 1999). It has been demonstrated that rodents sympatric to wild rabbits contribute to RHDV persistence and could collaborate not only in the dynamic of virus-host balance through this phenomenon, but also in the immunisation of populations where rabbits and rodents cohabit, similar to what occurs between kits refractory to RHD infection and susceptible adult rabbits (Calvete, 2011). In this study we were able to show how the faeces and carcasses of a mouse infected with RHDV through natural transmission are not subsequently capable of causing the disease in a susceptible rabbit, although an immune response is induced. These preliminary findings should lead to further investigation on the possibility of carrier mice causing disease in rabbits, as well as a study of the immunising role that rodents with RHDV (or other less lethal viral forms) can play, including their protective capacity. It is necessary to determine whether seroconversion occurs in infected rodents, as demonstrated in red foxes (*Vulpes vulpes*) and domestic house cats (*Felis catus*) fed with RHDV infected liver (Leighton *et al.,* 1995; Zheng *et al*., 2003) but not yet shown in the species studied in this thesis. In short, this study opens up a new field of research by extending the epidemiological information on where viruses are harboured between outbreaks, which was mostly attributed to kits carrying the virus or the inside of warrens (Cooke *et al*., 2000; Cooke, 2002). It also reopens discussion on the potential for mutation of RHDV that could enable it to cross species barriers and cause disease (Smith *et al.*, 1998; Smith, 1999).

An overview of the situation of wild rabbit populations must include discussion of the role of the viral diseases that have had such a major influence on the processes of rarefaction in these populations. Studies on autumn seropositivity to the myxoma virus (MV) and RHDV conducted in numerous areas of Extremadura show that rabbits have generally higher antibody levels to MV than to RHDV, probably due to summer outbreaks of myxomatosis. Autumn seropositivity to RHDV is positively and significantly correlated to rabbit relative abundance. The fraction of adults

seropositive to RHDV at this time of year is also higher. Because of this, rabbit hunting during this period can have a negative impact, as immunised population is eliminated, particularly in populations with low relative abundance where fewer rabbits are seropositive to RHDV.

Abundant, stable populations of wild rabbits have been shown to exist. Values obtained in July 2005, mostly from young individuals older than two months, revealed the presence of antibodies to RHDV in percentages of 10-54% in the north of Extremadura and higher values (60%-83%) in the south. In the case of myxomatosis, for the same period, values of 25%-56% seropositivity to MV were obtained in the south of Extremadura. These enzootic serological patterns to MV could warn us about susceptibility to epizootic outbreaks of myxomatosis in the populations studied. Enzootic outbreaks have been reported in rabbits in France and the Middle Valley of the Ebro (Spain), where antibody prevalence is from 90%-100% in adult rabbits. These outbreaks were observed when the prevalence of seropositive individuals in the population dropped to 50-20% due to recruitment of susceptible juveniles. Survivors of these outbreaks will increase their antibody levels after infection to the prevalence level of adults (Rogers *et al*., 1994; Arthur & Louzis, 1988; Marchandeau & Boucraut, 1999; Calvete & Estrada, 2000).

In general, for RHDV, the serological profile is similar to the profile detected by Cooke (1997) in Australian populations, with antibody prevalences of 80%-100% in 1995 and 1996. Simón *et al*. (1998) reported similar profiles in Navarra (Spain). The pattern is a decrease in antibody prevalence in the population as a result of reproduction, followed by new outbreaks of disease, after which prevalence reaches values close to 100% (Simón *et al*., 1998). These findings of 100% antibody prevalence to RHDV were detected in the populations of central mainland Spain used as donors for a translocation programme. In all cases, high levels of seropositivity have been shown to correlate to high rabbit densities due to the higher infection rate. This finding was also described by Dugast (1995), Marchandeau *et al*., (1998) and Marchandeau & Boucraut (1999). Thus the best way to control and encourage a population is to ensure density remains high, to favour high seropositivity rates. From our own data, all the areas with high antibody levels that were sampled in July 2005 showed categories of high abundance (following Blanco & Villafuerte, 1993; Guzmán *et al*., 2004), with relative abundance values for the same year of 72 latrines/hour in July (municipality of Talarrubias), 83 latrines/hour in August (municipality of Cáceres), and 87 latrines/hour in September (municipality of Plasenzuela).

Populations studied in the south of Extremadura (without preventive sanitary intervention through vaccinations and with high population densities) showed higher seropositivity prevalence to RHDV (around 80%) than to MV (around 50%). This reduces the importance of RHDV as a mortality

factor of a pathological nature during the time of year of the study (June-July). Myxomatosis is therefore the disease that could act as a destabilising factor in the populations due to the time of its appearance (June-August) and because it affects younger age classes. In this thesis it was seen that the presence of positive RHDV antibody levels shows complementarity to MV antibody levels. The mean positive antibody level is higher to MV than to RHDV.

When a population has low antibody levels, it can be vulnerable to a possible episode of RHD, as the fraction of unprotected rabbits is high. This circumstance could worsen if the recently described new variant RHDVb (Calvete *et al*., 2012; Sarto *et al*., 2013), which is capable of affecting young individuals less than 30 days old (Comenge & Mora, 2011), comes into contact with populations with low immunity levels. It is therefore essential to take measures intended not only to encourage dense rabbit populations and high titres to RHD in adults, but also to avoid the entry of external viruses that commonly occurs during translocation. The existence of 10.3% seropositive and PCR positive rabbits (carriers) out of a sample of 46 was shown in this thesis. These results for carrier rabbits are higher than the findings of Simón *et al*., (1998), which were 1.6%. We alsodetected 13.8% seronegative and PCR positive rabbits (persistently infected). This situation could be caused by inactivation of the virus during the course of a vigorous immune response, in which viral “vestiges” were detected even though the virus was no longer infectious.

It is also concluded that detecting healthy animals that were both PCR positive and seropositive indicates a population with some virus-host balance, making it advisable to preserve these populations. Thus practices such as summer hunting (culling) should be determined by susceptibility to hunting pressure based on criteria that are also epidemiological. Although culling has less effect on the reproductive fraction (higher fraction of young rabbits), low percentages of seropositivity to RHDV and/or low percentages of virus presence should provide a warning about susceptibility to hunting. However, studies are still necessary to indicate whether the titres found in this study are protective and to determine the nature of the viruses that were detected but not sequenced. Greater variability is found in the types of RHDV than previously thought (Moss *et al*., 2002). Serological evidence from France for the existence of a non-protective RHDV-like virus has been reported (Marchandeau *et al*., 2005). Similarly, in Italy and Germany rabbits were detected with viral variants that are genetically and antigenically different to the original virus (Capucci *et al*., 1998; Schirrmeier *et al*., 1999). These variants are termed non-pathogenic rabbit caliciviruses (RCV) (Capucci *et al*., 1996) and they could be non-pathogenic variants because the rabbits studied did not develop the disease, as they had antibodies. The possible role of these strange viruses in the impact of the disease and their level of protection have been addressed (Boots *et al*., 2004; Cooke *et al*.,

2002; Cooke, 2000; White *et al.*, 2001). It is not known what the virus type is that produces the antibodies detected in rabbits older than two months (and therefore not of maternal origin), with no signs of disease in the population of origin. Their presence in the populations observed, if they were different forms of the virus, could give these rabbits cross immunity to RHD and therefore greater resistance. If the presence of a new strain of less lethal non-haemagglutinating virus in these populations is confirmed and its effectiveness in reducing mortality from episodes of RHD is demonstrated, it would be highly worthwhile conserving and promoting the populations.

Population management based on translocations (introductions and reintroductions) and population reinforcements is governed by regulations, in particular those of the IUCN (1987). These regulations and the findings of certain studies on wild rabbit translocations (Calvete *et al.,* 1997; Letty *et al*., 2002; Calvete & Estrada, 2004) have rarely been applied to rabbit reintroduction programmes. It has been estimated that at least 500,000 rabbits are released every year in Spain and France (Calvete *et al.,* 1997) and therefore applying knowledge and monitoring populations to improve the results must be a central consideration. The reintroduction project in Granadilla (north of Extremadura) was designed to apply knowledge using some of the results obtained in this thesis. It therefore had to include the premises and recommendations discussed below. For the habitat, the capacity to receive introduced rabbits was assessed and the most suitable location was sought (Cabezas *et al*., 2011). Measures were taken to improve the quality of the habitat, and enclosures were built to reduce predation, avoid dispersal and monitor the population.

Management aspects and genetic and sanitary criteria were taken into consideration for the translocation. The characteristics of both the donor and the recipient population in the vicinity of the introduction area were recorded to assess the level of genetic compatibility. The programme was monitored at all phases to study what happened throughout the translocation.

The first aspect to be taken into account concerning the rabbits was the geographical origin of the targeted donors, based on information about the rabbits in the recipient area. From a morphological point of view, differences in size (weight and total length) between the *algirus* and *cuniculus* lineages were determined in this thesis. Results have recently been described for both subspecies (Castro *et al*., 2011). To some degree, this basic premise includes the hypothesis that the annual productivity pulse and climate conditions influence lagomorph reproduction. These factors lead us to consider that northern rabbits grow faster and are larger than rabbits from southern locations with dry climates (Williams & Moore, 1989; Bell & Webb, 1991). If we accept this hypothesis, for efficient translocation, donor rabbits must come from areas with similar bioecological characteristics to the recipient areas, where we can assume that the physiological variables of the

rabbits are suitable in neighbouring territories. Having an appropriately similar body condition is necessary for the rabbits to acclimatise. It has been shown that good rabbit physiological condition can modulate survival after release (Letty *et al*., 2002; Calvete *et al*., 2005; Cabezas *et al*., 2011). This means that it is important not only to start with individuals in optimum body condition, but also to maintain this condition throughout the reintroduction process. Controlling rabbits during capture, transport and handling until release, possibly including quarantine, can cause the loss of physical condition and negatively affect rabbit survival (Woodford & Rossiter, 1993; Letty *et al*., 2000; Calvete *et al*., 2005). This makes it essential to minimise the negative effects of inappropriate handling. In the Granadilla reintroduction programme, rabbits were not quarantined, as it was considered that this could cause a decline in body condition. The preference was for immediate action throughout the process, from capture in the area of origin to release in the recipient area.

From the point of view of the genetics of the viruses, although we initially considered the importance of the haplotype to be introduced and the consequences of introducing foreign viruses (Viggers *et al*., 1993; Wolf *et al*., 1996), all RHD viruses were regarded as a single population because they were from nearby geographical populations. A more or less constant pool of circulating viruses was assumed to exist in these environments. In dense populations, 23-24% of rabbits were found to have viruses (data from this thesis) and these viruses are probably related to Clade I (Alda *et al*., 2006). In the case of MV, current information indicates that there is no correlation between the geographical distances between populations in which viruses are present and genetic distances between the viruses affecting the populations (Alda *et al*., 2006). Thus although the natural presence of this type of virus in translocated rabbits was considered a survival risk in the short term, an attempt was made to minimise the risk through immunisation and the preservation of body condition, as in the case of RHDV.

In terms of the genetics of the translocated rabbits, studies indicate that it is necessary to have information available that combines data on nuclear DNA (Branco *et al*., 1999; Queney *et al*., 2001) (microsatellites) and mitochondrial DNA (Branco *et al*., 2000; Alda *et al*., 2006). In this way, information provided by microsatellites concerning the ability to adapt to the environment is included. However, in our reintroduction programme we took only information about lineages A and B into account when choosing the most suitable donor population. Rabbits from both the donor and the recipient area were in geographical proximity and had the genetic characteristics of intermingling populations of both mitochondrial lineages. They were therefore considered compatible. It was shown in this thesis that the rabbits studied in the contact area presented no differences, as described by Alda *et al*.,(2006). This shows homogenisation of the genetic characteristics due to

intermingling of the two types of rabbits. The origin of the translocated rabbits was strictly free-ranging, to avoid the consequences that rabbits raised in captivity have at the phenotypic and genetic level that affect their survival after release (Snyder *et al*., 1996).

In relation to release inside an enclosure that excluded terrestrial predators, survival increases considerably when rabbits are free from predation (Cabezas *et al.*, 2011) and the effect of the stress of a new habitat is minimised (Letty *et al*., 2000). In our case, the survival rate obtained five months after the first release, using a variety of scientific methods, was 45-49% based on the faeces count and 48.1% using the capture-recapture method (M/N = m/n). We considered this survival rate or success level high compared to the rate obtained in free releases, where the survival of translocated rabbits is around 9% in just the first month (Calvete & Estrada, 2004). The highest mortality detected was caused by aerial predators (19.2% in the first five months). No indications of viral diseases were observed at any stage of the study.

The failure to detect episodes of disease during the monitoring period may have been because the donor populations, with values of relative abundance in the high category, had high rates of seropositivity to MV and RHDV (100% in April). This circumstance is encouraging as it will favour survival by reducing mortality from RHD (Calvete *et al*., 2002), regardless of whether the antibodies in these seropositive rabbits came from earlier RHDV infections or were due to a non-pathogenic strain from the translocated rabbits. In the case of myxomatosis, other studies have also shown a positive relation between antibody concentrations and wild rabbit survival rates (Cooke & Berman, 2000; McPhee *et al*., 2002). Although the impact of the diseases on the translocated rabbits was monitored for only 12 months, all indications are that there were no outbreaks of either viral disease during the study period. The natural antibody values ensured a certain period of immunity, but we chose hyperimmunisation because rabbits can have better immunological status after vaccination from the greater protection provided by artificial induction of immunity (Calvete & Estrada, 2000). This action was based on the presence of a nearby native population with immune values to both diseases of 45.4% in the sampled rabbits, all of which were asymptomatic. This information may indicate that the native subpopulation was also (or had been) in contact with circulating viruses. The lower proportion of seropositive rabbits could be explained by the spatial isolation between these low density subpopulations, which would give them less contact with the viruses. It could also be explained by the existence of more virulent viral forms that would act as a limiting factor in population growth. With this information and data on periods of immunity in the wild, which can be from 12-18 months (Pagés, 1989; Argüello, 1991; Chasey, 1995; Dugast, 1995) or even shorter (<7 months) due to the fluctuating amount of viral antigen (Simón *et al*., 1993), we chose

hyperimmunisation for both diseases as a booster and to encourage a higher level of immunity as a preventive measure against a viral outbreak. This rise in the antibody level was demonstrated by monitoring five sentinel rabbits for eight months (data in this thesis). The situation of high immune status, the suitable habitat conditions both inside and outside the enclosure, and minimisation of the risk of predation inside the fencing positively affected rabbit survival, as other authors reported in their research (Cabezas *et al*., 2011).

Particular mention should be made of warren fumigation, which was not done in our study. Fumigation not only appears to have a limited effect (Osácar *et al*., 1996) due the large number of vectors in Mediterranean ecosystems and their varied phenology (Cooke, 1990), but could also affect sympatric rodents and therefore interfere in the epidemiology of RHDV,as seen in this doctoral thesis.

Relative abundance outside the enclosure, based on faeces counts per hour (27 latrines/hour) five months after openings had been made in the fencing in December 2009, leading to the gradual release of rabbits as they left of their own accord, was 193% higher than the count one year earlier, in April 2009 (14 latrines/hour). Thus introduction using technical criteria can be considered effective, following the recommendations of the protocols of indirect dung pellet counts (Kolb, 1994; Moreno & Villafuerte, 1995).

2. Epilogue

It would be advisable to implement, review and promote the lines of work that have evolved out of the preparation of this thesis, which are listed below as questions and considerations:

- Could rodents be used in rabbit hyperimmunisation programmes in a similar fashion to current research with rabbit kits?
- Could rodents be used as sentinels to obtain indirect information (types of viruses, levels of virus presence) about rabbits cohabiting with them?
- It would be useful to prepare monitoring programmes at a regional level that include epidemiological information on the evolution and status of viral diseases that affect wild rabbits.
- It would be very interesting to set up health monitoring plans that include immunological and genetic aspects (of both the viruses and the hosts) for rabbit farms, zoos, hunting grounds and natural areas.
- We consider it necessary to revise Royal Decree 1082/2009, of 3 July, which lays down the animal health requirements for translocating animals used in hunting, in relation to translocation of free-ranging rabbits that have antibodies to both viral diseases. As seen in this thesis, the presence of immunity in a population with no clinical signs of disease and no reports of recent outbreaks of disease is a feature that must be reinforced or at least maintained, as it could encourage natural recovery of the species.
- Hunting seasons should be revised by bioecological regions to take into account the physiological state of rabbits and their health status.
- We perceive a need to preserve high density populations with all their individual features and to establish a system of monitored populations that can be used as donors for reintroductions.
- It would be worthwhile taking a census of hunting farms and setting up an authorisation system for them based on quality criteria.
- It would be very interesting and helpful to create a wild rabbit monitoring facility or administrative office to coordinate experiences, combine information, encourage projects and programmes, offer advice and publicise matters related to wild rabbit management and conservation.

3. References

- Alda, F., Alcaraz, L., Hernández, M., Doadrio, I., García-Gartagoitia, J.L., Gaitero, T., 2006. Estudio genético e inmunológico del conejo silvestre: implicaciones epidemiológicas y poblacionales de las translocaciones y reintroducciones. Unpublished report. Departamento de Biodiversidad y Biología Evolutiva. Museo Nacional de Ciencias Naturales, Consejo Superior de Investigaciones Científicas. Madrid.
- Aparicio, F., Peiro, P.L., Castro, F., Villafuerte, R., 2011. Nuevo método de detección de uso de madrigueras: el tubo de huellas. In Díaz-Portero, M. A., Sánchez, J.F. Robles, M., (Eds). II Congreso internacional sobre el conejo de monte. Ponencias y comunicaciones. Proyecto LIFE+ 07 NAT/E/000742 "Priorimancha". Toledo 2011.
- Argüello, J.L., 1991. La enfermedad hemorrágica viral del conejo: vacunación y respuesta inmunológica. Revue Scientifique et Technique - Office International des Epizooties 10: 459–467.
- Arthur, C.P., Louzis, C., 1988. Myxomatose du lapin en France: une revue. Revue Scientifique et Technique – Office International des Epizooties 7: 939-957.
- Bell, D.J., Webb, N.J., 1991. Effects of climate on reproduction in the European wild rabbit (*Oryctolugus cuniculus*). Journal of Zoology 224: 639–648.
- Blanco, J.C., Villafuerte, R., 1993. Factores ecológicos que influyen sobre las poblaciones de conejos. Incidencia de la enfermedad hemorrágica. ICONA. Madrid.
- Boots, M., Hudson, P.J., Sasaki, A., 2004. Large shifts in pathogen virulence relate to host population structure. Science 303: 842–844.
- Branco, M., Machado, J.C., Ferrand, N., 1999. Extensive genetic polymorphism of peptidases A, B, C and D, in wild rabbit (*Oryctolagus cuniculus*) populations from the Iberian Peninsula. Biochemical Genetics 37: 237-249.
- Branco, M., Ferrand, N., Monnerot, M., 2000. Phylogeography of the European rabbit (*Oryctolagus cuniculus*) on the Iberian Peninsula inferred from RFLP analysis of the cytochrome b gene. Heredity 85: 307-317.
- Cabezas, S., Calvete, C., Moreno, S., 2011. Survival of translocated wild rabbits: importance of habitat, physiological and immune condition. Animal Conservation 14(6): 665-675.
- Calvete, C., 2011. ¿Es posible hiperinmunizar a las poblaciones de conejos frente a la enfermedad hemorrágica? Quercus 309: 18-22.
- Calvete, C., Villafuerte, R., Lucientes, J., Osácar, J.J., 1997. Effectiveness of traditional wild rabbit restocking in Spain. Journal of Zoology, London 241: 1-7.
- Calvete, C., Estrada, R., 2000. Epidemiología de enfermedad hemorrágica (VHD) y mixomatosis en el conejo silvestre en el valle medio del Ebro. Herramientas de gestión. Consejo de protección de la naturaleza en Aragón, Zaragoza, Spain.

- Calvete, C., Estrada, R., Villafuerte, R., Osácar, J.J., Lucientes, J., 2002. Epidemiology of viral haemorrhagic disease and myxomatosis in a free-living population of wild rabbits. Veterinary Record 150: 776–782.
- Calvete, C., Estrada, R., 2004. Short-term survival and dispersal of translocated European wild rabbits. Improving the release protocol. Biological Conservation 120(4): 507-516.
- Calvete, C., Angulo, E., Estrada, R., Moreno, S., Villafuerte, R., 2005. Quarantine length, blood biochemical parameters and survival of translocated European wild rabbits. Journal of Wildlife Management 69: 1063–1072.
- Calvete, C., Calvo, J.H., Sarto, P., 2012. Detection of a new variant of rabbit haemorrhagic disease virus in wild rabbits in Spain. XXXVII Symposium nacional de cunicultura organizado por la Asociación Española Nacional de Cunicultura (ASESCU). Barbastro. 24 & 25 May 2012.
- Capucci, L., Fusi, P., Lavazza, A., Lodovica, M., Rossi, C., 1996. Detection and preliminary characterization of a new rabbit calicivirus related to rabbit haemorrhagic disease virus but nonpathogenic. Journal of Virology 70: 8614-8623.
- Capucci, L., Fallacara, F., Grazioli, S., Lavazza, A., Pacciarini, M.L., Brocchi, E., 1998. A further step in the evolution of rabbit heamorrhagic disease virus: the appearance of the first consistent antigenic variant. Virus Research 58: 115–126.
- Castro, F., Ramírez, E., Ferreira, C., Aparicio, F., Álvaro, P.J., Manners, R.E., Steve Redpath, S., Villafuerte, R., 2011. ¿*Algirus* o *cuniculus*? Pequeñas y grandes diferencias. In: Díaz-Portero, M.A., Sánchez, J.F., Robles, M. (Eds). II Congreso internacional sobre el conejo de monte. Ponencias y comunicaciones. Proyecto LIFE+ 07 NAT/E/000742 "Priorimancha". Toledo, 24 & 25 November 2011.
- Chasey, D., Lucas, M.H., Westcott, D.G., Sharp, G., Kitching, A., Hughes, S.K., 1995. Development of a diagnostic approach to the identification of rabbit haemorrhagic disease. Veterinary Record 137: 158-160.
- Comenge, J., Mora, F.X., 2011. Brotes atípicos de enfermedad hemorrágica vírica en conejos en la Península Ibérica. Cunicultura 36(213): 7-10.
- Cooke, B.D., 1990. Rabbit burrows as environments for European rabbit fleas, *Spilopsyllus cuniculi* (Dale), in arid South Australia. Australian Journal of Zoology 38: 317-325.
- Cooke, B.D., 1997. Analysis of the spread of rabbit calicivirus from Wardang Island through Mainland Australia (Project CS236). Report, Meat Research Corporation, Sydney 1997.
- Cooke, B.D., Robinson, A.J., Merchant, J.C., Nardin, A., Capucci, L., 2000. Use of ELISAs in field studies of rabbit haemorrhagic disease (RHD) in Australia. Epidemiology Infection 124: 563-76.
- Cooke, B.D., Berman, D., 2000. Effect of inoculation route and ambient temperature on the survival time of rabbits, *Oryctolagus cuniculus* (L.), infected with rabbit haemorrhagic disease virus. Wildlife Research 27: 137–142.

- Cooke, B.D., 2002. Rabbit haemorrhagic disease: field epidemiology and the management of wild rabbit populations. Revue Scientifique et Technique. Office International des Épizooties 21: 347-358.
- Delibes-Mateos, M., Delibes, M., Ferreras, P., Villafuerte, R., 2008. Key role of European rabbits in the conservation of the Western Mediterranean basin hotspot. Conservation Biology 22: 1106–1117.
- Dugast, F., 1995. Étude de la prévalence sérologique de la maladie haemorragique virale dans une population de lapins de garenne. PhD thesis, Faculté de Médecine de Nantes, France.
- Fenner, F., Fantini, B., 1999. Biological control of vertebrate pests: the history of myxomatosis, an experiment in evolution. 1 edn. CABI Publishing, Wallingford.
- Guzmán, J.N., García, F.J., Garrote, G., Pérez de Ayala, R., Iglesias, M.C., 2004. El Lince ibérico (*Lynx pardinus*) en España y Portugal. Censo-diagnóstico de sus poblaciones. Naturaleza y Parques Nacionales: Serie Técnica. Dirección General para la Biodiversidad, Ministerio de Medio Ambiente, Madrid. 184 pp.
- Kitchen, A., Shackelton, L.A., Holmes, E.C., 2011. Family level phylogenies reveal modes of macroevolution in RNA viruses. Proceedings of the National Academy of Sciences of the United States of America PNAS, U S A 108: 238–43.
- Kolb, H., 1994. The age and post-juvenile growth of rabbits in the south-east of Scotland. Acta Theriologica 39: 49-57.
- Leighton, F.A., Artois, M., Capucci, L., Gavier-Widen, D., Morisse, J.P., 1995. Antibody response to rabbit viral hemorrhagic disease virus in red foxes (*Vulpes vulpes*) consuming livers of infected rabbits (*Oryctolagus cuniculus*). Journal of Wildlife Diseases 31: 541-544.
- Letty, J., Marchandeau, S., Clobert, J., Aubineau, J., 2000. Improving translocation success: an experimental study of antistress treatment and release method for wild rabbits. Animal Conservation 3: 211-219.
- Letty, J., Hivert, J., Queney, G., Aubineau, J., Monnerot, M., Marchandeau, S., 2002. Assessment of genetic introgression due to a wild rabbit restocking. Zeitschrift fur Jagdwissenschaft 48: 33-41.
- Marchandeau, S., Ricci, J.C., Chantal, J., 1998. Taux de prévalence sérologique du virus de la maladie virale haemorragique (VHD) du lapin de garenne (*Oryctolagus cuniculus*) et de ses formes apparentées au sein de différentes populations sauvages en France. Mammalia 62: 95- 103.
- Marchandeau, S., Boucraut, C., 1999. Epidemiology of myxomatosis and calicivirosis related to RVHD in a free-living population of European rabbits (*Oryctolagus cuniculus*). Gibier Faune Sauvage 16: 65-80.
- Marchandeau, S., Le Gall-Reculé, G., Bertagnoli, S., Aubineaua, J., Botti, G., Lavazza, A., 2005. Serological evidence for a non-protective RHDV-like virus. Veterinary Research 36: 53–62.

- McPhee, S.R., Berman, D., Gonzales, A., Butler, K.L., Humphrey, J., Muller, J., Waddington, J.N., Daniels, P., Koch, S., Marks, C.A., 2002. Efficacy of a competitive enzyme-linked immunosorbent assay (ELISA) for estimating prevalence of immunity to rabbit haemorrhagic disease virus (RHDV) in populations of Australian wild rabbits (*Oryctolagus cuniculus*). Wildlife Research 29: 635–647.
- Moreno, S., Villafuerte, R., 1995. Traditional management of scrubland for the conservation of rabbits *Oryctolagus cuniculus* and their predators in Doñana National Park, Spain. Biological Conservation 73: 81-85.
- Moss, S.R., Turner, S.L., Trout, R.C., White, P.J., Hudson, P.J., Desai, A., Armesto, M., Forrester, N.L., Gould, E.A., 2002. Molecular epidemiology of rabbit haemorrhagic disease virus. Journal of General Virology 83: 2461–2467.
- Muller, A., Freitas, J., Silva, E., Le Gall-Reculé, G., Zwingelstein, F., Abrantes, J., Esteves, P.J., Alves, P.C., Van der Loo, W., Kolodziejek, J., Nowotny, N., Thompson, G., 2009. Evolution of rabbit haemorrhagic disease virus (RHDV) in the European rabbit (*Oryctolagus cuniculus*) from the Iberian Peninsula. Veterinary Microbiology 135: 368- 373.
- Nyström, K., Le Moullac-Vaidye, B., Ruvoën-Clouet, N., Le Pendu, J., 2012. Shared human/rabbit ligands for rabbit hemorrhagic disease virus [letter]. Emerg Infect Dis [serial on the Internet]. 2012 Mar [date cited]. http://dx.doi.org/10.3201/eid1803.111402External Web Site Icon
- Osácar, J.J., Lucientes, J., Gajon, A., Moreno, C., Calvete, C., 1996. Efficacy of burrow fumigations against wild rabbit (*Oryctolagus cuniculus*) fleas (Siphonaptera) in Ebro's middle valley (NE Spain). 10th European SOVE Meeting, 2, 3, 4, 5 & 6 September 1996. Strasbourg.
- Pagés, A., 1989. Consideraciones técnicas de la sueroterapia y de la profilaxis vacunal en la enfermedad hemorrágica vírica del conejo (RHDV). Medicina Veterinaria 6: 285–291.
- Park, J.H., Lee, Y.S., Itakura, C., 1995. Pathogenesis of Acute Necrotic Hepatitis in Rabbit Hemorrhagic-Disease. Laboratory Animal Science 45: 445-449.
- Queney, G., Ferrand, N., Weiss, S., Mougel, F., Monnerot, M., 2001. Stationary distributions of microsatellite loci between divergent population groups of the European Rabbit (*Oryctolagus cuniculus*). Molecular Biology and Evolution 18: 2169-2178.
- Rogers, P.M., Arthur, C.P., Soriguer, R.C., 1994. The rabbit in continental Europe. The European rabbit: the history and biology of a successful colonizer. Thompson, H.V., King, C.M. (Eds), 22-63. Oxford University Press, Oxford.
- Sarto, P., Calvo, J.H., Calvo, A.J., Monroy, F., Calvete, C., 2013. Diagnóstico mediante PCR-dúplex de la nueva variante RHDVb del virus de la enfermedad hemorrágica en conejos. 42 Jornadas de Estudio. XV Jornadas sobre Producción Animal: Zaragoza, 14 & 15 May 2013.
- Schirrmeier, H., Reimann, I., Kollner, B., Granzow, H., 1999. Pathogenic, antigenic and molecular properties of rabbit haemorrhagic disease virus (RHDV) isolated from vaccinated rabbits: detection and characterisation of antigenic variants. Archives of Virology 144: 719–735.

- Simón, M.C., Gironés, O., Alonso, J.L., Muzquiz, J.L., García, J., Ortega, C., Muguruza, R., 1993. Enfermedad vírica hemorrágica en el conejo industrial: eficacia de una vacuna inactivada en la protección frente a la inoculación experimental. Medicina Veterinaria 10: 44–48.
- Simón, M.C., Ortega, C., Maynar, P., Muzquiz, J.L., De Blas, I., Muguruza, R., Gironés, O., Alonso, J.L., Sánchez, J., 1998. Studies in wild rabbit (*Oryctolagus cuniculus*) populations in Navarra, Spain. I. Epidemiology of rabbit viral haemorrhagic disease. Gibier Faune Sauvage, Game and Wildlife 15: 47-64.
- Smith, A.W., Skilling, D.E., Cherry, N., Mead, J.H., Matson, D.O., 1998. Calicivirus emergence from ocean reservoirs: Zoonotic and interspecies movements. Emerging Infectious Diseases 4: 13-20.
- Smith, A.W., 1999. Calicivirus models of emerging and zoonotic diseases. In 'Rabbit Control, RCD: Dilemmas and Implications. Proceedings of the Conference, 30-31 March 1998'. Pp. 37-42. (Royal Society of New Zealand Miscellaneous Series: Wellington, New Zealand).
- Snyder, N.F.R., Derrickson, S.R., Beissinger, S.R., Wiley, J.W., Smith, T.B., Toone, W.D., Miller, B., 1996. Limitations of captive breeding in endangered species recovery. Conservation Biology 10: 338-348.
- Van Regenmortel, M.H., Fauquet, C.M., 2000. Virus Taxonomy: Classification and Nomenclature of Viruses: Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press.
- Viggers, K.L., Lindenmayer, D.B., Spratt, D.M., 1993. The importance of disease in reintroduction programmes. Wildlife Research 20: 687–698.
- White, P.J., Norman, R.A., Trout, R.C., Gould, E.A., Hudson, P.J., 2001. The emergence of rabbit haemorrhagic disease virus: will a non-pathogenic strain protect the UK? Philos. Trans. Royal Society London B: Biological Science 356: 1087– 1095.
- Williams, C.K., Moore, R.J., 1989. Environmental and Genetic Influences on Growth of the Wild Rabbit, *Oryctolagus cuniculus* (L) in Australia. Australian Journal of Zoology 37(5): 591-59.
- Wolf, M.C., Griffith, B., Reed, C., Temple, S.A., 1996. Avian and mammalian translocations: Update and reanalysis of 1987 survey data. Conservation Biology 10, 1142–1154.
- Woodford, M.H., Rossiter, D.P.B., 1993. Disease risks associated with wildlife translocation projects. Revue Scientifique et Technique Office - International des Epizooties 12: 115–135.
- Zheng, T., Lu, G., Napier, A.M., Lockyer, S.J., 2003. No virus replication in cats fed with RHDV-infected rabbit livers. Veterinary Microbiology 95: 61.

CONCLUSIONS

CONCLUSIONS

1. Epidemiology of Rabbit Haemorrhagic Disease Virus

1.1. Wood mouse *Apodemus sylvaticus* and Algerian mouse *Mus spretus* naturally host rabbit haemorrhagic disease virus (RHDV) in their internal organs, specifically in the liver and the intestine. These mice cohabit sympatrically with wild rabbits, using rabbit warrens and the surrounding area as shelter.

1.2. Mice screened in Extremadura (n=51) showed a 9% level of RHDV in the case of *A*. *sylvaticus* (n=22) and 3.4% in the case of *M*. *spretus* (n=29) living in the wild. This is the first time this circumstance has been confirmed, not only in the Iberian Peninsula, but also in these species.

1.3. The strains of virus found in the mice are identical (in both *M*. *spretus* and *A*. *sylvaticus*) or very similar (in *A*. *sylvaticus*) to the strains found in sympatric populations of wild rabbit *Oryctolagus cuniculus* (n=31) in the study area of Plasenzuela (Extremadura, Spain) in July 2005.

1.4. Transmission of RHDV from infected rabbits to free-ranging rodents (*A. sylvaticus* and *M*. *spretus*) artificially cohabiting with them was experimentally confirmed in a room where an outbreak of RHD was produced. The mice hosted RHDV for some time without suffering from the disease or presenting behavioural alterations or macroscopic lesions consistent with these symptoms described for RHD in rabbits. RHDV has infectivity to these rodent species but not virulence. It is not known whether RHDV has immunogenicity and therefore gives rodents resistance or immunity.

1.5. Through experimentation, it was establishedthat the wood mouse (*A*. *sylvaticus*) may host the virus in its organism for at least 68 days after contact with RHDV in experimental conditions. The virus was detected in 87% of micefaeces seven days post contactand in 25% of faeces 68 days post contact. These unpublished findings strengthen the hypothesis of the involvement of these two mouse species in RHDV epidemiology when rabbits and rodents cohabit sympatrically.

1.6. In terms of the location of RHDV in mice, 87.5 % showed RHDV in faeces and 50% in the liver seven days after experimental transmission. This suggests a preference for the intestine as an ecological niche chosen for the virus in rodents.

1.7. Rodents infected with RHDV can cause a variety of responses in sympatric rabbits. A rabbit challenged with the carcass and faeces of a mouse that had been infected by infected rabbits showed an IgG humoral response. This confirms cross-species jumping and its possible

epidemiological impact. The challenged rabbit showed a titre of 1/32, which does not provide protection from subsequent exposure to RHDV. However, this result cannot be considered conclusive, as the sample size was not representative.

2. Epidemiology of RHD and myxomatosis

2.1. In summer 2005, 23.5% of the wild rabbits studied in a free-ranging, healthy population in Plasenzuela (Cáceres) and 24.1% of the rabbits studied in another population with similar characteristics in Reina (Badajoz) showed RHDV in their livers. In this same period in 2005, in a sample of 46 rabbits, 10.3% were found to be both seropositive and PCR positive (carriers). Additionally, 13.8% were found to be both seronegative and PCR positive (persistently affected). These results warn us of the risk of translocating rabbits from different geographical areas, as we may be introducing virulent strains or variants with negative impacts that are difficult to assess.

2.2. Immune status to RHD of two rabbit populations in Extremadura was analysed in July 2005. Rabbits from Plasenzuela (Cáceres) (n=24) showed 10% seropositivity and rabbits from Reina (n=31) showed 54% seropositivity. These results and the results of the presence of RHDV can indicate the epidemiological status of RHD at a given time. They can be applied to monitor trends and can therefore be used as a management tool.

2.3. Latrine/hour counting proved to be a good method of calculating the category of rabbit relative abundance. It allows data that is very useful for comparison to be collected relatively easily. Using this method, we determined that the two populations sampled are in the high density category, as the values were higher than 83 latrines/hour.

2.4. In 2004, in eight areas of Extremadura (five in Badajoz and three in Cáceres), autumn (October-December) was chosen as the period for taking serum samples from rabbits (n=107) killed during the hunting season. It was found that 88% of the rabbits had antibodies to the myxoma virus (MV), indicating that this virus was widespread and that individuals found at this time of year are survivors with high immune status to myxomatosis.

2.5. In autumn 2004, the rabbit populations screened showed higher percentages of individuals positive to MV than to RHDV, with very different and generally lower rates of rabbits seropositive to RHDV. Only two of the eight populations sampled (25%) showed high seropositivity percentages to RHDV (>80%). Seropositivity percentages were 53-60% in 37.5% of the population. The remaining proportion (three out of eight) had very low seropositivity

(<27%). It therefore appears that RHDV was less widespread in the population, or had appeared only recently, or had affected rabbit populations in such a way that the virus-host balance had not been established yet, or a long period had lapsed since the last contact with the virus.

2.6. No significant differences were observed by sex in the proportion of individuals seropositive to either MV or RHDV. For rabbit age, we did find differences in both age classes considered (young and adult), with a higher percentage of seropositive individuals among adults than among young rabbits (MV: adults 96%, young 62.5%; RHDV: adults 58%, young 12%).

2.7. Autumn seropositivity to RHDV in 2004 was positively and significantly correlated to rabbit relative abundance. Information about population immune status at specific times of the annual cycle may shed light on some management actions, as it can warn us of the vulnerability of certain populations to the entry of viruses through translocations and whether or not it is appropriate to hunt certain populations in decline with low percentages of protected individuals.

2.8. In three locations in Badajoz (Talarrubias-TA, Reina-RE, Valencia de las Torres-VT), wild rabbit populations studied in June and July 2003 showed high levels of naturally acquired immunity to RHDVand MV. Antibody levels were higher to RHDV (around 80%) than to MV (around 50%). The presence of positive titres to RHDV shows complementarity to positive titres to MV. This means that as an individual shows higher titration to MV, it will also show higher titration to RHDV. Significant differences were observed in the means of the antibody levels to MV and RHDV (n = 84) and the mean was higher to MV.

2.9. Rabbits screened in a confined wild rabbit population in Jerez de los Caballeros (JC) showed no immunity to either disease in June and July 2003. This circumstance advises caution in this type of control, as immune acquisition in such cases is essentially provided by appropriate, controlled vaccination and the population is therefore highly exposed to an epizootic disease.

3. Morphometry

3.1. In October 2003, a comparison of two lots of rabbits, one from the south of Badajoz (n=40) and the other from an area in Valladolid (n=40), showed differences in morphometry and body condition associated with latitudinal differences and the different habitats of the populations studied. The Valladolid rabbits had higher values for weight (1267.65+/-19.68 g) and length (408.03+/-24.72 mm) than the Badajoz rabbits (1107.22+/-98.68 g and 381.5+/-16.81 mm).

3.2. Body condition index was significantly higher for the Badajoz rabbits (20.05+/-2.47) than for the Valladolid rabbits (18.81+/-2.69). This could be the result of differences in weather conditions and habitat in the study areas. Food is available earlier in the south and rabbits reach optimum body condition before the start of the reproduction season.

4. Repopulations and translocations

4.1. Using individuals from nearby geographical areas, with known genetic structures and high levels of natural immunity, can improve translocation results. Five months after release into a 0.54 ha enclosure equipped with suitable shelter structures and trophic resources, survival rates of 95 rabbits introduced in 2009 were 45-49%.

4.2. Hyperimmunisation (to myxomatosis and RHD) of rabbits with natural immunity is a measure that can increase the natural survival rate in a controlled population, as it was seen that antibody levels increased and remained high for a longer period of time.

4.3. In the Granadilla recipient area, 45.4% of the rabbits sampled (n=11) were seropositive to both viral diseases and asymptomatic. This suggests that the native subpopulation was also in contact with the circulating viruses. A lower proportion of seropositive rabbits could be explained by the spatial isolation of these low density subpopulations, which would give them less contact with the viruses.

4.4. Management in introduction enclosures is appropriate for repopulating species in specific areas. Creating translocation units by forcing situations of high rabbit density based on introduction sites can be effective and would also help to interconnect larger areas. By using enclosures of this type to avoid terrestrial predation and applying appropriate sanitary control, we confirmed that the highest mortality detected was the result of aerial predation (19.2% in the first five months). No evidence of viral diseases was found during an eight-month study from 2009 to 2010.

4.5. Adapting the habitat both inside the introduction site and in the immediate vicinity appears to be decisive, but it is also essential to make the area optimal to receive translocated rabbits. After an opening had been made in the enclosure, rabbit abundance increased considerably (193%) from the previous year, from 14 to 27 latrines/hour.

La Portiña (Talavera de la Reina), 5 August 2013.

PHOTOS

1. Chapter I

The study area was in the municipality of Plasenzuela, where the landscape is dominated by grassland and *Retama sphaerocarpa,* interspersed with rocks and occasional holm oak (*Quercus ilex*) trees.

"Hypolithic" rodent traps were placed in the undergrowth near the mouths of burrows or right at the burrow entrances.

Adult specimens of *Apodemus sylvaticus* (top) and *Mus spretus* (bottom), showing the difference in size. A calliper was used to measure the tarsus in all cases, as in *M. spretus* it is <18mm.

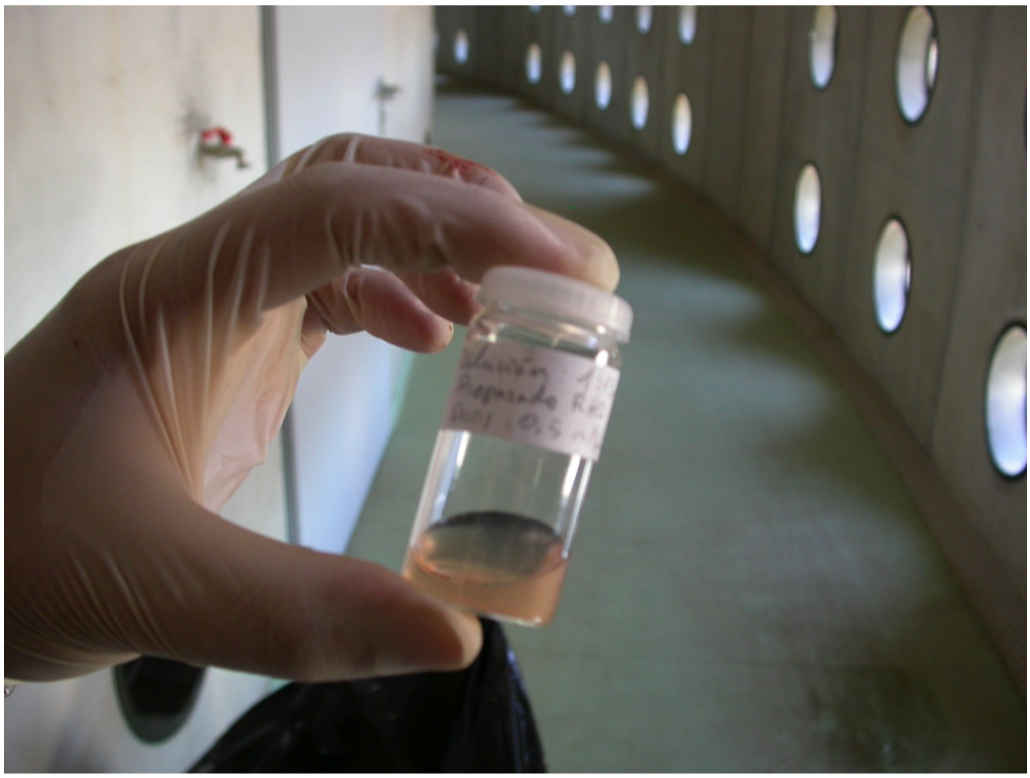
Close-up of the type of trap and bait used to catch the mice. The photo shows a specimen of *A. sylvaticus* captured using bait made from bread deep fried in re-used cooking oil.

Rabbits killed during culling (summer hunting). Liver samples were taken from these specimens for further processing.

Final state of samples before freeze storage.Several samples were taken from both rabbit and rodent livers. Samples were processed on alternate days and stored separately to avoid contamination.

2. Chapter II

The experimental transmission was conducted in biosecurity facilities at the former Jesús Usón Minimally Invasive Surgery Centre, Faculty of Veterinary Medicine, Cáceres, Spain.



Laboratorios Hipra S.A. provided the virulent reference strain of rabbit haemorrhagic disease virus: RHD-4764-183-ET (23/09/97).

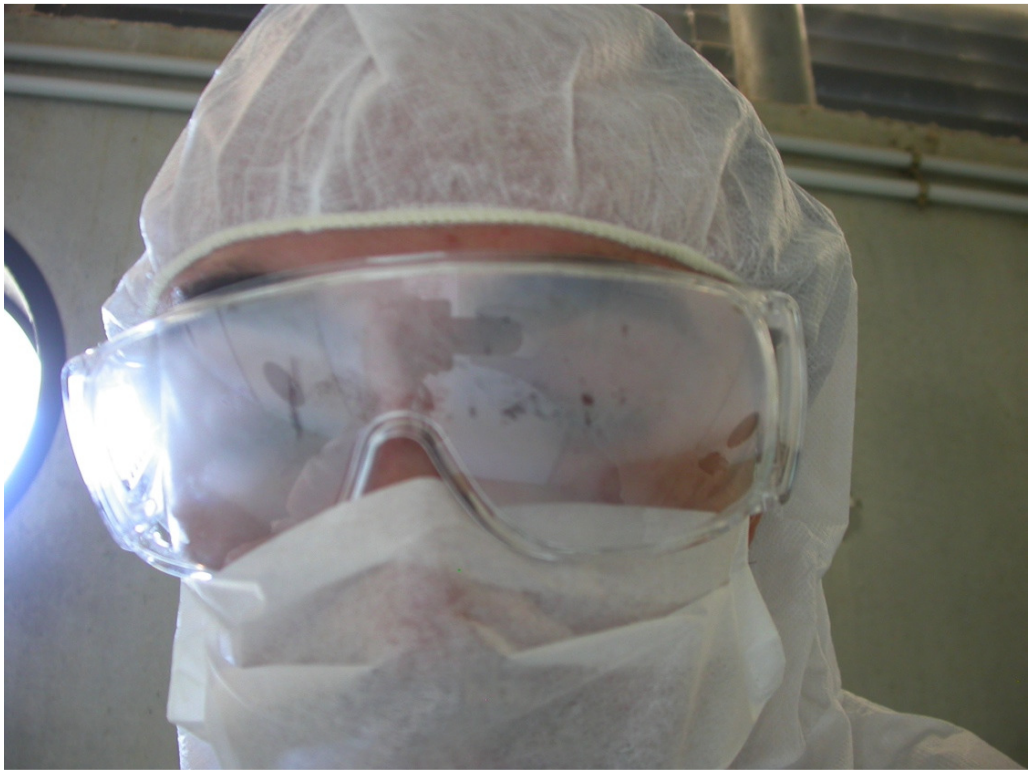
Rabbits on day 0 of the experiment, shortly after exposure to RHDV by subcutaneus injection (Coexistence phase 1).

Mice in the shelters made for the experiment during Coexistence phase 1. The top mouse is an *Apodemus sylvaticus*; the mouse below it is a *Mus spretus*.

Mice at the end of Coexistence phase 1, when no individuals had died and none had disease symptoms. The photo shows that the two species shared the same shelter.

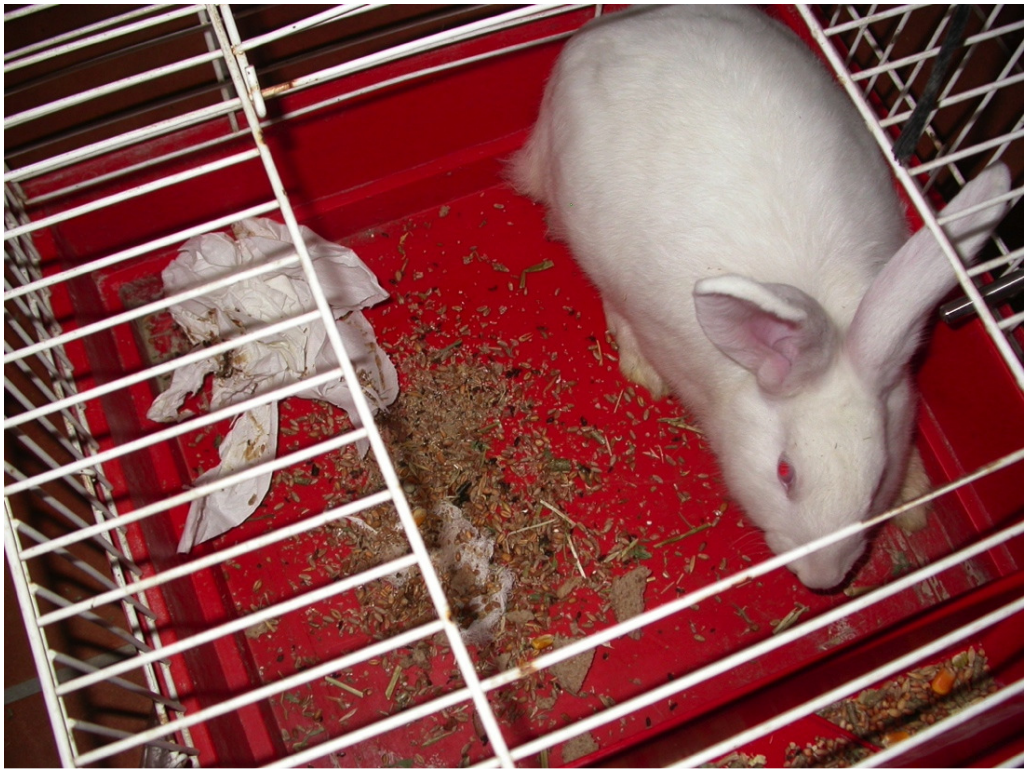
From the observation window of the coexistence room the process could be monitored during the seven days of the first phase.

One of the rabbits 48 hours after virus inoculation, when some rabbits showed strong symptoms of depression and lethargy as seen in the one pictured.

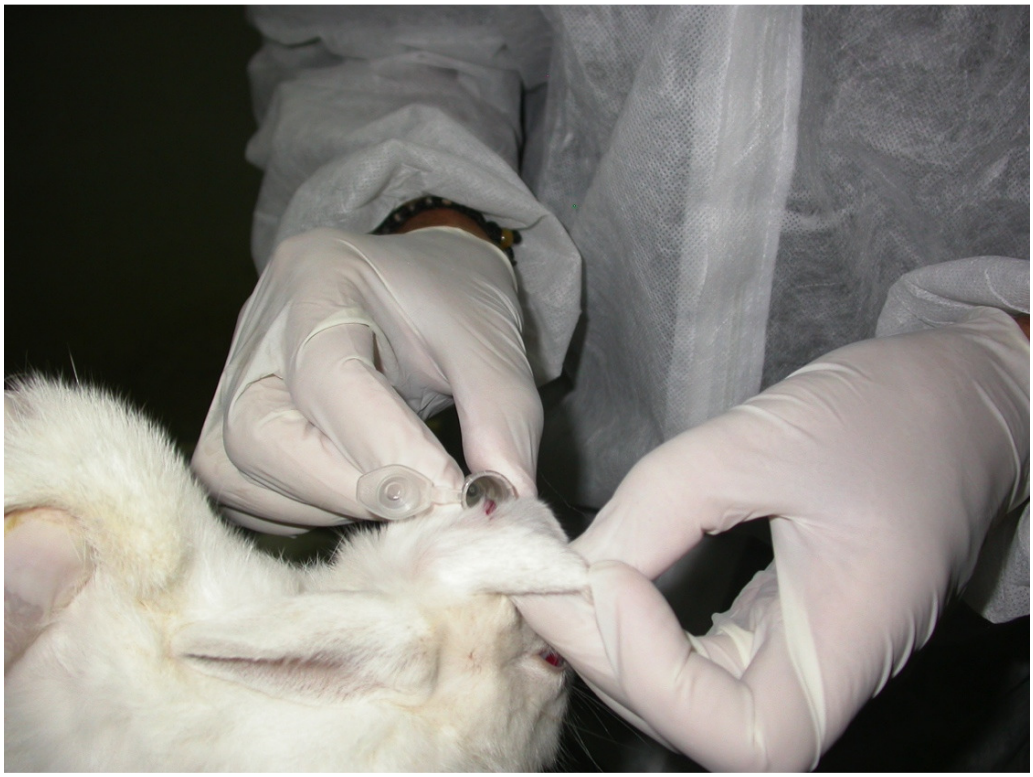


Entry into the room at the end of the coexistence phase was under strict biosecurity measures to prevent contamination and stop the virus exiting the facility.

The observation phase lasted 68 days. The mice were then taken from the coexistence room and housed in another room. The new room and the shelters and cages were previously disinfected with a virucide.



In Coexistence phase 2, the carcass and faeces of mouse M16 were placed inside the cage of the challenged rabbit.



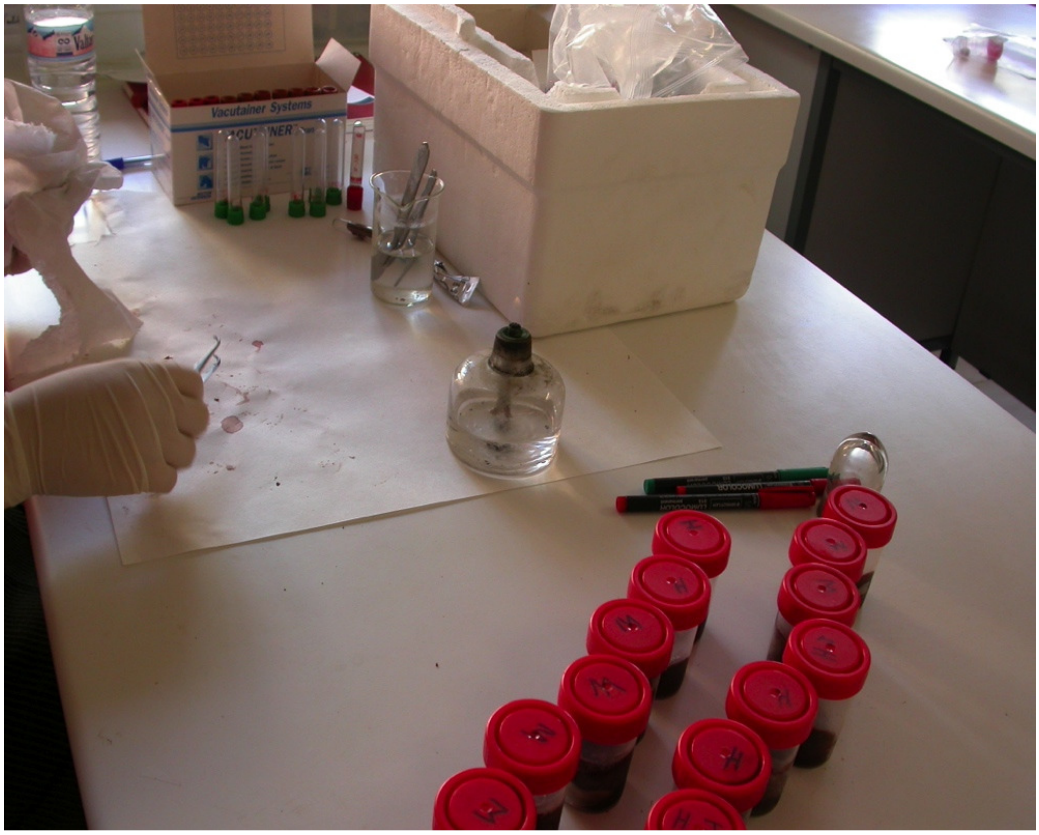
At the end of Coexistence phase 2, a blood sample was taken from the rabbit. After it was challenged with RHDV, the rabbit died 21 hours later.

3. Chapter III

Stuy area in Ocadal-Las Golondrinas, in the municipality of Cáceres. In July this area has very limited food and water resources.

Study area in Quintos del Viar, dominated by shrubs (mainly *Retama sphaerocarpa*).

Gloves were worn when obtaining serum and liver samples from the rabbits with a scalpel blade. New gloves and blades were used for each sample.



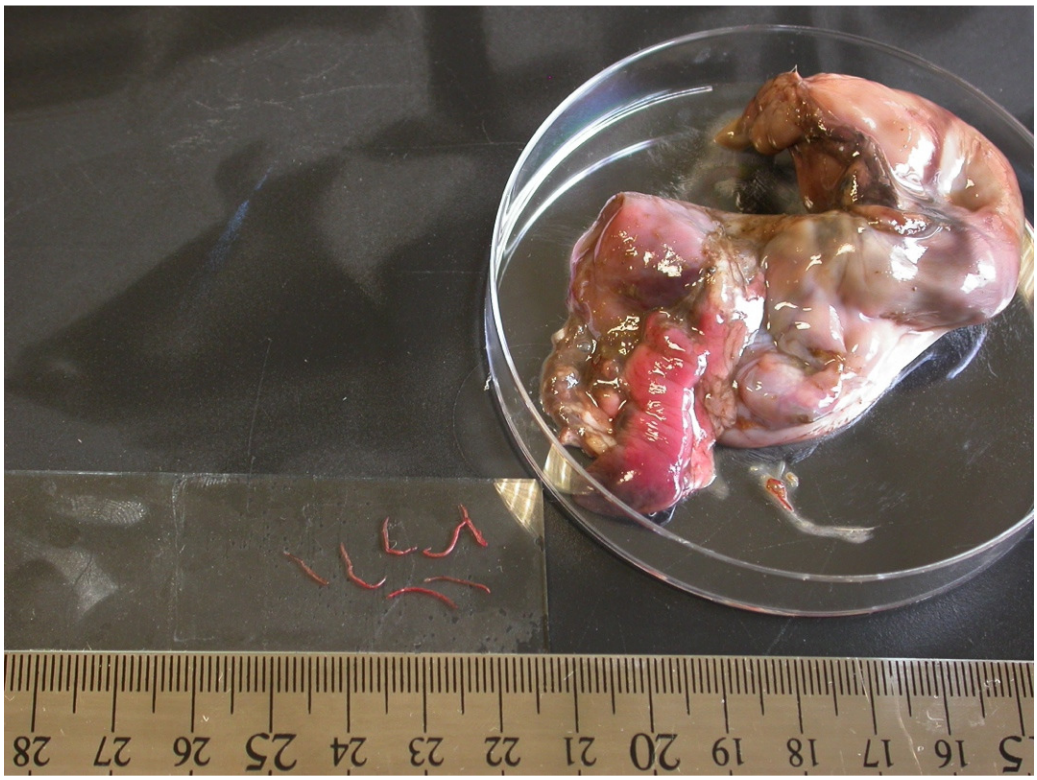
When the material arrived at the Faculty of Veterinary Medicine (Cáceres), the blood was centrifuged to obtain the serum and the liver samples were divided into smaller portions. All samples were frozen for transport to the laboratory.

4. Chapter IV

Hunted rabbits were sexed and their age was determined by palpating the epiphyseal cartilage in the metacarpals. In rabbits younger than eight months, a small pea-shaped lump can be felt in this area.

When possible, samples were taken from hunted rabbits *in situ* on the hunting grounds where they were captured. Blood samples were taken directly from the chest cavity, either by extraction with a syringe or through an opening in the cavity.

Some of the rabbits killed were taken to the laboratory to examine lesions consistent with rabbit haemorrhagic viral disease.



Necropsies showed the presence of parasites such as *Graphidium strigosum*, which is very common in rabbit stomachs.

5. Chapter V

Extracting rabbits from artificial burrows at a farm with full health management in Jerez de los Caballeros (Badajoz).

Extracting rabbits under partial health managment on the property of Serafin Mayoral, in Talarrubias (Badajoz).

La Rusal estate, in the municipality of Valencia de las Torres, where wild rabbits had no health management.

Extraction area in the municipality of Reina, where rabbits similarly had no health treatment.

The work to take samples from rabbits often required the combined efforts of a team from the University of Extremadura.

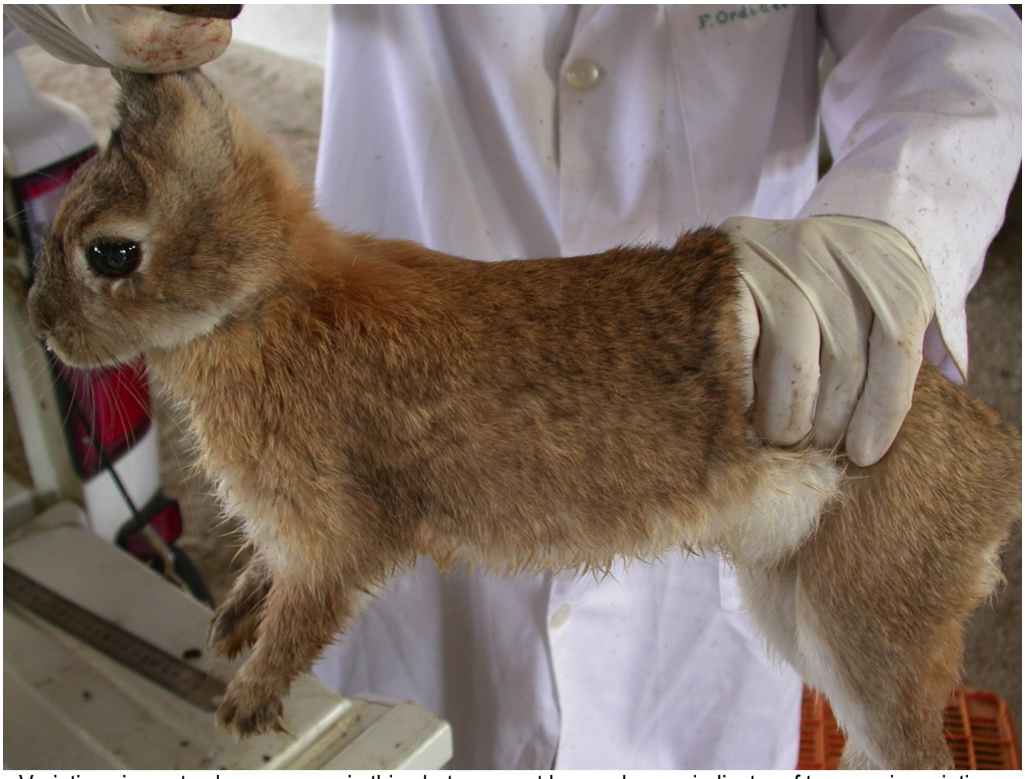
Extracting blood from the marginal ear vein. Blood was collected in an eppendorf vial for centrifuging at the laboratory.

After samples had been obtained, rabbits were identified with an ear tag and then released where they had been captured.

In Valencia de las Torres and Reina, rabbits were captured using trained ferrets.

6. Chapter VI

Wild rabbits captured for recording biometrics. La Rusal estate (Badajoz).



Variations in coat colour, as seen in this photo, cannot be used as an indicator of taxonomic variation.

Total length was measured from the tip of the nose to the start of the tail.

Close-up of hairless area on a female rabbit observed during sexing.

7. Chapter VI

Work to prepare the habitat inside the introduction enclosure for rabbits in the Granadilla restricted hunting area (Cáceres).

View of the introduction area after completion of the work to prepare the habitat.

Screening to determine immune status and genetics of a sample of rabbits from the recipient population closest to the introduction site, in the municipality of Granadilla.

Tunnel trap for catching rabbits in the area of origin of the donor population, in the province of Toledo.

Introduction of rabbits caught on the hunting grounds after transportation in boxes specially prepared for the journey.

Before release inside the enclosure, the translocated rabbits were vaccinated against RHD and myxomatosis.

APPENDIX

APPENDIX I: LETTER TO COLLABORATORS

UNIVERSIDAD DE EXTREMADURA

FACULTAD DE VETERINARIA

BIOLOGÍA Y ETOLOGÍA

Campus Universitario
Avenida de la Universidad, s/n
10071 - CÁCERES
Teléfonos: (927) 25 71 00/51/50
Fax: (927) 25 71 10

Estimados colaboradores:

Desde la Facultad de Veterinaria de Cáceres, llevamos un tiempo dedicados a la investigación científica de especies cinegéticas y, en concreto, algo más de dos años estudiando los factores que afectan a las poblaciones de conejo de monte en Extremadura. Gracias a su colaboración, hemos logrado analizar genéticamente más de 400 conejos y conocer el estado sanitario de más de 250 conejos. Pronto saldrán a la luz cuales son los resultados más relevantes de estos trabajos, pero necesitamos todavía más animales y seguir investigando.

Por todo ello, nos dirigimos a ustedes, cazadores, para pedirles su ayuda y conocimiento de campo para la toma de muestras.

Es necesario conocer con más detalle qué ocurre entre poblaciones de conejos vecinas, o lo que es lo mismo, entre cotos vecinos en un momento dado, y analizar variables muy importantes para conocer la dinámica poblacional del conejo (fundamentalmente, su estado inmunológico y la evolución de las enfermedades a lo largo del año). Nos hemos propuesto obtener esta información en esta temporada 2004-2005, hasta que termine la temporada de caza.

Por todo ello, les ruego sigan las instrucciones que abajo se detallan y distribuyan las fichas de encuesta entre aquellos cazadores que quieran colaborar:

Cada sobre enviado contiene:

Carta sellada: para que nos remitan a la Facultad con las FICHAS una vez rellenadas

Fichas de encuesta: UNA FICHA POR CAZADOR Y POR CADA DÍA QUE SALGA DE CAZA, de manera que cada colaborador pueda rellenar tantas como días hábiles salgan de caza. Si lo estiman oportuno, pueden hacer más copias.

Bolsas de muestras etiquetadas e individualizadas: para introducir una porción de hígado de 8 conejos, 1 zorro y 1 liebre. Se pueden recoger tantas muestras como se quiera y deberá anotarse la fecha, el sexo y la edad aproximada (más o menos de 9 meses). ESTAS MUESTRAS DEBERÁN GUARDARSE EN CONGELACIÓN HASTA SU RECOGIDA POR NOSOTROS (lo antes posible desde su notificación).

Nos pondremos en contacto telefónico con la dirección del acotado para poder solucionar cualquier duda y establecer la fecha de recogida de muestras. Para cualquier duda, sugerencia, observación o comentario pueden ponerse en contacto con el teléfono 927257151 (preguntar por Tomás Merchán).

Permitanme que una vez más, agradezca de antemano su inestimable ayuda, que es fundamental en la recuperación de nuestro conejo de monte.

Cáceres, 10 de Noviembre de 2004.

Tomás Merchán Sánchez
Responsable área Sanitaria "Proyecto Conejo"

Nota: como en otras ocasiones, la confidencialidad de los datos recabados en este estudio está garantizada, siendo estos, únicamente utilizados con fines científicos.

APPENDIX II: FIELD SHEET FOR ABUNDANCE SAMPLING

Muestreo población de conejos (*Oryctolagus cuniculus*) mediante índice de abundancia relativos

Coordenada de inicio	Coordenada final		FICHA
Fecha recorrido	Localización (paraje)	UTM 1X1	Municipio
Hora inicio	Hora final		Duración neta del muestreo

PRESENCIA DE CONEJO

Número de cagarruteros por tipos			
Pequeño	Mediano	Grande	TOTAL
Número de escarbaduras			
Número de conejos vistos			

HÁBITAT DE MUESTREO

Categorías de cobertura: 0(0%) 1 (1-10%) 2(11-20%) 3(21-40%) 4(41-60%) 5(61-80%) 6(81-100%)								
Anchura banda muestreada	Distancia a borde matorral(refugio)	% roca	% suelo desnudo	% pastizal	% cultivo	% matorral	% arbolado	Observaciones

CATEGORÍAS DE ABUNDANCIA RELATIVA DE CONEJOS EN FUNCIÓN DEL Nº DE LETRINAS/HORA DE MUESTREO

(*Guzman et al.*, 2004)

Categorías	Nº de letrinas/hora
Ausente	0
Bajo	0-33
Medio	33-66
Alto	66-100
Muy alto	>100

APPENDIX III: PROTOCOL FOR COLLECTION AND DELIVERY OF BIOLOGICAL MATERIAL FOR GENETIC AND IMMUNOLOGICAL ANALYSES

PROTOCOLO DE RECOGIDA Y ENTREGA DE MATERIAL BIOLÓGICO PARA ANÁLISIS GENÉTICO E INMUNOLÓGICOS

RECOGIDA DE MUETRAS DURANTE LA CAZA

MUESTRAS DE SANGRE

- Recoger la sangre por punción cardiaca lo más rápidamente posible después de la muerte del animal (como máximo 10 – 15 minutos tras la muerte del animal).

- Para la extracción de sangre por punción cardiaca, colocar el animal en decúbito dorsal o decúbito lateral derecho y estirar el miembro anterior izquierdo, de manera que con una inclinación de 30 ° aproximadamente, y a la altura del 3º - 5º espacio intercostal, se introduce la aguja en dirección rostro – medial, probando a diferentes niveles aspirando hasta extraer de 3 a 5 ml. de sangre.

- Pasar inmediatamente la sangre obtenida a un tubo con EDTA (3-4 ml) y a un recipiente eppendorf, etiquetar las muestras y refrigerar rápidamente de 0 a 4ºC. Mantener las muestras en estas condiciones, sin romper la cadena de frío, hasta su entrega en el Departamento.

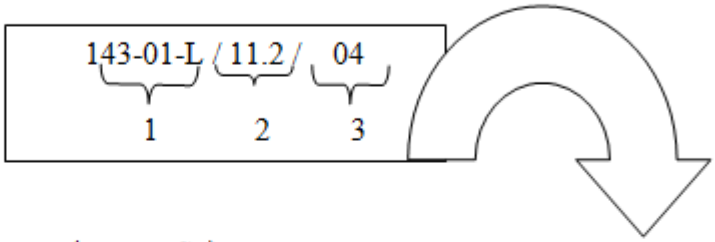
- Hasta el momento de su entrega, podrá usarse una nevera portátil con bloques de congelación (deberá vigilarse que esta temperatura permanezca constante), pero la muestra nunca debe CONGELARSE

1

ETIQUETADO DE MUESTRAS PARA ANÁLISIS GENÉTICOS E INMUNOLÓGICOS

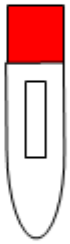
- Tanto el recipiente de la muestra de oreja como el de las muestras de sangre, deberán estar perfectamente identificados y reflejados en la ficha de toma de muestras por coto de la forma abajo esquematizada, y se individualizarán en una bolsa todas las muestras recogidas por coto

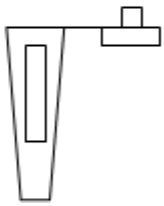
ETIQUETA IDENTIFICATIVA
(EJEMPLO)



1.- Matrícula del coto
2.- Fecha de la toma de muestra (mes y año)
3.- Número de la muestra en ese coto

Cuando las muestras provengan del mismo individuo, deberá coincidir la identificación, y la sangre se mantendrá siempre en refrigeración





Muestra tejido (oreja) **Tubo EDTA** **eppendorf**

NORMAS PARA LA RECOGIDA Y CONSERVACIÓN DE ANIMALES MUERTOS

- Recoger los conejos encontrados muertos sin lesiones externas evidentes de enfermedad o aquellos con síntomas evidentes de padecimiento de enfermedad.(No recoger animales en avanzado estado de putrefacción)

- Colocar en bolsas de plástico juntamente a la ficha de identificación. Por cada animal, aún encontrando un grupo de animales en un mismo coto, habrá que rellenar una ficha de recogida de muestras y en su reverso, cumplimentar por completo el cuestionario referido a continuación.

- Mantener en refrigeración o congelar los animales hasta su entrega en la Unidad de Biología y Etología.

2

CUESTIONARIO A RELLENAR EN CASO DE ENCONTRAR ANIMALES MUERTOS

(rodear lo que proceda)

Antigüedad de la muestra (muerto recientemente)
¿Hay elevada mortalidad de la/s especie/s .....en el paraje de toma de muestra? (si/no)
¿Hay una elevada densidad de la/s especie/s.....en ese punto? (si/no)
Aspecto general del animal
.....
.....
Comportamiento anormal de la especie consistente en:
.....

PRINCIPALES USOS DEL SUELO Y VEGETACIÓN PREDOMINANTE EN LA ZONA DONDE SE ENCONTRÓ EL ANIMAL (especificar).
Pastizal
Matorral
Siembras de secano
Siembras de regadío
Pinar
Robledal
Encinar
Alcornocal
Otros

CONDICIONES ATMOSFÉRICAS LOS DOS DÍAS ANTERIORES A LA RECOGIDA DEL ANIMAL

Temperatura	**Lluvia**
Bajo 0ª C	No llovió
0 a 10 ° C	Escasa
10 a 20 ° C	Abundante
20 a 30 ºC	
mas de 30º C	

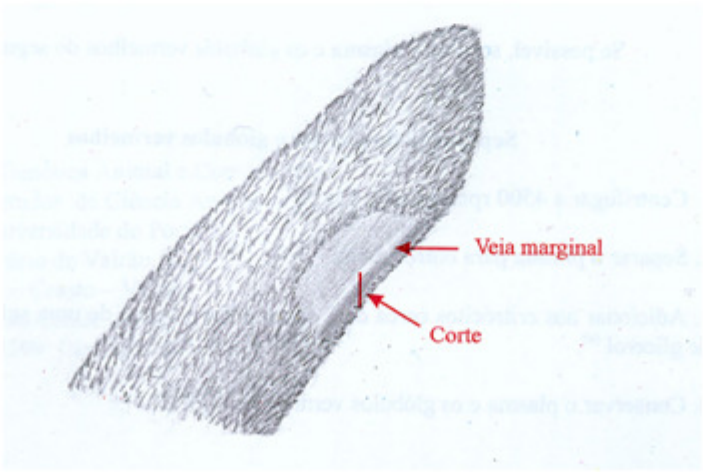
3

RECOGIDA DE MUETRAS DE ANIMALES MORIBUNDOS /VIVOS

- Recoger los conejos encontrados moribundos o vivos, sin lesiones externas evidentes de enfermedad o aquellos con síntomas evidentes de padecimiento de las mismas, y mediante punción cardiaca o por corte en la vena marginal del pabellón auricular, recoger la sangre y pasarla inmediatamente a un tubo con EDTA y un tubo eppendorf, etiquetar las muestras y refrigerar rápidamente de 0 a 4 C. Mantener las muestras en estas condiciones, sin romper la cadena de frío, hasta su entrega en el Departamento.

- Después de realizar esta labor, se procederá a la recogida de muestra de tejido del pabellón auricular y su posterior identificación mediante la ficha de recogida de muestras, que en el reverso tiene una serie de cuestiones que habrá que cumplimentar por entero.

- En animales vivos, después de friccionar la oreja con algodón empapado en alcohol, se hace un pequeño corte con una hoja de bisturí según se aprecia en el esquema, llenando lo máximo posible un tubo con EDTA y un tubo eppendorf. Para obtener sangre, también podrá realizarse la sección del tercio distal de la oreja, que se efectuará mediante un corte limpio con cuchilla de bisturí. Deberá efectuarse siempre que previamente se desinfecte con alcohol el lugar de corte y se utilice un cuchilla nueva o desinfectada



4

CUESTIONARIO A RELLENAR EN CASO DE ENCONTRAR ANIMALES MORIBUNDOS / VIVOS

(rodear lo que proceda)

¿Hay elevado nº de animales enfermos en el paraje de donde se encuentra el animal? (si/no)
¿Hay una elevada densidad de la/s especie/s……..en ese punto? (si/no)
Aspecto general del animal
……
……
……

MIXOMATOSIS
Edema subcutáneo de cabeza o región anal (si/no)
Presencia de nódulos fibróticos en las mismas zonas (si/no)
Conjuntivitis (si/no)
RHD
Neumonía hemorrágica (si/no)
Traqueítis hemorrágica y exuberante (si/no)
Hipertrofia de timo (si/no)

Comportamiento anormal de la especie consistente en
……

PRINCIPALES USOS DEL SUELO Y VEGETACIÓN PREDOMINANTE EN LA ZONA DONDE SE ENCONTRÓ EL ANIMAL (especificar).
Pastizal
Matorral
Siembras de secano
Siembras de regadío
Pinar
Robledal
Encinar
Alcornocal
Otros

CONDICIONES ATMOSFÉRICAS LOS DOS DÍAS ANTERIORES A LA RECOGIDA DEL ANIMAL

Temperatura	**Lluvia**
Bajo 0ª C	No llovió
0 a 10 ° C	Escasa
10 a 20 ° C	Abundante
20 a 30 °C	

5

APPENDIX IV: ORIGINAL PUBLICATIONS AND OTHER CONTRIBUTIONS

Infection, Genetics and Evolution 11 (2011) 1469–1474

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Short communication

Detection of rabbit haemorrhagic disease virus (RHDV) in nonspecific vertebrate hosts sympatric to the European wild rabbit (*Oryctolagus cuniculus*)

Tomás Merchán [a,*], Gregorio Rocha [a], Fernando Alda [b], Eliane Silva [c], Gertrude Thompson [d], Sebastián Hidalgo de Trucios [e], Albert Pagés [f]

[a] Departamento de Ingenieria del Medio Agronómico y Forestal, University of Extremadura, Avda. Virgen del Puerto 2, 10600 Plasencia, Spain
[b] Instituto de Investigación en Recursos Cinegéticos (CSIC-UCLM-JCCM), Ronda de Toledo s/n, 13005 Ciudad Real, Spain
[c] Multidisciplinary Unit of Biomedical Investigation (UMIB), University of Porto, P-4099-003 Porto, Portugal
[d] Department of Veterinary Clinics, Abel Salazar Biomedical Sciences Institute (ICBAS), University of Porto, P-4099-003 Porto, Portugal
[e] Grupo en Recursos Cinegéticos y Biodiversidad (RCB), Veterinary School, University of Extremadura, 10071 Caceres, Spain
[f] Hipra Laboratories S.A., La Selva 135, 17170 Amer, Spain

ARTICLE INFO

Article history:
Received 8 February 2011
Received in revised form 30 April 2011
Accepted 2 May 2011
Available online 11 May 2011

Keywords:
Apodemus
Central Spain
Mus
Oryctolagus cuniculus
Rabbit haemorrhagic disease virus
Reservoir

ABSTRACT

Since its detection in China in 1984, rabbit haemorrhagic disease (RHD) has been the subject of numerous studies. Yet, the evolutionary origin of rabbit haemorrhagic disease virus (RHDV) is still under debate. For example, some aspects related to the epidemiology of the disease are still unknown, such as where the virus is hosted between RHD outbreaks. To detect the presence of RHDV in rabbit-sympatric micromammals, 51 rodents (29 *Mus spretus* and 22 *Apodemus sylvaticus*) and 31 rabbits (*Oryctolagus cuniculus*) from the same location in central Spain were analyzed. In those samples in which the virus was detected, a fragment of the VP60 protein gene from the RHDV capsid was sequenced and the phylogenetic relationships between them and other strains of RHDV in the Iberian Peninsula were analyzed. In total, five viral strains were identified in *A. sylvaticus*, *M. spretus* and *O. cuniculus*. All strains were found to be well supported within the clade of RHDV found in rabbits in the Iberian Peninsula. Moreover, one of the strains was found in all three species under study, which suggests the capability of RHDV to infect other mammals apart from the rabbit which have not yet been investigated. The transmission of the virus is discussed as well as its ecoepidemiological implications.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Rabbit haemorrhagic disease (RHD), first discovered in China in 1984 (Liu et al., 1984), has a high rate of morbidity (100%) and mortality (40–100%) in adult European wild rabbits (Henning et al., 2005). Following its discovery in Spain in 1989, many populations of European wild rabbit have suffered high mortality rates of close to 80% (Villafuerte et al., 1994). Since then, rabbit numbers have declined at a relatively constant rate (Moreno et al., 2007). RHD is caused by a positive-sense, single-stranded RNA virus, a member of the genus *Lagovirus* within the family Caliciviridae (Parra and Prieto, 1990). This genus also includes the European brown hare syndrome virus (EBHSV), as well as the non-pathogenic rabbit calicivirus (RCV), which causes asymptomatic seroconversion in rabbits and is considered a potential apathonic ancestor of RHDV (Capucci et al., 1996). Recently a new calicivirus has been described, named the Michigan rabbit calicivirus (MRCV), which causes subclinical infections and whose genome shows an average similarity of 79% with RHDV (Bergin et al., 2009). However, further studies have questioned this finding and have proposed instead that MRCV is not a novel calicivirus but a new variant of the nonpathogenic RCV-like group (Abrantes and Esteves, 2010).

Since its identification, RHDV has been the subject of numerous studies. Yet, some aspects, such as the evolutionary origin of the virus, are still unknown or under debate (Forrester et al., 2006; Kerr et al., 2009). Various hypotheses have been put forward to explain the origin of RHDV, such as (i) the transference of EBHSV from the European brown hare (*Lepus europaeus*) to the rabbit (Nowotny et al., 1997), (ii) that changes were produced in a non-pathogenic virus, rendering it virulent (Moss et al., 2002), or (iii) the emergence of RHDV from another virus infecting another species (Fenner and Fantini, 1999).

All lagoviruses, with the exception of EBHSV, which solely affects hares (Löliger and Eskens, 1991), are specific to *Oryctolagus cuniculus* in domestic and wild forms, although the latter prove

* Corresponding author. Tel.: +34 696 12 19 81; fax: +34 927 42 52 09.
E-mail addresses: tmerchan@unex.es (T. Merchán), gregorio@unex.es (G. Rocha), alda.fernando@gmail.com (F. Alda), eli@mail.icav.up.pt (E. Silva), gat1@mail.icav.up.pt (G. Thompson), shidalgo@unex.es (S.H. de Trucios), apmante@gmail.com (A. Pagés).

1567-1348/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.meegid.2011.05.001

more sensitive to the virus (Argüello et al., 1988; Pagés, 1989). Despite the fact that RHDV and EBHSV show a genetic similarity of 71% and are also related antigenically, as shown by the presence of common internal epitopes (Wirblich et al., 1995), cross-infection does not occur (Lavazza et al., 1996). Equally, experimental infection of diverse species of laboratory and wild animals with RHDV has produced no symptoms of the disease (Gavier-Widen et al., 1997; Nowotny et al., 1999; Smid et al., 1991). Nor have other species of American leporids (eastern cottontail *Sylvilagus floridanus*, volcano rabbit *Romerolagus diazi*, and black-tailed jackrabbit *Lepus californicus*) been clinically affected upon experimental exposure to RHDV (Gregg et al., 1991). Likewise, non-target mammals during periods of viral activity in New Zealand were not affected (Parkes et al., 2004). The replication of RHDV in other hosts apart from the rabbit has not been established. In Australia, seroconversion has been detected as a response to the viral antigene in mice inoculated with the virus (Gould et al., 1997) as well as in kiwis (*Apteryx australis*) exposed to contaminated material (Buddle et al., 1997). Similarly, in red foxes (*Vulpes vulpes*) that had ingested rabbits infected with RHDV, post-infection antibody titers were detected, without the viral replication ever fully manifesting (Leighton et al., 1995).

The main natural transmission mechanism of RHDV is direct contact between infected rabbits through respiratory and oral routes and skin lesions (Xu et al., 1988). Subclinically ill or infected rabbits are the principal sources of infection (Xu and Chen, 1989). Moreover, rabbits with persistent infection (Löliger and Eskens, 1991), carriers (Cancellotti and Renzi, 1991; Cooke, 2002), as well as young rabbits under 20 days old who manage to survive the disease (Rosell et al., 1989), can also act as reservoirs of the virus. However, possible alternative hosts of the virus and their role in the epidemiology of the disease have scarcely been studied. Some field and laboratory studies have shown how predatory mammals and birds play a role in the transmission of RHDV (Chasey, 1994; Gavier-Widen and Morner, 1993; Simón et al., 1994), acting as mechanical reservoirs or vectors. Schirrmeier et al. (1990) conducted an experiment with rodents and insects, suggesting that these did not act as reservoirs; they did, however, act as possible passive transmitters of the virus. Likewise, other studies prove the importance of the role played by insects in transmission (Asgari et al., 1998; Gehrmann and Kretzschmar, 1991; Lenghaus et al., 1994), as well as that of the decomposing remains of the infected rabbits themselves (McColl et al., 2002), or their warrens in housing the RHDV (Calvete et al., 2002).

In this paper, the possible presence of RHDV is analyzed in two species of rodent (wood mouse *Apodemus sylvaticus* and Algerian mouse *Mus spretus*) and in the European wild rabbit, which all share the same habitat in a specific area of central Iberian Peninsula. This association is vital to extend knowledge on the role that other wild species (which have not yet been investigated) may play both in the origin and in the epidemiology of this disease.

2. Material and methods

2.1. Study area and sampling methods

The study area is situated in the municipality of Plasenzuela in Extremadura, Spain (39°22′N 6°02′W). It is classified as a high rabbit relative abundance area (Blanco and Villafuerte, 1993), with a hunting bag of 780 rabbits per year (Junta Extremadura, 2005; pers. comm.). The land is a rich thicket of mostly *Retama sphaerocarpa* shrubs, interspersed with rocks and occasional holm oak trees (*Quercus ilex*). The climate is typically Mediterranean; mild winters and very hot summers of variable temperature and precipitation (average of <500 mm per annum).

Rodents were captured alive, from 5th July to 25th July 2005, using wire mesh traps ("Hipólito" traps) (Carro et al., 2007). A trapping grid ($N = 10$) covering 5 ha was set up within the study area. Fried bread was used as bait. Wire mesh traps were placed at the entrance or in the vicinity of active rabbit warrens (<1 m from the entrance). Rows of ten traps per hectare, spaced up to 20 m apart, were set at night and checked each morning. The capture effort was maintained for 20 nights and traps were not relocated until a minimum of 10 micromammals per hectare was reached. In total, 51 micromammals (29 Algerian mice, *M. spretus*, Ms1–Ms29 and 22 wood mice, *A. sylvaticus*, As1–As22) were captured. No other species was captured. Each mouse captured was sacrificed "in situ" by cervical dislocation. Each animal was placed in an individual bag, properly identified and refrigerated for transportation to the laboratory, where they were frozen at −20 °C.

Additionally, 31 rabbits (*O. cuniculus*, Oc1–Oc31) were shot by hunters during an exceptional legal hunting period made available from the 15th of July to the 15th of August 2005 in the same area of study. Samples were handled and stored as described above for mice.

2.2. Laboratory methods

The RHDV sampling consisted in the extraction of the entire or parts of the liver from the animals captured. This task was carried out at different times and using sterile laboratory material, under strict biosecurity measures to avoid any cross-contamination. Furthermore, and to the same end, the different species (*O. cuniculus*, *A. sylvaticus* and *M. spretus*) were treated in separate laboratories. All samples were processed on premises belonging to the Caceres Veterinary School of the University of Extremadura (Spain).

Viral RNA was extracted from an organ homogenate of 0.1 g in 1 ml phosphate buffered saline (PBS) using TRI® Reagent LS (Sigma). cDNA was synthesized using random priming and M-MLV reverse transcriptase (Invitrogen). To test for positive samples, nested PCR was used to partially amplify RHDV capsid protein gene VP60 (Moss et al., 2002). The reaction mixture (25 μl), for PCR I and PCR II, contained 2.5 μl of 10× PCR buffer, 0.6 μl of each primer (10 mM), 100 μM of each dNTP, 2.0 μl $MgCl_2$ (25 mM), 1 U Taq DNA polymerase (Fermentas) and 1 μl of cDNA or product from first PCR. Primers RHDV1 and 4 were used for the first PCR and primers RHDV2 and 3 for the nested reaction. For both PCRs the cycling conditions were 30 cycles of 95 °C for 40 s, 50 °C for 40 s, 72 °C for 2 min and a final elongation of 72 °C for 10 min, in a thermocycler C 1000 (Bio-Rad). The size of the obtained PCR products was determined by ethidium bromide staining of a 2% agarose gel electrophoresis. The expected size of the final PCR product was 573 bp corresponding to the region covering positions 6151–6724 of the major capsid protein VP60 of RHDV of the Spanish RHDV strain AST89 (Z49271). The molecular analyses were carried out in the Clinical Veterinary Practice Department laboratories of the Abel Salazar Biomedical Sciences Institute (ICBAS), University of Porto (Portugal). PCR amplicons were purified and sequenced in both directions, in an ABI 3730XL (Applied Biosystems) using BigDye® terminator kit v3.1 (Applied Biosystems) with primers RHDV2 and 3. These analyses were performed by STAB VIDA (Portugal). All sequences obtained were deposited in Genbank with the following access numbers: HQ198365–HQ198366, HQ198368–HQ198371, HQ413340–HQ413341.

2.3. Phylogenetic analysis of RHDV VP60

Chromatograms were checked and assembled using Sequencher 4.6 (Gene Codes Corporation). Consensus nucleotides were translated into amino acids using MacClade 4.05 (Maddison

and Maddison, 2000) and manually aligned with other homologous sequences available in GenBank from the Iberian Peninsula (Alda et al., 2010; Müller et al., 2009) and representatives from RHDV Genogroups 1–6 previously described (Le Gall-Recoulé et al., 2003). Rabbit calicivirus (RCV) (X96868) was used as an outgroup.

In order to identify the most appropriate evolutionary model by the Akaike corrected information criterion (AICc), the program jModeltest 0.1.1 was used (Posada, 2008). Phylogenetic relationships among all the RHDV strains were inferred using Bayesian Inference (BI) in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), simulating four simultaneous Markov chains (MCMC) for 4×10^6 generations each, and using a sampling frequency of 100 generations. Convergence between run parameters from paired simultaneous runs was adjusted considering an adequate sampling based on average standard deviation of split frequencies being <0.01 (Huelsenbeck and Ronquist, 2005). The program Tracer v1.5 (Rambaut and Drummond, 2007) was used to determine burn-in length considering the set of trees saved prior to log likelihood stabilization and convergence.

3. Results

3.1. Detection of RHDV in mice and rabbits

All sampled rodents appeared healthy. RHDV positivity was detected by molecular analysis in the liver of 3 mice (5.8%): 2 *A. sylvaticus* (As15 and As26) and 1 *M. spretus* (Ms8). The rabbits analyzed also appeared healthy, showing no sign of the disease. Out of the 31 rabbits caught, the virus was detected in 7 (23.5%), of which 6 provided analysable sequences (Oc1–Oc6).

3.2. Phylogenetic relationships of RHDV isolated in rabbits and mice

Along with the homologous sequences available in GenBank from the Iberian Peninsula and representative from the main RHDV lineages, we constructed an alignment of 82 sequences. The best evolutionary model estimated by jModelTest 0.1.1 for our data was TVM + G, and the gamma shape parameter was $G = 0.366$.

In all of the animals analyzed, 5 strains of virus were found, according to the sequences obtained from the VP60 gene. The phylogenetic analysis performed was congruent with previous studies, and indicated that all Iberian samples form a monophyletic group (Alda et al., 2010; Müller et al., 2009). All of the virus strains detected in this study were found within this Iberian lineage (Genogroup 1, Fig. 1). Three rabbits (Oc1, Oc3 and Oc6), one *A. sylvaticus* (As15) and one *M. spretus* (Ms8) showed the same sequence of RHDV VP60 capsid gene, identical to a strain identified in Portugal in 2004 (EU192134, Müller et al., 2009). This strain was very similar to those detected in two other rabbits (Oc4 and Oc5), which differed one nucleotide from each other (*p*-distance = 0.002), and they came together to form a highly supported clade, which was the sister group of another series of strains isolated in Spain between 2004 and 2006 (Fig. 1). The other two identified strains of RHDV, corresponding to one rabbit (Oc2) and one *A. sylvaticus* (As26) grouped together (*p*-distance = 0.028) and formed a differentiated and supported clade containing strains isolated at a much earlier point in time, i.e. between 1994 and 2005 in Spain and Portugal (Fig. 1).

4. Discussion

To our knowledge, this is the first time that the natural presence of RHDV in wild micromammals *A. sylvaticus* and *M. spretus* has been detected. These species were captured in the immediate vicinity of warrens in areas of high relative abundance of rabbits. Unfortunately, the reduced sample size in this study does not allow to establish significant differences regarding infection rates of RHDV in *A. sylvaticus* (9%) and *M. spretus* (3.4%). Nevertheless, we might hypothesize that infection rates most likely depend on the abundance of each species in the area of study and/or on the use they make of the space. In this case, *A. sylvaticus* is the more widely distributed in Mediterranean environments (Castién and Gosálbez, 2001), and it is found more narrowly tied to the European wild rabbit and its shelters (Díaz, 1992). Therefore, a greater prevalence of RHDV in *A. sylvaticus* may be influenced by these factors.

The fact that the viruses detected in mice were identical (Ms8 and As15) or very similar (As26) to the viruses isolated in rabbits in the same vicinity and to the rest of the viruses that circulate in the Iberian Peninsula (Fig. 1) indicates that the strains of RHDV circulating in nature are capable of infecting different species of mammals. This finding is of great interest in view of the fact that if the non-pathogenic circulation of RHDV were demonstrated in wild mice, this would substantiate the theory that the origin of the RHDV may be in the transmission of this virus to the rabbit from another species (Fenner and Fantini, 1999). On the other hand, the prevalence and transmission of the virus within mice is not well known but it could be related to the fact that they use rabbit warrens as shelter structures (Delibes-Mateos et al., 2008), which may facilitate contact with the virus, through infected rabbits (ill or reservoir individuals).

The co-occurrence of these species of micromammals during outbreaks of the disease in the rabbits could indicate that mice may be infected passively by the impregnation of viral particles in legs and hairs, which are ingested during grooming. Another source of infection could be the ingestion of parts of the remains of rabbits that have died of the disease, especially those dead in the interior of their warrens (Cooke, 1996). Yet another possible means of infection is the faecal–oral route, as happens among rabbits (Xu and Chen, 1989). Rabbit faeces can carry the viruses both because the first viral replication occurs in the intestinal crypts of Lieberkühn (Gregg et al., 1991) and because further viruses are conducted to the intestines through the bile duct, following intrahepatic replication (Marcato et al., 1991). In this way, species that ingest the rabbit faeces, such as *A. sylvaticus and M. spretus* (Valverde, 1967), might be infected.

In this study, it has not been possible to ascertain whether the murids with virus presented an immune response, such as that induced in predatory animals or scavengers after ingesting the remains of ill or dead animals (Leighton et al., 1995; Parkes et al., 2004; Simón et al., 1994). Nor has it been possible to prove whether they can suffer from the disease or whether they have some sort of clinical manifestation due to the presence of RHDV, although at the time of their capture none showed any symptoms of the disease. Nevertheless, it would be important to determine the viability of RHDV and how long it can endure in natural murid populations. These data could inform on the intraspecific and interspecific transmission and diffusion of the virus (Merchán et al., in preparation) and on the cycles of occurrence of RHD, which could be influenced by the existence of associated micromammalian communities. It is unknown whether these micromammals have the capability to excrete and transmit the RHD virus, but were this the case, the endurance and spread of RHDV could be affected. Furthermore, as an evolutionary consequence of continuous contact with the virus, the rodents could participate in inducing a certain immune response in some individuals within sympatric rabbit populations, if the latter are infected by carrier mice.

For this reason, the presence of rodent communities in sympatry with the European wild rabbit could facilitate the permanence and generalization of these viral forms. If cross-infection between species were proved conclusively, this could suggest the existence of an ecological relationship of passive

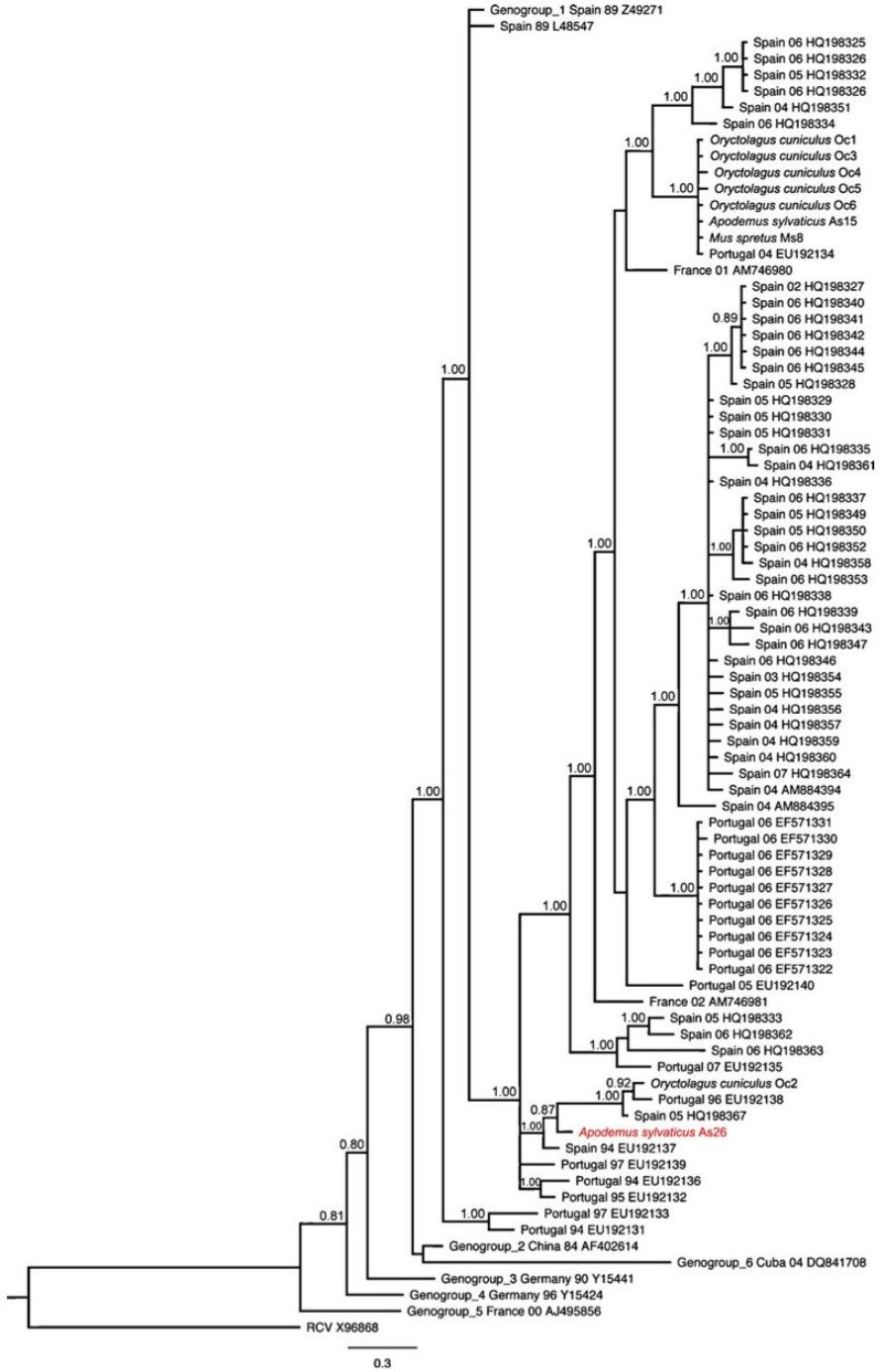


Fig. 1. Phylogenetic tree obtained by Bayesian inference for the partial VP60 gene sequences of RHDV. Numbers above branches indicate posterior probabilities above 0.80 for BI. Year and region of isolation are indicated for all samples obtained from GenBank. The sequences were grouped into genogroup 1–6 adapted to the classification used by Le Gall-Recoulé et al. (2003). RHDV isolated from non-rabbit hosts are indicated and highlighted in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

collaborators in the increase of the rabbits' resistance to RHDV infections.

To conclude, we consider it important to preserve the communities of micromammals in sympatry with the European rabbit in light of the complex relationship that the populations of this species maintain with RHDV. This report clearly manifests the need for further studies to fully determine the potential of micromammals as environmental preservers of RHDV or as transmitters of less fatal varieties of the virus, especially considering the implications on the origin and the dynamics of the disease that this may have.

Acknowledgements

This project was funded by the DGIDTI of Regional Government of Extremadura with file number: 2PR02A035. Animal trapping was authorised by the Government of Extremadura (exp. no. CN04/2328). Sincerest gratitude to the collaborators in the field work Begoña García, Francisco Cid and Francisco Ordiales. Thanks, also, to Elizabeth Nestor for reviewing the English text.

References

Abrantes, J., Esteves, P.J., 2010. Not-so-novel Michigan rabbit calicivirus. Emerg. Infect. Dis. 16, 1331–1332.

Alda, F., Gaitero, T., Suárez, M., Merchán, T., Rocha, G., Doadrio, I., 2010. Evolutionary history and molecular epidemiology of rabbit haemorrhagic disease virus in the Iberian Peninsula and Western Europe. BMC Evol. Biol. 10, 347.

Argüello, J.L., llanos, A., Pérez, L.I., 1988. Enfermedad vírica hemorrágica del conejo en España. Med. Vet. 5, 645–650.

Asgari, S., Hardy, J.R., Sinclair, R.G., Cooke, B.D., 1998. Field evidence for mechanical transmission of rabbit haemorrhagic disease virus (RHDV) by flies (Diptera: Calliphoridae) among wild rabbits in Australia. Virus Res. 54, 123–132.

Bergin, I.L., Wise, A.G., Bolin, S.R., Mullaney, T.P., Kiupel, M., Maes, R.K., 2009. Novel calicivirus identified in rabbits, Michigan, USA. Emerg. Infect. Dis. 15, 1955–1962.

Blanco, J.C., Villafuerte, R., 1993. Factores ecológicos que influyen sobre las poblaciones de conejo: incidencia de la enfermedad hemorrágica. Empresa de Transformación Agraria S.A., Madrid.

Buddle, B.M., de Lisle, G.W., McColl, K., Collins, B.J., Morrissy, C., Westbury, H.A., 1997. Response of the North Island brown kiwi, *Apteryx australis mantellli* and the lesser short-tailed bat, *Mystacina tuberculata* to a measured dose of rabbit haemorrhagic disease virus. N.Z. Vet. J. 45, 109–113.

Calvete, C., Estrada, R., Villafuerte, R., Osacar, J.J., Lucientes, J., 2002. Epidemiology of viral haemorrhagic disease and myxomatosis in a free-living population of wild rabbits. Vet. Rec. 150, 776–782.

Cancellotti, F.M., Renzi, M., 1991. Epidemiology and current situation of viral haemorrhagic disease of rabbits and the European brown hare syndrome. Rev. Sci. Tech. Off. Int. Epiz. 10, 409–421.

Capucci, L., Fusi, P., Lavazza, A., Lodovica, M., Rossi, C., 1996. Detection and preliminary characterisation of a new rabbit calicivirus related to rabbit haemorrhagic disease virus but nonpathogenic. J. Virol. 70, 8614–8623.

Carro, F., Pérez Aranda, D., Lamosa, A., Schmalenberger, H.P., Pardavila, X., Gegundez, M.I., Soriguer, R.C., 2007. Eficiencia de tres tipos de trampas para la captura de micromamíferos. Galemys 19, 73–81.

Castién, E., Gosálbez, J., 2001. Pequeños mamíferos forestales: influencia de las actividades forestales sobre las comunidades de Insectívoros y Roedores. In: Camprodon, J., Plana, E. (Eds.), Conservación de la biodiversidad y gestión forestal: su aplicación en la fauna vertebrada. Edicions de la Universitat de Barcelona, Barcelona, pp. 353–364.

Chasey, D., 1994. Possible origin of rabbit haemorrhagic disease in the United Kingdom. Vet. Rec. 135, 496–499.

Cooke, B.D., 1996. Field epidemiology of rabbit calicivirus disease in Australia. In: ESVV Symposium on Caliciviruses, University of Reading, Reading, September 15–17, 1996.

Cooke, B.D., 2002. Rabbit haemorrhagic disease: field epidemiology and the management of wild rabbit populations. Rev. Sci. Tech. Off. Int. Epiz. 2, 347–358.

Delibes-Mateos, M., Delibes, M., Ferreras, P., Villafuerte, R., 2008. Key role of European rabbits in the conservation of the Western Mediterranean basin hotspot. Conserv. Biol. 22, 1106–1117.

Díaz, M., 1992. Rodent seed predation in cereal crop areas of Central Spain: effects of physiognomy, food availability, and predation risk. Ecography 15, 77–85.

Fenner, F., Fantini, B., 1999. Biological Control of Vertebrate Pests: The History of Myxomatosis an Experiment in Evolution. CABI Publishing, Wallingford.

Forrester, N.L., Trout, R.C., Turner, S.L., Kelly, D., Boag, B., Moss, S., Gould, E.A., 2006. Unravelling the paradox of rabbit haemorrhagic disease virus emergence, using phylogenetic analysis; possible implications for rabbit conservation strategies. Biol. Conserv. 131, 296–306.

Gavier-Widen, D., Morner, T., 1993. Descriptive epizootiological study of European brown hare syndrome in Sweden. J. Wildlife Dis. 29, 15–20.

Gavier-Widen, D., Berndtsson-Treiberg, L., Mejerland, T., Rivera, E., Morner, T., 1997. Experimental infection of silver foxes (*Vulpes vulpes*) and blue foxes (*Alopex lagopus*) with calicivirus of leporids. In: Chasey, D, Gaskell, R.M, Clarke, I.N (Eds.), Proceedings of the 1st International Symposium on Caliciviruses, UK, pp. 172–175.

Gehrmann, B., Kretzschmar, C., 1991. Ein experimentelleler Beitrag zur Epizootiologie der Viralen Hamorrhagischen Septikamie der Kaninchen (rabbit haemorrhagic disease, RHD) Ubertragung durch Fliegen. Berl. Munch. Tierarztl. 104, 192–194.

Gregg, D.A., House, C., Meyer, R., Berninger, M., 1991. Viral haemorrhagic disease of rabbits in Mexico: epidemiology and viral characterization. Rev. Sci. Tech. Off. Int. Epiz. 10, 435–451.

Gould, A.R., Kattenbelt, J.A., Lenghaus, C., Morrissy, C., Chamberlain, T., Collins, B.J., Westbury, H.A., 1997. The complete nucleotide sequence of rabbit haemorrhagic disease virus (Czech strain V351): use of the polymerase chain reaction to detect replication in Australian vertebrates and analysis of viral population sequence variation. Virus Res. 47, 7–17.

Henning, J., Meers, J., Davies, P.R., Morris, R.S., 2005. Survival of rabbit haemorrhagic disease virus (RHDV) in the environment. Epidemiol. Infect. 133, 719–730.

Huelsenbeck, J.P., Ronquist, F., 2005. Bayesian analysis of molecular evolution using MrBayes. In: Nielsen, R. (Ed.), Statistical Methods in Molecular Evolution. Springer, New York, pp. 183–232.

Kerr, P.J., Kitchen, A., Holmes, E.C., 2009. Origin and phylodynamics of rabbit hemorrhagic disease virus. J. Virol. 83, 12129–12138.

Lavazza, A., Scicluna, M.T., Capucci, L., 1996. Susceptibility of hares and rabbits to the European brown hare syndrome virus (EBHSV) and rabbit haemorrhagic disease virus (RHDV) under experimental conditions. Zentralblatt fur Veterinarmedizin, Reihe B. J. Vet. Med. Ser. B 43, 401–410.

Le Gall-Recoulé, G., Zwingelstein, F., Laurent, S., de Boisséson, C., Portejoie, I., Rasschaert, D., 2003. Phylogenetic analysis of rabbit haemorrhagic disease virus in France between 1993 and 2000, and the characterisation of RDHV antigenic variants. Arch. Virol. 148, 65–81.

Leighton, F., Artois, M., Capucci, L., Gavier-Widen, D., Morisse, J., 1995. Antibody response to rabbit viral hemorrhagic disease virus in red foxes (*Vulpes vulpes*) consuming livers of infected rabbits (*Oryctolagus cuniculus*). J. Wildlife Dis. 31, 541–544.

Lenghaus, C., Westbury, H., Collins, B., Ratnamohan, N., Morrissy, C., 1994. Overview of the RHD project in Australia. In: Munro, R.K., Williams, R.T. (Eds.), Rabbit Haemorrhagic Disease: Issues in Assessment for Biological Control, Bureau of Resource Sciences. Australian Government Publishing Service, Canberra, pp. 104–129.

Liu, S.J., Xue, H.P., Pu, B.Q., Qian, N.H., 1984. A new viral disease of rabbits. Anim. Husband. Vet. Med. 16, 253–255.

Löliger, H.C., Eskens, U., 1991. Incidence, epizootiology and control of viral haemorrhagic disease of rabbits and the European brown hare syndrome in Germany. Rev. Sci. Tech. Off. Int. Epiz. 10, 423–430.

Maddison, D.R., Maddison, W.P., 2000. MacClade 4: Analysis of Phylogeny and Character Evolution. Sinauer Associates, Sunderland, MA.

Marcato, P.S., Benazzi, C., Vecchi, G., Galeotti, M., Della Salda, L., Sarli, G., Lucidi, P., 1991. Clinical and pathological features of viral haemorrhagic disease of rabbits and the European brown hare syndrome. Rev. Sci. Tech. 10, 371–392.

McColl, K.A., Morrissy, C.J., Collins, B.J., Westbury, H.A., 2002. Persistence of rabbit haemorrhagic disease virus in decomposing rabbit carcases. Aust. Vet. J. 80, 298–299.

Moreno, S., Beltrán, J.F., Cotilla, I., Kuffner, B., Laffite, R., Jordán, G., Ayala, J., Quintero, C., Jiménez, A., Castro, F., Cabezas, S., Villafuerte, R., 2007. Long-term decline of the European wild rabbit (*Oryctolagus cuniculus*) in south-western Spain. Wildlife Res. 34, 652–658.

Moss, S.R., Turner, S.L., Trout, R.C., White, P.J., Hudson, P.J., Desai, A., Armesto, M., Forrester, N.L., Gould, E.A., 2002. Molecular epidemiology of rabbit haemorrhagic disease virus. J. Gen. Virol. 83, 2461–2467.

Müller, A., Freitas, J., Silva, E., Le Gall-Reculé, G., Zwingelstein, F., Abrantes, J., Esteves, P.J., Alves, P.C., van der Loo, W., Kolodziejek, J., 2009. Evolution of rabbit haemorrhagic disease virus (RHDV) in the European rabbit (*Oryctolagus cuniculus*) from the Iberian Peninsula. Vet. Microbiol. 135, 368–373.

Nowotny, N., Bascunana, C.R., Ballagi-Pordany, A., Gavier-Widen, D., Uhlen, M., Belak, S., 1997. Phylogenetic analysis of rabbit haemorrhagic disease and European brown hare syndrome viruses by comparison of sequences from the capsid protein gene. Arch. Virol. 142, 657–673.

Nowotny, N., Ros Bascunana, C., Ballagi-Pordany, A., Belak, S., Gavier-Widen, D., Uhlen, M., 1999. Phylogeny and variability of rabbit haemorrhagic disease virus, and the present situation of rabbit haemorrhagic disease in Europe. Rabbit control: RCD dilemmas and implications. J. R. Soc. N.Z. Misc. 55, 47–51.

Pagés, A., 1989. Aspectos epidemiológicos y laboratoriales de la enfermedad hemorrágica de los conejos (RHD) en España. Med. Vet. 6, 153–158.

Parkes, J., Heyward, R.P., Henning, J., Motha, M.X.J., 2004. Antibody responses to rabbit haemorrhagic disease virus in predators, scavengers, and hares in New Zealand during epidemics in sympatric rabbit populations. N.Z. Vet. J. 52, 85–89.

Parra, F., Prieto, M., 1990. Purification and characterization of a calicivirus as the causative agent of a lethal haemorrhagic disease in rabbits. J. Virol. 64, 4013–4015.

Posada, D., 2008. jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25, 1253–1256.

Rambaut, A., Drummond, A., 2007. Tracer v1. 4. , http://beast.bio.ed.ac.uk/Tracer.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3 Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Rosell, J.M., Badiola, J.I., Pérez, A., Badiola, J.J., García, J.A., Vargas, M.A., 1989. Enfermedad vírica hemorrágica del conejo. I. Epizootiológica y clínica. Med. Vet. 6, 275–284.

Schirrmeier, H., Granzow, H., Bergmann, H., Schlüter, H., 1990. Experimentelle Untersuchungen zur Hämorrhagischen Septikämie der Kaninchen. Monatsh. Veterinarmed. 45, 193–197.

Simón, M.C., Muguruza, R., Alonso, J.L., Muzquiz, C., Girones, O., Haffar, A., 1994. Recherche du virus de la maladie hemorragique virale du lapin (RHD) chez le renard et role des canides domestiques dans la transmission de la maladie. Rec. Med. Vet. 170, 841–845.

Smid, B., Valicek, L., Rodak, L., Stepanek, J., Jurak, E., 1991. Rabbit haemorrhagic disease: an investigation of some properties of the virus and evaluation of an inactivated vaccine. Vet. Microbiol. 26, 77–85.

Valverde, J.A., 1967. Estructura de una Comunidad de Vertebrados Terrestres. Consejo Superior de Investigaciones Científicas, Madrid.

Villafuerte, R., Calvete, C., Gortázar, C., Moreno, S., 1994. First epizootic of rabbit hemorrhagic disease in free living populations of *Oryctolagus cuniculus* at Doñana National Park, Spain. J. Wildlife Dis. 30, 176–179.

Wirblich, C., Sibilia, M., Boniotti, M.B., Rossi, C., Thiel, H.J., Meyers, G., 1995. 3C-like protease of rabbit hemorrhagic disease virus: identification of cleavage sites in the ORF1 polyprotein and analysis of cleavage specificity. J. Virol. 69, 7159–7168.

Xu, W., Du, N., Liu, S., 1988. A new virus isolated from hemorrhagic disease in rabbits. In: Proc. 4th World Rabbit Cong, Budapest, pp. 456–461.

Xu, Z.J., Chen, W.X., 1989. Viral hemorrhagic disease in rabbits: a review. Vet. Res. Commun. 13, 205–212.

Alda et al. BMC Evolutionary Biology 2010, 10:347
http://www.biomedcentral.com/1471-2148/10/347

BMC Evolutionary Biology

RESEARCH ARTICLE Open Access

Evolutionary history and molecular epidemiology of rabbit haemorrhagic disease virus in the Iberian Peninsula and Western Europe

Fernando Alda[1,2*], Tania Gaitero[3], Mónica Suárez[3], Tomás Merchán[4], Gregorio Rocha[4], Ignacio Doadrio[1]

Abstract

Background: Rabbit haemorrhagic disease virus (RHDV) is a highly virulent calicivirus, first described in domestic rabbits in China in 1984. RHDV appears to be a mutant form of a benign virus that existed in Europe long before the first outbreak. In the Iberian Peninsula, the first epidemic in 1988 severely reduced the populations of autochthonous European wild rabbit. To examine the evolutionary history of RHDV in the Iberian Peninsula, we collected virus samples from wild rabbits and sequenced a fragment of the capsid protein gene VP60. These data together with available sequences from other Western European countries, were analyzed following Bayesian Markov chain Monte Carlo methods to infer their phylogenetic relationships, evolutionary rates and demographic history.

Results: Evolutionary relationships of RHDV revealed three main lineages with significant phylogeographic structure. All lineages seem to have emerged at a common period of time, between ~1875 and ~1976. The Iberian Peninsula showed evidences of genetic isolation, probably due to geographic barriers to gene flow, and was also the region with the youngest MRCA.
Overall, demographic analyses showed an initial increase and stabilization of the relative genetic diversity of RHDV, and a subsequent reduction in genetic diversity after the first epidemic breakout in 1984, which is compatible with a decline in effective population size.

Conclusions: Results were consistent with the hypothesis that the current Iberian RHDV arose from a single infection between 1869 and 1955 (95% HPD), and rendered a temporal pattern of appearance and extinction of lineages. We propose that the rising positive selection pressure observed throughout the history of RHDV is likely mediated by the host immune system as a consequence of the genetic changes that rendered the virus virulent. Consequently, this relationship is suggested to condition RHDV demographic history.

Background

Viruses containing RNA as their genetic material usually have a great capacity to adapt. This is so because the viral polymerases responsible for replicating the genome have a high error rate and therefore, many genomic variants are created at a high generation rate [1,2]. This high mutation rate of RNA viruses makes them excellent models for addressing evolutionary processes such as epidemic invasions, since their ecological and evolutionary dynamics occur at similar time scales [3-5].

RNA viruses are the causal agents of many emerging diseases and consequently the appearance, or reappearance of diseases caused by these viruses is not infrequent [2]. However, other ecological, social, health or behavioural factors besides their high rate of genetic variation can play an important role in the emergence of a disease [6].

Rabbit haemorrhagic disease (RHD) is a recent disease that was detected for the first time in 1984 in China, and attributed to rabbits (*Oryctolagus cuniculus*) imported from the former German Democratic Republic. The disease was described to cause sudden death without apparent deterioration of the rabbit's body condition, although haemorrhaging in the lungs was a

* Correspondence: alda.fernando@gmail.com
[1]Dpto. Biodiversidad y Biología Evolutiva, Museo Nacional de Ciencias Naturales (CSIC). José Gutiérrez Abascal 2, 28006 Madrid, Spain
Full list of author information is available at the end of the article

BioMed Central

© 2010 Alda et al; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Alda et al. BMC Evolutionary Biology 2010, 10:347
http://www.biomedcentral.com/1471-2148/10/347

frequent observation [7,8]. Hereafter, the disease rapidly spread to other Asian [9] and European [10] countries. The first report of RHD in Europe was in Italy in 1986 [11], and already in 1988 it was detected in wild rabbits in Spain [12] and in domestic rabbits in Russia, the Middle East, Africa, America and India [10].

The aetiological agent of RHD is a single stranded positive sense RNA virus belonging to the family Caliciviridae [13,14]. Since its discovery, many research efforts have been devoted to its study. However many issues remain unclear or controversial, such as its evolutionary origin or the causes of the rapid succession of epidemics in such a short period of time [15,16].

Several hypotheses have been proposed to explain the origins of rabbit haemorrhagic disease virus (RHDV), for example: (*i*) spread of brown hare syndrome virus - a closely related calicivirus - to the rabbit [17], (*ii*) changes in the properties of a non pathogenic virus that make it virulent; or (*iii*) the virus arose from a virus infecting another species [18]. The detection of RHDV specific antibodies and RNA fragments in rabbit serum samples from 1955 to 1980 [19-21], however, seem to support the idea that the virus was already circulating in Europe in an avirulent form before the first epidemic was detected in China in 1984 [16].

Several causes have also been proposed to explain the rapid expansion of RHD. Initially it was assumed that China was the origin from where the virus spread causing severe epidemics [18,22]. However, subsequent phylogenetic studies have demonstrated that the Chinese strains of the virus originated in Europe and currently circulating RHDV strains do not have a single origin. Rather, the virus seems to have originated at least twice in the past: firstly in Europe, without causing the disease, and later in the rabbits exported to China [16,23].

These studies have also detected a large number of RHDV evolutionary lineages that show low genetic divergence both within and among phylogenetic groups [17,19,24,25]. Some of these lineages do not present a clear geographic structure, but they do reflect a temporal structure whereby some lineages become extinct or less frequent, while others derived from them are able to persist and cause new outbreaks of the disease [16,17,24]. Despite showing no significant genetic differences in different geographic regions, RHDV does differ in its epidemiology and virulence [15,26-29].

Some regions, notwithstanding the drastic reductions suffered by rabbit populations after a first epidemic, have seen a decline in the initially high virulence of RHDV and this has enabled populations to gradually recover [15,26]. In the Iberian Peninsula, however, where rabbits are of great ecological and economic significance [30,31], RHD still affects many regions in which rabbit populations have been decimated or even extinguished [28,32].

This heterogeneity in the way RHDV affects rabbits in different geographic regions may indicate that there are factors such as the host, climate or population size that determine the epidemiology of the virus [15,28]. Regarding the host, in the Iberian Peninsula, the European rabbit is an autochthonous species. Analyses of mitochondrial and nuclear markers have revealed two highly divergent lineages in the European rabbit within the Iberian Peninsula that correspond to subspecies *O. c. algirus* and *O. c. cuniculus* [33-36]. These two lineages are the result of two divergent populations that evolved separately for a long time in two glacial refugia, one located in the southwest of the Iberian Peninsula and another in the northeast. A post-glacial expansion might have created a contact zone in the center of the Iberian Peninsula [37]. Furthermore, the northern *O. c. cuniculus* expanded outside of the Iberian Peninsula and originated all European rabbits, as well as those of North Africa, America, Australia, New Zealand and all the domestic breeds. This expansion originated an intense bottleneck effect that diminished considerably the genetic diversity in the *O. c. cuniculus* populations outside of the Iberian Peninsula [34,38-40]. Consequently, the rabbit in the Iberian Peninsula shows the largest genetic diversity across the world distribution of the species [34,41]. This genetic distinctiveness might pose different selective pressures and evolutionary constraints for RHDV compared to other regions. However, so far, comparative studies including the two rabbit lineages are scarce [16,42].

The objectives of the present study were: to examine the evolutionary history of RHDV in the Iberian Peninsula and to infer the virus' demographic history, with special focus on its Western European distribution. Given the genetic characteristics of this virus, we would expect to observe: (*i*) a high genetic diversity and (*ii*) a temporal genetic structure due to its high mutation rate. In terms of the possible origin of the virus and disease in the Iberian Peninsula, we could speculate that (*iii*) if a virulent form of the RHDV was introduced, the age of the most recent common ancestor should correspond to the time elapsed since the appearance of the disease, while if the virus already existed in an avirulent form, its age will be older. Also, according to the recent and rapid geographic expansion of RHDV we would expect (*iv*) its demographic history to reflect significant growth of its populations and that (*v*) if the intensity of the epidemics has decreased, or at least the number of hosts, a subsequent decline should also be observed.

Alda et al. BMC Evolutionary Biology 2010, 10:347
http://www.biomedcentral.com/1471-2148/10/347

Methods

Sampling and RNA isolation, amplification and sequencing

Rabbits were collected in Spain between 2003 and 2007 (Additional File 1). About 125 mg of lung tissue were homogenized in 1.25 ml of sterile PBS (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH7.4) and centrifuged at 2500 × *g* for 15 min. The supernatant was collected and stored at -20°C upon RNA extraction.

Viral RNA was extracted from 100 μl of the lung homogenate using TriPure reagent (Roche) following the manufacturer's instructions. A fragment of the major capsid protein gene VP60 was amplified in all samples using the primers RHDV1, RHDV2, RHDV3 and RHDV4 [19].

The primers RHDV1 and RHDV4 were used to RT-PCR amplify a 698 bp fragment corresponding to positions 6096-6794 of the RHDV [43]. The RT-PCR reaction was conducted under the conditions indicated in the AccessQuick RT-PCR System kit (Promega) and the thermocycling program consisted of 45 min at 48°C for cDNA synthesis, followed by 2 min at 95°C, 40 cycles of 1 min at 95°C, 40 s at 64°C, 1 min at 72°C, and a final extension of 5 min at 72°C. Next, we performed a nested PCR with the primers RHDV2 and RHDV3 to amplify a 573 bp fragment corresponding to positions 6135-6719 of the RHDV. The PCR reaction contained 200 μM of dNTPs, 0.2 μM of each primer, 1 μg/μl of BSA, 1 U of *Taq* polymerase (Eppendorf), 2.5 μl of PCR buffer 10X (500 mM KCl, 100 mM Tris-HCl pH8.3, 15 mM Mg^{2+}) and 0.5-2 μl of a 1:100 dilution of the RT-PCR product. The PCR program involved an initial denaturing step of 2 min at 95°C, 40 cycles of 1 min at 95°C, 1 min at 64°C and 1 min at 72°C, and a final extension step of 5 min at 72°C. All the RNA extraction and amplification processes were carried out in a laboratory specifically equipped for this purpose. All reactions included positive and negative controls. PCR products were purified using ExoSAP-IT (GE Healthcare) and sequenced using the BigDye Terminator v3.1 kit (Applied Biosystems) and the primers RHDV2 and RHDV3 in an automated sequencer ABI3730.

Phylogenetic analysis

Chromatograms were checked visually and the consensus sequence was constructed for each sample using Sequencher 4.6 (Gene Codes Corporation). Nucleotide sequences were translated into amino acids using MacClade 4.05 [44] and manually aligned with other homologous sequences available in GenBank.

Recombination has been previously described in RHDV [45,46]. Although this seems to be a rather rare phenomenon that does not adversely affect rate estimations [16], we did not include sequences that had been identified as putative recombinants [16,45,46]. The alignment inferred from all of the available data (AD, n = 151, 563 bp) was subdivided in smaller data sets comprising sequences for the main geographic areas previously analyzed: Germany (GER), France (FRA1), United Kingdom (UK), China (CHI) and Iberian Peninsula (IB) (Additional File 2).

For a wider comparison, we included available data on non-overlapping VP60 sequences which could be of interest because of their temporal or geographic origins. Thus, an alignment of French RHDV isolates, consisting of non-overlapping sequences of 501 bp at the 3' end of the VP60 gene, was built (FRA2, n = 21) (Additional File 2) [24,47].

To evaluate the genetic variability of RHDV for each of these data sets from the main geographic areas we calculated their gene diversity (H_d), nucleotide diversity (π) and number of polymorphic sites (S) using DnaSP 5.10.1 [48].

Phylogenetic relationships among all the RHDV strains (AD, n = 151) were inferred using different phylogenetic methods. As an outgroup we used four rabbit calicivirus (RCV) sequences (X96868, GQ166866, EU871528, NC011704), a seemingly avirulent, antigenically and genetically related to RHDV [49].

The evolutionary model that best fitted our data, according to the Akaike Information Criterion, was calculated in jModelTest 0.1.1 [50]. Firstly, we performed a Maximum Likelihood (ML) analysis, and used the best evolutionary method for our data as obtained in jModelTest 0.1.1, and allowed the program PhyML 3.0 [51] to optimize the tree topology and the values of gamma and proportions of invariant sites. Support for the ML trees was assessed by 1000 bootstrap replicates. Secondly, a Bayesian Inference (BI) analysis was performed using MrBayes 3.1.2 [52], simulating four simultaneous Markov chains (MCMC) for 4×10^6 generations each, and using a sampling frequency of 100 generations. The first 250,000 generations were discarded as burn-in. The BI analysis was performed considering one and three partitions of the data corresponding to the 1^{st}, 2^{nd} and 3^{rd} codon positions each assigned different substitutions models. Bayesian posterior probabilities were obtained to assess the robustness of the BI trees.

Lastly, to test for the presence of statiscally significant geographical clustering in RHDV, we performed randomization tests on three tree-shaped statistics: the parsimony score (PS), the association index (AI) and the monophyletic clade statistic (MC) using BaTS 1.0 [53]. Randomizations were performed across the posterior distribution of trees obtained from MrBayes 3.1.2, hence accounting for phylogenetic uncertainty.

Alda et al. BMC Evolutionary Biology 2010, 10:347
http://www.biomedcentral.com/1471-2148/10/347

Estimation of evolutionary rates, dates and past population demographic history

The overall population dynamics of RHDV (AD) was inferred in BEAST 1.5.3 [54] using the coalescent Bayesian Skyline Model [55] and an uncorrelated lognormal relaxed clock. Four independent analyses were performed under the best-fit substitution model and the SRD06 partition model, recommended for protein coding genes [54,56], and run for 5×10^7 generations and a sampling frequency of 1000 steps. All output generated were analyzed in Tracer 1.5 to test for convergence and mixing, and were used to estimate the substitution rate and time to the most recent common ancestor (tMRCA) of RHDV for each of the major lineages found and all the geographic regions.

We explored the changes through time of the relative genetic diversity ($N_e\tau$, where τ is the average generation time) of RHDV using the Bayesian Skyline Plot (BSP) method [55]. Median and 95% highest posterior density intervals (HPD) were obtained with Tracer 1.5.

We also tested for demographic changes using summary statistic based methods. The effective population size parameter (θ_0) under a growth-decline population model (θ_1 fixed at 1,000,000) was calculated for RHDV in DnaSP 5.10.1. Three time periods were defined based on the results obtained in the BSP analysis: avirulent period (before 1984), epidemic breakout (after 1984) and recent period (after 2000). Because samples sizes differed among time periods and, consequently, effective population size estimates might be biased, we constructed for the "after 1984" and "after 2000" periods 10 data sets with 9 random samples. These results were averaged and compared with the minimum sample size of RHDV before 1984.

Selection analyses

To estimate selection pressures before and after the first epidemic of RHDV in 1984, we calculated the ratio between non-synonymous (d_N) and synonymous (d_S) substitutions for each individual codon using the fixed-effects likelihood (FEL) and random effects likelihood (REL) methods [57]. These analyses were performed in the online package Datamonkey [58]. Absence of recombination points was checked using GARD [59]. We established a value of $\alpha = 0.1$ for FEL and BF = 20 for REL. In both cases, the d_N/d_S ratio was calculated using a neighbour-joining tree based on the best-fit evolutionary model.

As indicated above, to account for the effect of different sample sizes in the analysis, we analyzed 10 data sets with 9 random samples isolated after 1984, and compared with the results from RHDV isolated before the first epidemic.

Results

RHDV was detected in 47 wild rabbits from Spain and it was possible to obtain a 563 bp sequence from each sample. All the sequences were analyzed unambiguously and we detected no evidence of infection by more than one viral strain in the same individual (GenBank accession numbers: HQ198325-HQ198371, Additional File 1).

Along with the homologous sequences available in GenBank, we constructed an alignment of 151 sequences (AD). From this alignment we extracted the data sets for Iberian Peninsula (IB, $n = 71$), France (FRA1, $n = 8$), United Kingdom (UK, $n = 49$), Germany (GER, $n = 7$) and China (CHI, $n = 10$). The alignment of non overlapping sequences from France (FRA2) contained 21 sequences isolated between 1988 and 2003 (Additional File 2).

The 151 RHDV sequences analyzed constituted 122 unique haplotypes ($H_d = 0.993 \pm 0.003$). The geographic regions that showed the highest genetic diversity were France and Germany. In contrast, among the 71 Iberian samples we found 45 haplotypes, which represented the lowest RHDV diversity ($H_d = 0.968 \pm 0.010$) (Additional File 3).

The best evolutionary model estimated by jModelTest 0.1.1 for our data was TrN+I+G. The gamma shape parameter was G = 0.574 and the proportion of invariable sites I = 0.172.

The phylogenetic methods used rendered congruent topologies (Figure 1). Both analyses indicated three main lineages (Lineage I, II and III) in which most of the RHDV samples were included. Lineage I included RHDV strains isolated in different European regions (Figure 1), some of which had been isolated in the UK before the description of the disease in 1984 and others isolated during the first outbreaks in France, Germany and Spain (AST/89 and MC-89). Also, this lineage included all the Iberian RHDV samples isolated in Spain and Portugal from 1994 to 2007 (Figure 1). Six Iberian clades were described based on previous findings (Genogroup 1 [24], IB1, IB2 and IB3 [42]) and the new isolates from this study (IB4, IB5, IB6). No clear geographic structure was observed among the Iberian samples, although most of the clades were restricted in time, with the exception of IB3 that was the most widespread clade both in time and space (Figure 1, Additional File 1). In Lineage II, we exclusively found European strains of RHDV, mainly from the UK, Germany and France. Conversely, Lineage III included strains from different continents. German strains were found at a basal position with respect to all the Chinese strains, except a sample from 1984, which seemed more related to those of Lineage I and II, but without bootstrap or posterior probability support. This lineage also contained samples from the United States, France and Reunion Island in the Indian Ocean. Conversely, several RHDV strains from different

Alda *et al. BMC Evolutionary Biology* 2010, **10**:347
http://www.biomedcentral.com/1471-2148/10/347

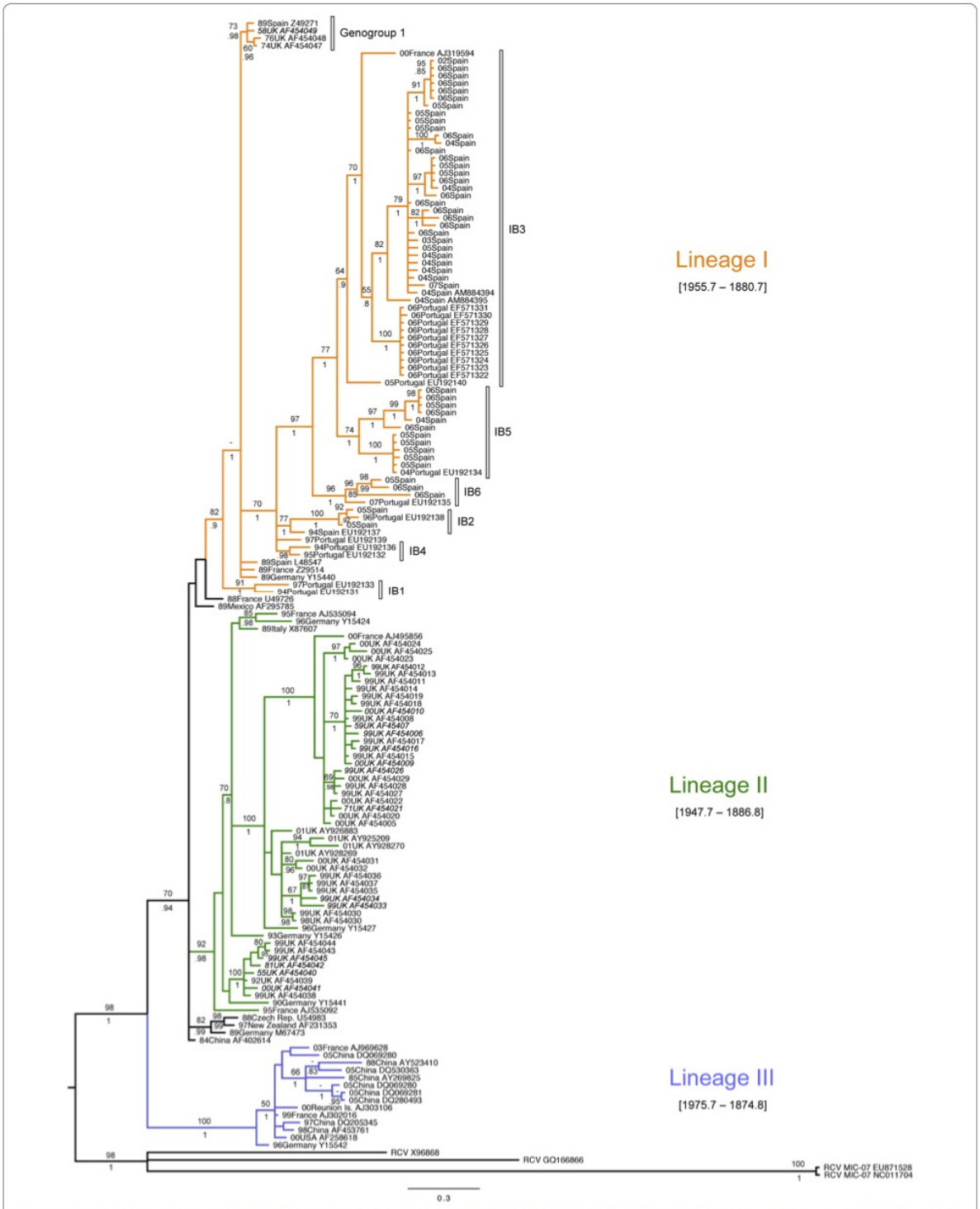


Figure 1 Phylogenetic tree obtained by Bayesian inference for all the RHDV strains analyzed. Numbers above branches indicate bootstrap values above 50 for ML analysis, and posterior probabilities above 0.80 for BI are shown below branches. Year and region of isolation is indicated for all samples. 95% HPD for the tMRCA of the main lineages found are indicated. Iberian clades within Lineage I are shown based on previous studies [24,42] and new data. Names in italics indicate avirulent RHDV strains.

Alda et al. BMC Evolutionary Biology 2010, 10:347
http://www.biomedcentral.com/1471-2148/10/347

regions (France, Germany, Czech Republic, China, Mexico and New Zealand) and strains prior to 1989 were not included in any of the three main lineages identified (Figure 1).

Overall, the test performed for the presence of phylogeographic structure using the PS and AI statistics, strongly rejected the hypothesis of panmixia (observed PS of 21.986, expected PS of 71.718 [$P < 0.0001$]; observed AI of 1.133, expected AI of 10.175 [$P < 0.0001$]). Furthermore, the MC statistic indicated that the correlation between phylogeny and taxa location was only significant for the samples from the Iberian Peninsula (observed MC_{IB} of 41.046, expected MC_{IB} of 3.681 [$P = 0.01$]), UK (observed MC_{UK} of 21.040, expected MC_{UK} of 2.757 [$P = 0.01$]) and China (observed MC_{CHI} of 6.354, expected MC_{CHI} of 1.175 [$P = 0.01$]), that appeared exclusively in Lineages I, II and III, respectively, with the exception of the "ancient" strains from the UK that were found in Lineage I. On the other hand, the RHDV isolates from France and Germany that appeared in all lineages, and in many cases were closely related to strains from other regions (Figure 1), did not show a significant geographic correlation.

The coalescence based Bayesian analysis implemented in BEAST 1.5.3 revealed that the MRCA for all the RHDV data dated back to ~1884 (95% HPD 1941.3-1730.7) and estimated an evolutionary rate of 5.48×10^{-4} substitutions/site/year (95% HPD 2.79×10^{-4}-8.10×10^{-4}). The three main clades found showed similar 95% HPD intervals for their MRCA, from ~1875 to ~1976 (Figure 1), suggesting a close time of emergence for these lineages.

When the main geographic regions were considered, the youngest tMRCA was recovered for the Iberian RHDV (~1936, 95% HPD 1955.7-1869.2) followed by the isolates from the UK (~1898, 95% HPD 1940.3-1822.5). The remnant regions showed wider 95% HPD intervals that were similar to the age of the complete RHDV data set (Figure 2).

Bayesian Skyline Plot analysis of the demographic history of RHDV indicated that, as a whole, the relative genetic diversity of RHDV increased in the 1950's followed by a stationary phase until the mid 1980's. Thereafter, RHDV relative genetic diversity started to decline, especially in the late 1990's when this reduction was more drastic (Figure 3). Because samples were not balanced across years, the same analysis was performed removing those sequences dated before 1984 and presumably avirulent strains [15]. The plot recovered showed the same demographic and temporal pattern, although the stationary phase was not as clearly differentiated (data not shown).

The effective population size parameter θ_0 estimated for the three main periods identified in the BSP also

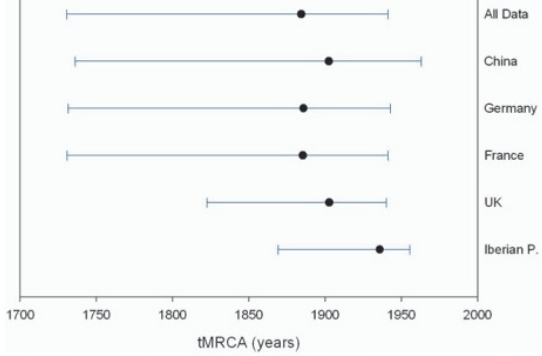


Figure 2 Estimates of the tMRCA for the main geographical regions analyzed. Dots indicate median tMRCA values and blue lines represent 95% HPD.

showed evidence of change in N_e of RHDV across time. An increase of N_e was observed between the avirulent period, before 1984 (θ_0 = 11.597), and the samples after the first epidemic breakout, after 1984 (θ_0 = 20.620 ± 2.407, average value based on 10 data sets of 9 random sequences). Subsequently, a reduction of N_e was observed for the most recent isolates after 2000 (θ_0 = 14.713 ± 5.812).

Adaptive selection analyses suggested an increase in the selective pressure of RHDV after the first epidemic breakout (Table 1). Before 1984 no codons were identified as positively selected by any method, and 29 codons were considered under negative selection by FEL, but not by the REL method. On the other hand, after 1984, codon 137 of the alignment was identified as positively selected considering all the data after 1984 and in 9 out

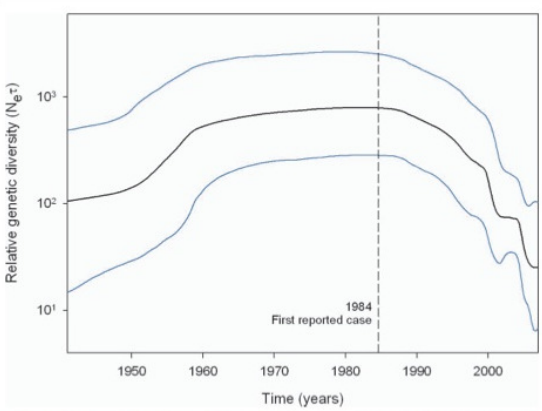


Figure 3 Bayesian Skyline plot analysis of RHDV. Graphical representation of relative genetic diversity ($N_e\tau$) changes across time of RHDV. Black line indicates the median and blue lines represent 95% HPD.

Alda et al. BMC Evolutionary Biology 2010, 10:347
http://www.biomedcentral.com/1471-2148/10/347

Table 1 Adaptive selection analyses (FEL and REL) of RHDV performed in Datamonkey

	d_N/d_S	FEL		REL	
		Positive selected	Negative selected	Positive selected	Negative selected
Before 1984					
All data	0.173	0	29	0	0
After 1984					
All data	0.162	1	100	N/A	N/A
Average (10 data sets)	0.158 ± 0.017	1.600 ± 0.843	49.500 ± 5.126	2.400 ± 5.542	24.200 ± 28.389

of 10 random data sets analysed. Other codons (e.g. codon 155) were identified as positively selected, but not consistently among the random data sets. The FEL method also detected 100 negatively selected codons in all the samples after 1984 and 49.500 ± 5.126 codons under negative selection considering the 10 random data sets (Table 1).

Discussion and Conclusion

Genetic variability and origin of RHDV in the Iberian Peninsula

Although haplotype diversity was high and similar among regions, the nucleotide diversity of the RHDV strains varied among geographic regions. In the Iberian Peninsula, UK and China, nucleotide diversity was much lower than in France or Germany, and was equivalent to half the overall nucleotide diversity (Additional File 3). The low genetic diversity observed in these regions could suggest that the virus originated from a single introduction (e.g. as it is known to have occurred in China [7]), or that it is the outcome of geographic isolation, (e.g.the case of the British Isles). Thus, the genetic diversity of the virus in these regions could have been reduced as a consequence of a founder event in the former case, or of drift in the latter [60].

To date in the Iberian Peninsula, it was unknown if (*i*) RHDV was already circulating in an avirulent form, (*ii*) there was a single introduction of RHDV or (*iii*) subsequent contact with other strains occurred. The phylogenetic data obtained here indicate that all the field strains of RHDV in the Iberian Peninsula have a common ancestor and are closely related to strains AST/89 and MC-89 isolated during the first RHD outbreaks in Spain (Figure 1). The age of the MRCA of the Iberian strains is the youngest of all the regions analyzed (Figure 2), but still predates the first RHD outbreak in 1984 (~1936, 95% HPD: 1955.7-1869.2). Hence, it may be deduced that the virus introduced in the Iberian Peninsula came from a more ancient lineage already circulating in Europe. This fact was confirmed by the samples included in Lineage I together with AST/89 and MC-89, isolated in different regions of Europe since 1958 (Figure 1).

Dispersal of RHDV is fast and effective in short distances [61] because it is primarily transmitted by direct contact between sick animals or indirectly by dead animals or contaminated food [8]. However, long distances or geographic barriers, such as those existing in Great Britain or the Iberian Peninsula, can affect its dispersal [42]. The transport of rabbits or their products can also play an important role in passive dispersal of the virus [18]. According to the lack of any geographic structure of the Iberian strains (Additional File 1), it seems that movement of rabbits could have promoted RHDV dispersal within the Iberian Peninsula [32,62]. However, considering the significant global geographic association of all the Iberian strains, this is probably not the case for the import and export of rabbits abroad. Only one viral strain isolated in France in 2000 was found in the Iberian RHDV group (Figure 1) [42], which could have crossed the mountains transported by insects or the wind [63,64]. Therefore, the Pyrenees might also represent an effective barrier for RHDV, as it has been shown for many other organisms [65].

Owing to the rapid evolutionary rate of RNA viruses, their molecular phylogenies reveal both spatial and temporal patterns [4]. In France, six genetic groups have been described for RHDV that are mostly consistent with their dates of isolation but not their geographical location [24,47]. In the Iberian Peninsula, some clades spanned a wide range of time isolates, but most were restricted in time (Figure 1, Additional File 1). For example, clade IB1 was basal to all the other Iberian clades, including those strains isolated in 1989. Although this relationship was not supported by the ML analysis, it might indicate that, in the past, other related strains might have occurred (e.g. Genogroup 1, IB1, IB4) but are currently extinct. This temporal pattern, whereby lineages successively become extinct and others appear, is typical of RNA viruses [66,67], including calicivirus [68], and is mainly conditioned by positive selection [69].

Phylogenetic relationships of RHDV

In general, the phylogenetic relationships resolved here are in line with the findings of studies that have analyzed different portions of the genome or geographic regions [15-17,19,23,24,42,47].

Three divergent groups were recovered from our phylogenetic reconstruction (Figure 1). These three groups

Alda et al. BMC Evolutionary Biology 2010, 10:347
http://www.biomedcentral.com/1471-2148/10/347

(Lineages I, II and III) closely matched the lineages obtained in a phylogeny based on 43 complete sequences of the VP60 gene, in which two major lineages were described, one of which was divided in two well supported groups [45]. Thus, Lineages I, II and III described here would correspond to groups Ia, Ib and II respectively recovered in the phylogeny based on the complete VP60 gene sequence [45]. However, in other works in which partial sequences of the VP60 gene were examined, a much larger number of RHDV groups were defined, many of which are not supported [15,19,42,70,71].

As explained above, the genetic structure of RHDV has usually been described as being related to its year of isolation [17,19,24,41] and within temporal groups, a link to its place of origin emerges [41]. In our data, however, we observed a global geographical distribution of the lineages rather than a temporal structure of RHDV sequences (Figure 1).

The only lineage that did not contain samples before the first RHD outbreak was Lineage III, and we could consider that all the RHDV samples in this lineage arose from already virulent strains. On the other hand, Lineage I and II included either samples isolated before the first outbreak or avirulent strains. However, these lineages were not older than Lineage III. All lineages showed similar tMRCA estimates, suggesting a common time of emergence for all of them, between ~1875 and ~1976. This observed emergence pre-dates the documented emergence of RHD, a possibility that Kerr and colleagues had already suggested [16].

The RHDV strains from the Iberian Peninsula, UK and China appeared, almost exclusively, in Lineages I, II and III respectively. However, other strains such as those from France and Germany appeared in all the main clades (Figure 1). The presence of strains from different evolutionary lineages in the same region could be attributable to a greater extent to ancestral polymorphism and/or to higher flow of viruses in certain areas of central Europe [16], compared to more isolated regions such as the Iberian Peninsula and the British Isles. Furthermore, these isolated regions were those with the youngest tMRCA (Figure 2), suggesting that RHDV might have originated in continental Eurasia [16] and subsequently colonized the islands and southern peninsulas. However, different sampling sizes might affect the accuracy of these estimates, and conclusions should be drawn with caution.

Demographic history and natural selection

The substitution rate estimated for the complete RHDV data set (5.48×10^{-4} substitutions/site/year) was very similar to that reported recently for a similar data set (7.7×10^{-4} substitutions/site/year, 95% HPD: 3.9×10^{-4} - 11.0×10^{-4} [16]). However, these rates were more than an order of magnitude lower than the rate estimated for the same gene region using a ML method (1.3×10^{-3} substitutions/site/year, 0.59×10^{-3} - 2.1×10^{-3} [72]), and more than two orders of magnitude lower than the rate estimated for the capsid gene of other calicivirus [68]. Although the estimated substitution rate is within the range observed for single stranded RNA viruses, it more closely resembles the lower substitution rates recorded for viruses transmitted by arthropod vectors [72,73]. The relatively low substitution rate of this virus could therefore explain the long term persistence of certain strains in some populations many years after their introduction [74].

The BSP method, which allows the fitting of different demographic scenarios across time [55], distinguished three stages in the history of RHDV. First, a growth stage between 1950 and 1960; second, a stationary stage between 1960 and 1984 and finally a stage of decline beyond 1984 that became steeper at the end of the 1990s (Figure 3). The different demographic trends before and after the description of the first RHD outbreak match the different patterns of natural selection for each time range. Thus, during the growth and stationary stages, we detected no evidence of significant positive selection, whereas after 1984 evidence did emerge of positive selection, as well as an increase in the number of codons under negative selection over the VP60 fragment analyzed (Table 1).

In our data, codon 137, which is located in region E of the major capsid gene VP60, showed the strongest evidence of positive selection. The presence of positive selection in this region agrees with previous studies of RHDV and other calicivirus and with the fact that this region contains the main antigenic determinants [68,75]. Furthermore, the association between positive selection and antigenicity suggests that RHDV evolution is mainly driven by the host immune response [75].

Thus, the great virulence that characterized the first outbreaks of RHD would have caused a strong immune response in the rabbit, generating great selective pressure. In this way and as indicated by the selection analyses performed [75], there is ongoing selective pressure on certain RHDV strains, and consequently the fixation of favourable mutations or the purging of deleterious variants would result in the strong decrease in the relative genetic diversity recently observed for the virus [76,77].

Additional material

Additional file 1: MCC tree obtained in Beast for Lineage I and time span for each of the Iberian clades. Map and list of the Iberian samples analyzed in this study.

Additional file 2: Origin and year of isolation of RHDV samples analyzed in this study.

Alda *et al. BMC Evolutionary Biology* 2010, **10**:347
http://www.biomedcentral.com/1471-2148/10/347

Additional File 3: Descriptive statistics of the genetic variability in RHDV.

Acknowledgements
The authors wish to thank F. Guil and the members of Fundación CBD-Habitat for their help providing samples. L. Alcaraz assisted with the laboratory work and A. Burton, M. Díez-León and A. Christofferson reviewed the English text. We also thank R. Zardoya for helpful discussions and three anonymous referees for their valuable suggestions. FA benefitted from a FPU pre-doctoral grant from the Spanish Ministry of Education and Science. This study was funded by projects MAM/2484/2002-65/2002 and 010203030003.

Author details
[1]Dpto. Biodiversidad y Biología Evolutiva, Museo Nacional de Ciencias Naturales (CSIC). José Gutiérrez Abascal 2, 28006 Madrid, Spain. [2]Instituto de Investigación en Recursos Cinegéticos (CSIC-UCLM-JCCM). Ronda de Toledo s/n, 13071 Ciudad Real, Spain. [3]Dpto. Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid. Avda. Puerta de Hierro s/n, 28040 Madrid, Spain. [4]Dpto. Ingeniería del Medio Agronómico y Forestal, Universidad de Extremadura. Av. Virgen del Puerto 2, 10600 Plasencia, Spain.

Authors' contributions
FA conceived the study, obtained and analyzed the molecular data and wrote the manuscript. TG and MS designed laboratory protocols and obtained molecular data. TM and GR collected samples and molecular data. ID provided funding and logistic support for the study. The final draft was read and approved by all the authors.

Received: 5 June 2010 Accepted: 10 November 2010
Published: 10 November 2010

References

1. Domingo E, Martinez-Salas E, Sobrino F, de la Torre JC, Portela A, Ortin J, Lopez-Galindez C, Perez-Brena P, Villanueva N, Najera R, *et al*: **The quasispecies (extremely heterogeneous) nature of viral RNA genome populations: biological relevance - a review.** *Gene* 1985, **40(1)**:1-8.
2. Holmes EC: **Error thresholds and the constraints to RNA virus evolution.** *Trends Microbiol* 2003, **11**:543-546.
3. Biek R, Drummond AJ, Poss M: **A virus reveals population structure and recent demographic history of its carnivore host.** *Science* 2006, **24**:845-852.
4. Holmes EC: **The phylogeography of human viruses.** *Mol Ecol* 2004, **13(4)**:745-756.
5. Real LA, Henderson JC, Biek R, Snaman J, Jack TL, Childs JE, Stahl E, Waller LA, Tinline R, Nadin-Davis S: **Unifying the spatial population dynamics and molecular evolution of epidemic rabies virus.** *Proc Natl Acad Sci USA* 2005, **102(34)**:12107-12111.
6. Nichol ST, Arikawa J, Kawaoka Y: **Emerging viral diseases.** *Proc Natl Acad Sci USA* 2000, **97(23)**:12411-12412.
7. Liu SJ, Xue HP, Pu BQ, Quian NH: **A new viral disease in rabbit.** *Anim Husbandry Vet Med* 1984, **16**:253-255.
8. Xu ZJ, Chen WX: **Viral haemorrhagic disease in rabbits: a review.** *Vet Res Commun* 1989, **13**:205-212.
9. Park JH, Kida H, Ueda K, Ochiai K, Goryo M, Itakura C: **Etiology of rabbit haemorrhagic disease spontaneously occurring in Korea.** *J Vet Med B Infect Dis Vet Public Health* 1991, **38**:749-753.
10. Morisse JP, Le Gall G, Boilletot E: **Hepatitis of viral origin in Leporidae: introduction and aetiological hypotheses.** *Rev Sci Tech Off Int Epiz* 1991, **10**:283-295.
11. Cancellotti FM, Renzi M: **Epidemiology and the current situation of viral haemorrhagic disease of rabbits and the European brown hare syndrome in Italy.** *Rev Sci Tech Off Int Epiz* 1991, **10**:409-422.
12. Argüello JL, Llano A, Pérez-Ordoyo García LL: **Enfermedad vírica hemorrágica del conejo en España.** *Med Vet* 1998, **5(12)**:645-650.
13. Ohlinger VF, Haas B, Meyers G, Weiland F, Thiel HJ: **Identification and characterization of the virus causing rabbit haemorrhagic disease.** *J Virol* 1990, **64**:3331-3336.
14. Ohlinger VF, Thiel HJ: **Identification of the viral haemorrhagic disease virus of rabbits as a calicivirus.** *Rev Sci Tech Off Int Epiz* 1991, **10**:311-323.
15. Forrester NL, Trout RC, Turner SL, Kelly D, Boag B, Moss S, Gould EA: **Unravelling the paradox of rabbit haemorrhagic disease virus emergence, using phylogenetic analysis; possible implications for rabbit conservation strategies.** *Biol Cons* 2006, **131(2)**:296-306.
16. Kerr PJ, Kitchen A, Holmes EC: **Origin and phylodynamics of rabbit hemorrhagic disease virus.** *J Virol* 2009, **83(2)**:12129-12138.
17. Nowotny N, Bascunana CR, Ballagi-Pordany A, Gavier-Widen D, Uhlen M, Belak S: **Phylogenetic analysis of rabbit haemorrhagic disease and European brown hare syndrome viruses by comparison of sequences from the capsid protein gene.** *Arch Virol* 1997, **142(4)**:657-673.
18. Fenner F, Fantini B: **Biological control of vertebrate pests: the history of myxomatosis; an experiment in evolution.** Wallingford: CABI Publishing;, 1 1999.
19. Moss SR, Turner SL, Trout RC, White PJ, Hudson PJ, Desai A, Armesto M, Forrester NL, Gould EA: **Molecular epidemiology of rabbit haemorrhagic disease virus.** *J Gen Virol* 2002, **83(10)**:2461-2467.
20. Rodak L, Smid B, Valicek L, Vesely T, Stepanek J, Hampl J, Jurak E: **Enzyme-linked immunosorbent assay of antibodies to Rabbit Haemorrhagic Disease Virus and determination of its major structural proteins.** *J Gen Virol* 1990, **71(5)**:1075-1080.
21. Trout RC, Chasey D, Sharp G: **Seroepidemiology of rabbit haemorrhagic disease (RHD) in wild rabbits (*Oryctolagus cuniculus*) in the United Kingdom.** *J Zool* 1997, **243**:846-853.
22. Angulo E, Cooke B: **First synthesize new viruses then regulate their release? The case of the wild rabbit.** *Mol Ecol* 2002, **11(12)**:2703-2709.
23. Forrester NL, Abubakr MI, Abu Elzein EME, al-Afaleq AI, Housawi FMT, Moss SR, Turner SL, Gould EA: **Phylogenetic analysis of rabbit haemorrhagic disease virus strains from the Arabian Peninsula: Did RHDV emerge simultaneously in Europe and Asia?** *Virology* 2006, **344(2)**:277-282.
24. Le Gall-Reculé G, Zwingelstein F, Laurent S, de Boisseson C, Portejoie Y, Rasschaert D: **Phylogenetic analysis of rabbit haemorrhagic disease virus in France between 1993 and 2000, and the characterisation of RHDV antigenic variants.** *Arch Virol* 2003, **148**:65-81.
25. Matiz K, Ursu K, Kecskemeti S, Bajmocy E, Kiss I: **Phylogenetic analysis of rabbit haemorrhagic disease virus (RHDV) strains isolated between 1988 and 2003 in eastern Hungary.** *Arch Virol* 2006, **151(8)**:1659-1666.
26. Lugton IW: **A cross-sectional study of risk factors affecting the outcome of rabbit haemorrhagic disease virus release in New South Wales.** *Aust Vet J* 1999, **77**:322-328.
27. Villafuerte R, Calvete C, Gortázar C, Moreno S: **First epizootic of rabbit hemorrhagic disease in free living populations of *Oryctolagus cuniculus* at Doñana National Park, Spain.** *J Wildl Dis* 1994, **30(2)**:176-179.
28. Calvete C: **Modeling the effect of population dynamics on the impact of rabbit hemorrhagic disease.** *Conserv Biol* 2006, **20(4)**:1232-1241.
29. Parkes J, Norbury GL, Heyward RP, Sullivan G: **Epidemiology of rabbit haemorrhagic disease (RHD) in the South Island, New Zealand, 1997-2001.** *Wildl Res* 2002, **29**:543-555.
30. Delibes-Mateos M, Redpath SM, Angulo E, Ferreras P, Villafuerte R: **Rabbits as a keystone species in southern Europe.** *Biol Cons* 2007, **137(1)**:149-156.
31. Ferrer M, Negro JJ: **The near extinction of two large European predators: super specialists pay a price.** *Conserv Biol* 2004, **18(2)**:344-349.
32. Delibes-Mateos M, Ferreras P, Villafuerte R: **Rabbit populations and game management: the situation after 15 years of rabbit haemorrhagic disease in central-southern Spain.** *Biodivers Conserv* 2008, **17**:559-574.
33. Branco M, Ferrand N, Monnerot M: **Phylogeography of the European rabbit (*Oryctolagus cuniculus*) in the Iberian Peninsula inferred from RFLP analysis of the cytochrome b gene.** *Heredity* 2000, **85(4)**:307-317.
34. Ferrand N, Branco M: **The evolutionary history of the European rabbit (*Oryctolagus cuniculus*): Major patterns of population differentiation and geographic expansion inferred from protein polymorphism.** In *Phylogeography of European Refugia.* Edited by: Weiss S, Ferrand N. Netherlands: Springer; 2007:207-235.
35. Geraldes A, Ferrand N, Nachman MW: **Contrasting patterns of introgression at X-linked loci across the hybrid zone between subspecies of the European rabbit (*Oryctolagus cuniculus*).** *Genetics* 2006, **173(2)**:919-933.
36. Queney G, Ferrand N, Weiss S, Mougel F, Monnerot M: **Stationary distributions of microsatellite loci between divergent population groups**

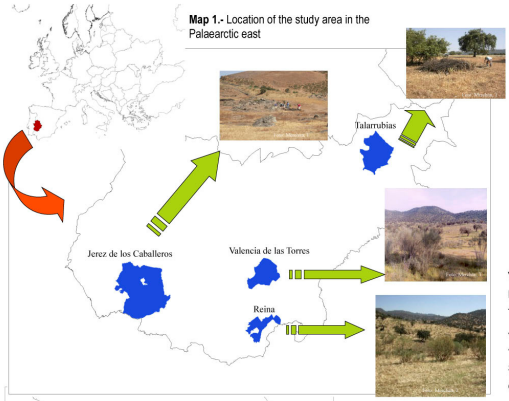
of the European rabbit (*Oryctolagus cuniculus*). *Mol Biol Evol* 2001, **18(12)**:2169-2178.
37. Branco M, Monnerot M, Ferrand N, Templeton AR: **Postglacial dispersal of the European rabbit (*Oryctolagus cuniculus*) on the Iberian Peninsula reconstructed from nested clade and mismatch analyses of mitochondrial DNA genetic variation.** *Evolution* 2002, **56(4)**:792-803.
38. Esteves PJ, Lanning D, Ferrand N, Knight KL, Zhai SK, van der Loo W: **Allelic variation at the V_Ha locus in natural populations of rabbit (*Oryctolagus cuniculus*, L.).** *Journal of Immunology* 2004, **172(2)**:1044-1053.
39. Queney G, Vachot AM, Brun JM, Dennebouy N, Mullsant P, Monnerot M: **Different levels of human intervention in domestic rabbits: Effects on genetic diversity.** *J Hered* 2002, **93(3)**:205-209.
40. van der Loo W, Mougel F, Sanchez MS, Bouton C, Castien E, Fonseca A, Ferrand N, Soriguer R, Monnerot M: **Cytonuclear disequilibria in wild populations of rabbit (*Oryctolagus cuniculus* L.) suggest unequal allele turnover rates at the b locus (IGKC1).** *Immunogenetics* 1999, **49(7-8)**:629-643.
41. Queney G, Ferrand N, Marchandeau S, Azevedo M, Mougel F, Branco M, Monnerot M: **Absence of a genetic bottleneck in a wild rabbit (*Oryctolagus cuniculus*) population exposed to a severe viral epizootic.** *Mol Ecol* 2000, **9(9)**:1253-1264.
42. Muller A, Freitas J, Silva E, Le Gall-Reculé G, Zwingelstein F, Abrantes J, Esteves PJ, Alves PC, van der Loo W, Kolodziejek J: **Evolution of rabbit haemorrhagic disease virus (RHDV) in the European rabbit (*Oryctolagus cuniculus*) from the Iberian Peninsula.** *Vet Microbiol* 2008, **135(3-4)**:368-373.
43. Rasschaert D, Huguet S, Madelaine M, Vautherot JF: **Sequence and genomic organization of a rabbit hemorrhagic disease virus isolated from a wild rabbit.** *Virus Genes* 1995, **9**:121-132.
44. Maddison DR, Maddison WP: **MacClade 4: Analysis of phylogeny and character evolution.** Sunderland, MA: Sinauer Associates; 2000.
45. Abrantes J, Esteves PJ, van der Loo W: **Evidence for recombination in the major capsid gene VP60 of the rabbit haemorrhagic disease virus (RHDV).** *Arch Virol* 2008, **153(2)**:329-335.
46. Forrester NL, Moss SR, Turner SL, Schirrmeier H, Gould EA: **Recombination in rabbit haemorrhagic disease virus: possible impact on evolution and epidemiology.** *Virology* 2008, **376**:390-396.
47. Le Gall-Reculé G, Arnauld C, Boilletot E, Morisse JP, Rasschaert D: **Molecular epidemiology of rabbit haemorrhagic disease virus outbreaks in France during 1988 to 1995.** *J Gen Virol* 1998, **79**:11-19.
48. Librado P, Rozas J: **DnaSP v5: A software for comprehensive analysis of DNA polymorphism data.** *Bioinformatics* 2009, **25**:1451-1452.
49. Capucci L, Fusi P, Lavazza A, Pacciarini ML, Rossi C: **Detection and preliminary characterization of a new rabbit calicivirus related to rabbit hemorrhagic disease virus but non pathogenic.** *J Virol* 1996, **70**:8614-8623.
50. Posada D: **jModelTest: Phylogenetic Model Averaging.** *Mol Biol Evol* 2008, **25(7)**:1253-1256.
51. Guindon S, Gascuel O: **PhyML: A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood.** *Syst Biol* 2003, **52(5)**:696-704.
52. Ronquist F, Huelsenbeck JP: **MrBayes 3: Bayesian phylogenetic inference under mixed models.** *Bioinformatics* 2003, **19**:1572-1574.
53. Parker J, Rambaut A, Pybus OG: **Correlating viral phenotypes with phylogeny: Accounting for phylogenetic uncertainty.** *Infection Genetics and Evolution* 2008, **8**:239-246.
54. Drummond AJ, Rambaut A: **BEAST: Bayesian evolutionary analysis by sampling trees.** *BMC Evol Biol* 2007, **7**:214.
55. Drummond AJ, Rambaut A, Shapiro B, Pybus OG: **Bayesian coalescent inference of past population dynamics from molecular sequences.** *Mol Biol Evol* 2005, **22**:1185-1192.
56. Shapiro B, Rambaut A, Drummond AJ: **Choosing appropriate substitution models for the phylogenetic analysis of protein-coding sequences.** *Mol Biol Evol* 2006, **23(1)**:7-9.
57. Kosakovsky Pond SL, Frost SD: **Not so different after all: a comparison of methods for detecting amino acid sites under selection.** *Mol Biol Evol* 2005, **22(5)**:1208-1222.
58. Kosakovsky Pond SL, Frost SD: **Datamonkey: rapid detection of selective pressure on individual sites of codon alignments.** *Bioinformatics* 2005, **21(10)**:2531-2533.
59. Kosakovsky Pond SL, Posada D, Gravenor MB, Woelk CH, Frost SD: **GARD: a genetic algorithm for recombination detection.** *Bioinformatics* 2006, **22(24)**:3096-3098.
60. Hedrick PW: **Genetics of populations.** Boston, MA: Jones and Bartlett;, 3 2005.
61. Lavazza A, Capucci L: **How many caliciviruses are there in rabbits? A review on RHDV and correlated viruses.** In *Lagomorph biology: evolution, ecology and conservation.* Edited by: Alves PC, Ferrand N, Hackländer K. Berlin Heidelberg: Springer-Verlag; 2008:263-278.
62. Delibes-Mateos M, Ramírez E, Ferreras P, Villafuerte R: **Translocations as a risk for the conservation of European wild rabbit *Oryctolagus cuniculus* lineages.** *Oryx* 2008, **42**:259-264.
63. Asgari S, Hardy JRE, Sinclair RG, Cooke B: **Field evidence for mechanical transmission of rabbit haemorrhagic disease virus (RHDV) by flies (Diptera: Calliphoridae) among wild rabbits in Australia.** *Virus Res* 1998, **54**:123-132.
64. McColl KA, Merchant JC, Hardy J, Cooke BD, Robinson A, Westbury HA: **Evidence for insect transmission of rabbit haemorrhagic disease virus.** *Epidemiology and Infection* 2002, **129**:655-663.
65. Gómez A, Lunt DH: **Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula.** In *Phylogeography of Southern European Refugia - Evolutionary perspectives on the origins and conservation of European biodiversity.* Edited by: Weiss S, Ferrand N. Dordrecht: Kluwer Academic Publishers; 2006:155-188.
66. Grenfell BT, Pybus OG, GJ R, Wood JLN, Daly JM, Mumford JA, Holmes EC: **Unifying the epidemiological and evolutionary dynamics of pathogens.** *Science* 2004, **303**:327-332.
67. Wittke V, Robb TE, Thu HM, Nisalak A, Nimmannitya S, Kalayanrooj S, Vaughn DW, Endy TP, Holmes EC, Aaskov JG: **Extinction and rapid emergence of strains of Dengue 3 Virus during an interepidemic period.** *Virology* 2002, **301(1)**:148-156.
68. Coyne KP, Gaskell RM, Dawson S, Porter CJ, Radford AD: **Evolutionary mechanisms of persistence and diversification of a Calicivirus within endemically infected natural host populations.** *J Virol* 2007, **81(4)**:1961-1971.
69. Bello G, Casado C, Garcia S, Rodriguez C, del Romero J, Carvajal-Rodriguez A, Posada D, Lopez-Galindez C: **Lack of temporal structure in the short term HIV-1 evolution within asymptomatic naive patients.** *Virology* 2007, **362(2)**:294-303.
70. Forrester NL, Trout RC, Gould EA: **Benign circulation of rabbit haemorrhagic disease virus on Lambay Island, Eire.** *Virology* 2007, **358(1)**:18-22.
71. Le Gall-Reculé G, Zwingelstein F, Laurent S, Portejoie Y, Rasschaert D: **Molecular epidemiology of European brown hare syndrome virus in France between 1989 and 2003.** *Arch Virol* 2006, **151(9)**:1713-1721.
72. Jenkins GM, Rambaut A, Pybus OG, Holmes EC: **Rates of molecular evolution in RNA viruses: a quantitative phylogenetic analysis.** *J Mol Evol* 2002, **54**:156-165.
73. Duffy S, Shackelton LA, Holmes EC: **Rates of evolutionary change in viruses: patterns and determinants.** *Nat Rev Genet* 2008, **9(4)**:267-276.
74. Forrester NL, Boag B, Moss SR, Turner SL, Trout RC, White PJ, Hudson PJ, Gould EA: **Long-term survival of New Zealand rabbit haemorrhagic disease virus RNA in wild rabbits, revealed by RT-PCR and phylogenetic analysis.** *J Gen Virol* 2003, **84**:3079-3086.
75. Esteves PJ, Abrantes J, Carneiro M, Müller A, Thompson G, van der Loo W: **Detection of positive selection in the major capsid protein VP60 of the rabbit haemorrhagic disease virus (RHDV).** *Virus Res* 2008, **137**:253-256.
76. Rambaut A, Pybus OG, Nelson MI, Viboud C, Taubenberger JK, Holmes EC: **The genomic and epidemiological dynamics of human influenza A virus.** *Nature* 2008.
77. Charlesworth B: **Effective population size and patterns of molecular evolution and variation.** *Nat Rev Genet* 2009, **10**:195-205.

doi:10.1186/1471-2148-10-347
Cite this article as: Alda *et al.*: Evolutionary history and molecular epidemiology of rabbit haemorrhagic disease virus in the Iberian Peninsula and Western Europe. *BMC Evolutionary Biology* 2010 **10**:347.

Antibody prevalence to myxoma virus and RHD analysis in wild rabbit (*Oryctolagus cuniculus)* populations under different type of management in south Extremadura (Spain)

Merchán, T., Rocha, G., Gómez, F., Cortázar, G., Hidalgo de Trucios, S., Ordiales, F., Serrano, S.

Grupo de Investigación en Recursos Cinegéticos y Biodiversidad (RCB) Facultad de Veterinaria. Universidad de Extremadura (Spain)



The appearance of myxoma virus (MV) disease in the 50´s, followed by the Rabbit Haemorrhagic Disease (RHDV) emergence in the 80´s (Argüello *et al.*, 1988), caused an important reduction in both density and distribution of wild rabbit (*Oryctolagus cuniculus*, L 1758) in the Iberian Peninsula.

Despite the huge influence of these pathologies on the ecology of wild rabbit's, the knowledge of the epidemiology for both diseases in wild populations is scarce in Spain and null in the case of Extremadura. Consequently, this study analysers the positive antibody prevalence to MV and RHDV in four wild rabbit populations characterised by high densities, similar habitat and free of hunting activities, in Badajoz County. These populations varied in terms of sanitary control from full to inexistent management.

STUDY AREAS

The study was carried out in four areas situated in the west-central Iberian Peninsula (see map 1). These study areas have similar ecological characteristics, which are Mediterranean dehesas (oak woodland pasture) dominated by *Quercus sp., Retama sphaerocarpa, Cistus sp.* and *Ulici erioclad- Cistetum ladaniferi.* According to Rivas Martínez (1983), these areas are characterised for high evapotranspiration with hot and dry summers without any rain, and moderate cold and rainy winters. The landscape is characterised by flatlands with some prominences settled on Plio-quaternary promoting the presence of wild rabbit.

MATERIAL AND METHODS

ANALYSED POPULATIONS AND THEIR SANITARY CONTROL:

Valencia de las Torres	(VT)	null
Reina	(RE)	null
Talarrubias	(TA)	incomplete
Jerez de los Caballeros	(JC)	exhaustive*

•controlled population isolated through fencing

SAMPLE SIZE: 93 rabbits of all ages

CAPTURE DATE: during reproductive period (june-july)

CAPTURE METHODS: in vivo, hunt with ferret and nets" and manual extraction in artificial

OBTENTION OF SAMPLES:

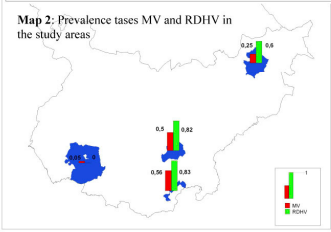
•Previous clinic evaluation of animal checking for disease symptoms

•Blood samples were taken from the ear's marginal vein

sample processing:

The samples were analysed by ELISA methods

Values over 2 for MV and 1.5 for RHDV were considered positive antibody prevalence



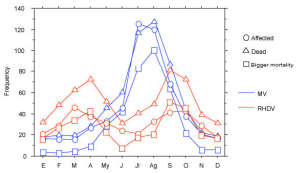


Figure 1.- phenology of MV and RDHV in the study areas

RESULTS AND DISCUSSION

Rabbit Haemorrhagic Disease (RHDV)

The prevalence rates (positive antibody prevalence cases/ total analysed cases per population) were higher for RHDV than MV (**Map 2**), for all the study areas except for JC. The prevalence rates obtained follow a similar pattern to the one described by Simón *et al.* (1998) in the case of Navarra, with values varying from 80-100%. This fact could be explained for RHDV latest appearance and for its epidemiological pattern with at least two epizootic outbreaks, partially due to persistent epidemiological reservoirs, not identified yet, keeping the virus in certain areas. For the study area JC, the lack of positive cases might be due to animal isolation avoiding any contact with external rabbits carrying the illness.

In **Figure 1**, the phenology of both enzootics is represented from epidemiological data collected in a previous study of 474 game preserves in Badajoz province for 2002-2003. Comparing this information with the positive antibody levels results obtained for June-July, when the lowest RHDV´s incidence, the percentage of positive animals is the highest probably due to the lack of sampled animals are those that have overcome the outbreak of March and have had enough time to acquire a sufficient immunisation level. This immunisation level suggests a recent contact with the virus, being in theory all these animals immune to the illness.

Weighing the mean antibody titles against RHDV (level of immunity), statistically significant differences are found (Kruskal-Wallis test, H = 39,63; p < 0,0001; d. f. = 3, 89) between the four populations (**Figure 2**). These variations among populations could be explained by different sanitary management. The population of JC (with exhaustive sanitary control) compared to the rest of study areas shows differences. Nevertheless, this fact does not occur comparing the zones in pairs (Mann-Whitney U = 290 RE *vs* VT: p = 0,7582, n = 52; M-W U = 160 RE *vs* TA: p = 0,5583, n = 38; M-W U = 316T *vs* VT: p = 0,6670, n = 54). Consequently, the different management procedures that take place in VT, TA and RE, do not significantly affect the immunity levels, except in JC. This fact suggests that studied population could be gaining some natural immunity levels, which may be supported by high population density (Calvete y Estrada, 2000) or by a change in illness´ epizootiology (CooKe, 1994).

Preventive sanitary control are carried out in two of the study areas (TA and JC) where the last vaccination for MV and RHDV took place a year ago, while in the other two areas (VT and RE) no sanitary control is implemented. In TA the results obtained for the mean antibody level and prevalence, suggest a low sanitary control effectiveness (though it is difficult to measure). In this area the immunity and prevalence levels are slightly lower to those in VT and RE. Meanwhile, in JC a positive respond for sanitary intervention is found (without any positive case), which is due to population isolation caused by fencing. The confined animals could suffer the infection if they are not vaccinated and are in contact with carriers or ill wild rabbits.

Myxoma virus (MV)

MV is considered responsible for the direct or indirect mortality of at least the 50% of juvenile since they leave the burrows (Calvete *et al.*, 1995), this fact helps to understand illness´ phenology in Badajoz province (**Figure 1**). The higher illness incidence takes place between June and July (according to Soriguer (1980), in Southeast Spain the usual period is July-October), at the time that the last young animals emerge just before the summer. Therefore it is expected that the sampled animals, mainly adults (since most youngster have died once infected, Ross *et al.*, 1989; Rogers *et al.*, 1994), showing high seropositive prevalence. However, prevalence rates are lower than in the case of RHDV, possibly due to the longer period of time since the appearance of this epizooty (developing some natural resistance) and due to the intervention of vectors in the epidemiology, therefore has a direct influence on their success. At the time of the study new outbreak is foreseeable, which is seen in **Figure 1**, corroborating the findings of other authors when population's prevalence decreases to 50-20% (Arthur y Luzis, 1988; Rogers y cols. 1994; Calvete y Estrada, 2000).

Mean antibody levels (immunity level) is shown in **Figure 3,** where significant differences in immunity levels (Kruskal-Wallis´ Test, H = 22,13; p < 0,0001; d. f. = 3, 89) against MV are found for the 4 study areas, due once more to the sampled population of JC. Comparing the means of the study areas by pairs, do not show significant differences (Mann-Whitney U = 300 RE *vs* VT: p = 0,90, n = 52; M-W U = 137 RE *vs* TA: p = 0,178, n = 38) except VT against TA (M-W U = 236 TA *vs* VT: p = 0,05, n = 54). Accordingly, the effect of sanitary treatments for MV seems to determine the presence of positive antibody levels in each population.

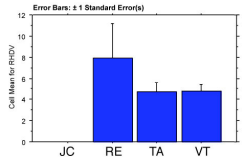


Figure 2.- mean immunity level for RHDV in the study areas

Immunologic responses against RHDV and MV

Comparing just those positive cases for RHDV and MV simultaneously per animal, we find that there is a positive correlation between both illness (as suggest Calvete *et al* en 1995 as complementarily instead of activity between illness), in such way that greater values for MV are associated to higher values for RHDV in each animal (R = 0.081 p = 0.0004, n = 30) (**Figure 4**). Nonetheless, considering the study areas separately none of them is significantly correlated, except RE and VT which present a marginal statistic significance (RE: R = 0.4 p = 0.06, n = 18; VT: R = 0.255 p = 0.06, n = 34) (**Figures 5 and 6**).

Comparing the mean values of positive cases for MV and RHDV we obtained significant differences (T-Test= 4.04, p = 0.0001, n = 84) (**Figure 7**), where the mean positive values for MV are higher than those for RHDV. This result could be explained by longer evolution time since the appearance of the MV, furthermore recent and continuated contact with the virus could have the same effect as a revaccination increasing antibody levels for MV. Also has to be considered the fact that a high percentage of sampled animals were adults eliminating the decreasing influence that young individuals would have on mean antibody levels, obtaining higher values for this reason.

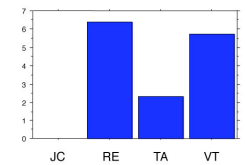


Figure 3.- mean immunity levels vs. MV in the study areas

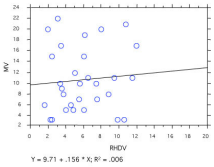


Figure 4.- simple regression: positive prevalence vs. MV and RHDV

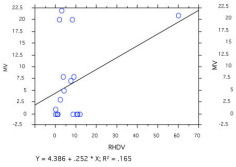


Figure 5.- Simple regression in Reina

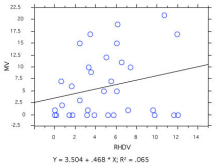


Figure 6: Simple regression in V.Torres

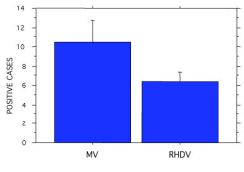


Figure 7.- Means of positive cases for MV and RHDV, with 95% Confidence interval

CONCLUSIONS

- The populations of three studied areas (Valencia de la Torres, Reina and Talarrubias) show high antibody levels for MV and RHDV in June-July, and this fact could increase wild rabbit surviving in the following months reaching a probable second epizootic outbreak (RHDV) in optimal immune conditions.
- The null immunity detected in the population of animals Jerez de los Caballeros (JC) suggests the need be very carefull with this kind of management, as the population´s exposure to suffer a epootic outbreak is high. Nevertheless the animals once vaccinated could present a better immunologic situation (Calvete & Estrada, 2000) to reinforce closer populations.
- In areas free of any restriction in terms of mobility, the high natural immunity levels suggest appropiate measures in order to keep high density populations and to consider vaccination actios which could significate a counterproductive action (Calvete & Estrada, 2003). It is essential to know the populations´ natural immunological level adquired for MV and RHDV to determine the vaccination suitability.
- The seropositive prevalences observed in the study areas where mobility is not restricted are higher for RHDV (80%) than for MV (50%), which reduces the importance of RHDV as the pathological mortality factor. Consequently, MV acts as population destabiliser due to its time of appearance (June-July) and to its effects on juveniles.
- The existence of RHDV positive antibody levels are related to MV levels, where the mean positive values for MV are higher.

Bibliography:

ARGUELLO, J. L.; LLANOS, A.; PÉREZ, L. I. (1988). Enfermedad Vírica Hemorrágica del conejo en España. *Medicina Veterinaria*, 5: 645- 650.

ARTHUR, C. P.; LOUZIS, C. (1988). Myxomatose du lapin en France: une revue. Revue Scientifique et Thecnique. O.I.E., 7:939-957.

CALVETE, C. et al.- (1995). La enfermedad Hemorrágica del conejo. Trofeo, 304:22-28.

CALVETE, C.;ESTRADA, R. (2000). Epidemiología de la Enfermedad Hemorrágica Vírica (VHD) y Mixomatosis en el conejo silvestre en el valle medio del Ebro. –Herramientas de gestión-. Publicaciones del Consejo de protección de la Naturaleza en Aragón.

CALVETE, C.;ESTRADA, R. (2003).Las Campañas de vacunación contra la Mixomatosis y Enfermedad Hemorrágica (RHD) tienen un impacto negativo sobre la supervivencia a corto plazo de los conejos silvestres. Resúmenes VI Jornadas SECEM, Ciudad Real, pp.30.

COOKE, B. D. (1994). Rabbit haemorrhagic disease in wild rabbits. Animal and plant control commission. Informe inédito, 16 pp.

PEINADO LORCA, M.; RIVAS-MARTÍNEZ, S. (1987). La vegetación de España. Universidad de Alcalá.

RIVAS-MARTÍNEZ, S. (1983). Pisos bioclimáticos de España, Lazaroa, 5:33-43.

ROGERS, P. M.; ARTHUR, C. P.; SORIGER, R. C. (1994). The rabbit in Continental Europe, The European rabbit. The history and biology of a successful colonizer, Edited by: Thompson, H.V.&King, C.M. 22-63.

ROSS, J.; TITTENSOR, A. M.; FOX, A. P.; SANDERS, M. F. (1989). Myxomatosis in farmland rabbit populations in England and Wales. Epidemiology Infectious, 103:333-357.

SIMÓN, M. C.; ORTEGA, C.; MAYNAR, P.; MUZQUIZ, J. L.; DE BLAS, I.; GIRONÉS, O.; ALONSO, J. L; SÁNCHEZ, J. (1998). Studies in wild rabbit (*Oryctolagus cuniculus*) populations in Navarra (Spain). I. Epidemiology of rabbit viral haemorrhagic disease. Gibier Faune Sauvage, Game Wildlife, 15(1): 47-64.

SORIGUER, R. C. (1980). Mixomatosis en una población de conejos de Andalucía occidental. Evolución temporal, epidemia invernal y resistencia genética. I Reunión iberoamericana de Zoología y Conservación de Vertebrados. La Rábida, 241- 250.

Acknowledgments

We are very grateful to land property managers, for their invaluable help in the field work and facilities.

Financial support for the study was provided from the Government of Extremadura by mean a collaboration agreement between Extremadura University and the Agricultural and Environmental Ministry in the Regional Government (Ref. nº 379/01), and by The Education, Science and Technology Ministry in the Regional Government (FEDER Funds: II PRIDTI exp.: 2PR02A035)


2nd World
Lagomorph
Conference
July 26-31 2004 | Vairão - Portugal
information and registration-form available at:
http://cibio.up.pt/lagconf
organized by:
Research Center in Biodiversity and Genetic Resources
in collaboration with:
Research Institute of Wildlife Ecology, Univ. of Veterinary Medicine Vienna
Lagomorph Specialist Group, IUCN
CIBIO
LAGOMORPH
SPECIALIST GROUP

Weight, length and body condition differences between two wild rabbit (*Oryctolagus cuniculus*, L. 1758) populations from two far western Spain areas.

Díez, Carlos*; Merchán, Tomás; Pérez, Jose Antonio*; Cortázar, Gloria**; Bartolomé, Daniel*; Gómez, Paco**;Alonso, Marta Elena*;Hidalgo de Trucios, Sebastián**.**

*Dpto. Producción Animal II. Facultad de Veterinaria. Universidad de León. (España).

**Grupo de Investigación en Recursos Cinegéticos y Biodiversidad. Facultad de Veterinaria. Universidad de Extremadura. (España).

dp2cdv@unileon.es. tmerchan@unex.es.

INTRODUCCTION AND OBJECTIVES.

Two wild rabbit (*Oryctolagus cuniculus*, L. 1758) populations were studied in order to determine weight and body length differences. In the other hand, it was calculated a body condition index. These populations were respectively from the North of Valladolid County and the South of Badajoz County.

MATERIAL AND METHODS .

Eighty adult wild rabbits were captured, forty in each area, 10 males and 30 females, all of them older than nine months. Captures were made during October of the year 2003. Two methods were used to capture alive animals, "hunt with ferret", and manual extraction in artificial burrows.

When animals were caught, were sexed, and their weights (P) and total body lengths (L.T.) were registered. The total length settled down from the beginning of the muzzle, until the birth of the tail, with the animal on foot and following the dorsal half line. On the other hand, an index of physical condition settled down (I.C.F. = (P/L.T.3)·106) (ALVES, 1996) that determines that the relative variations to the corporal weight are related with the nutritional state of the individuals.

Statistical analysis was made using SPSS v.11.0 for WINDOWS.

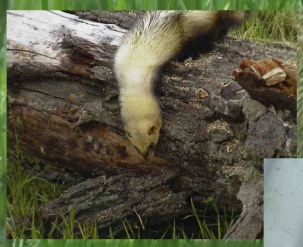


Photo 1.- Ferret used to catch rabbits into their burrows.

Photo 2.- Material used to determine weight and total length.

	Weight (g)	Total length (cm)	Body condition index
Rabbits from Badajoz	1107,22 ± 98,68	381,5 ± 16,81	20,05 ± 2,47
Rabbis from Valladolid	1267,65 ± 19,68	408,03 ± 24,72	18,81 ± 2,69

Table 1.- Results in each area (Medium values ± standar deviation).

RESULTS AND DISCUSSION.

It was found that the animals from Valladolid presented significantly bigger weight and L.T that those from Badajoz, being this difference similar when we compared both sexes, although these were not significant between sexes in each area respectively.

These differences are common because wild rabbit as a lot of species is characterized by a gradual variation of size in function of the latitude where they live (BERGMANN, 1847).

On the other hand, when we analyze the body condition index, turn out to be significantly bigger for the animals coming from Badajoz. This result may be because meteorological conditions and habitat conditions were different in both study areas. When this study was carried out (October), animals in south of Spain are nearer to reproductive time, they have bigger food quality abundance at this moment, and then they reach a good body condition before.

Figure 1.- Study areas.

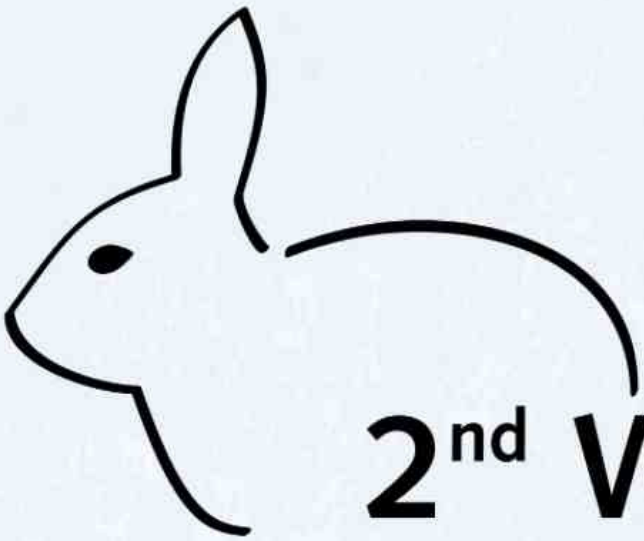
BIBLIOGRAPHY.

ALVES, M.G. (1996). Incidência da doença hemorrágica viral do coelho-bravo, *Oryctolagus cuniculus*. na beira interior. Relatorio Final de Estágio.Vila Real.

ALVES, P.C. (1994). Estudo da reproduçao e do estado da condiçao física de duas populaçaes portuguesas de coelho-bravo, *Oryctolagus cuniculus*. Dissertaçao de mestrado em ecologia aplicada. F.C.U.P. Porto 86 p.p.

BERGMANN, C. (1847). Ubre die Verhänltnisse der Wärmeoekonomie der Thieze zu ihrer Grösse. Göttinger Studien Pt. 1 : 595-708.

CAMPS, J. (1994). Sinecología del conejo ibérico. Federcaza 101, 42-51.



2nd World Lagomorph Conference

July 26-31 2004 | Vairão - Portugal

information and registration-form available at:

http://cibio.up.pt/lagconf

organized by:

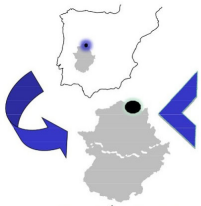
Research Center in Biodiversity and Genetic Resources

in collaboration with:

Research Institute of Wildlife Ecology, Univ. of Veterinary Medicine Vienna

Lagomorph Specialist Group, IUCN

Experiencia de implantación de conejo de monte (*Oryctolagus cuniculus*) en un área cercada en la Zona de Caza Controlada de Granadilla (Cáceres).



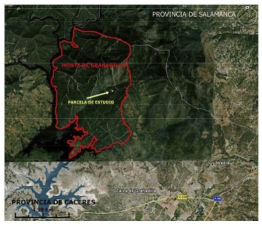


Figura 1.- Área de estudio.

Merchán, T., and Rocha, G.

Universidad de Extremadura

La aparición de del virus de la mixomatosis (MV) en la década de los 50, seguida de la irrupción del virus de la enfermedad hemorrágica del conejo (RHDV) en la de los 80 (Argüello *et al*., 1988), causó una importante reducción en la densidad y distribución del conejo de monte (*Oryctolagus cuniculus*, L 1758) en la Península Ibérica. Por lo tanto, con el fin de incrementar el valor ecológico de las áreas donde el conejo se ha enrarecido, son necesarias labores de la reintroducción basadas en criterios técnicos sanitarios y genéticos.

ÁREA DE ESTUDIO

El monte de Granadilla perteneciente al Término Municipal de Zarza de Granadilla, se encuentra situado al norte de la provincia de Cáceres con una superficie de 5.188 ha. Se localiza aproximadamente a 40º 13´ 46" de latitud norte y 6º 3´ 19" de longitud oeste, en el centro oeste de la Península Ibérica (Figura 1). El área de estudio presenta una vegetación potencial típica mediterránea, dominada por *Quercus sp., Retama sphaerocarpa*, *Cistus sp.* y *Ulici erioclad-Cistetum ladaniferi*, según Rivas Martínez (1983), pero debido a la política fotestal de la década de los 70 y 80, la zona se encuentra fuertemente transformada en pinar y monte bajo de brezo y jara. El paisaje se caracteriza por presentar ciertas pendientes salpicadas de pequeños valles así como planicies de cresta con pastizal que puede favorecer la presencia de conejo de monte.

OBJETIVOS

- **CARACTERIZACIÓN PREVIA DE LAS ZONAS DE ACTUACIÓN Y ANÁLISIS DE IDONEIDAD DEL HÁBITAT.**
- **ANÁLISIS DEL ESTATUS GENÉTICO Y SEROLÓGICO DE LAS POBLACIONES RECEPTORAS Y DONANTES.**
- **ESTABLECIMIENTO DE UN NÚCLEO DE ALTA DENSIDAD DE CONEJOS DONDE PREVIAMENTE SE HAYAN REALIZADO LAS OPORTUNAS ACTUACIONES SOBRE EL MEDIO.**
- **MONITORIZACIÓN SANITARIA INCLUYENDO ANÁLISIS SEROLÓGICOS DE LA NUEVA POBLACIÓN IMPLANTADA O FUNDADORA.**

MATERIAL Y METODOS

Para la realización de nuestro estudio, partimos de la utilización de una parcela construida tiempo atrás (1999). Dicha parcela se encuentra ubicada en el paraje denominado "Cuatro caminos", lugar que presenta zonas de pinar y zonas de monte mediterráneo. Muy cercano a esta zona, se pueden encontrar pequeñas subpoblaciones de conejo de monte (IKA = 0,93 conejos/Km.) que permitirían el trasiego de individuos con el área descrita. Con el fin de recuperar el conejo en la zona se llevó a cabo las siguientes actuaciones: impermeabilización frente a la predación terrestre el cercón de 0,54 has, eliminación del 50% matorral interior, construcción de majanos, caballones de tierra para facilitar la creación de madrigueras, preparación de zona de siembra y colocación de comederos/bebederos. En el exterior del vallado, y protegido de malla frente a ungulados, se llevó a cabo la modificación del hábitat siguiendo el mismo esquema descrito, con el objeto de que ésta fuera el área de dispersión temprana y aumentar así la capacidad de acogida del área inmediata, tras la salida al exterior. Todos los animales introducidos fueron sexados, vacunados, desparasitados externamente e identificados mediante crotal auricular y procedían de un área situada en la misma cuenca hidrográfica de la provincia de Toledo.

RESULTADOS Y DISCUSIÓN

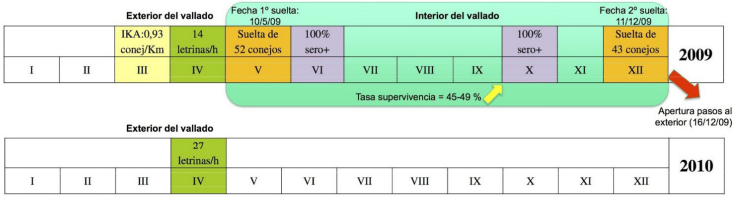


Table 1.- Titulación de anticuerpos, genética y otros datos de la población **donante** (4/4/2008).

Muestra	sexo	edad	RHD	Titulación	Mixomatosis	Titulación	Patrón de restricción	Ancestro sobre mtDNA
1	H	> 9 m	**POSITIVA**	(1/256)	**POSITIVA**	(1/512)	A	**B**
2	M	< 9 m	**POSITIVA**	(1/512)	**POSITIVA**	(1/512)	D	**B**
3	M	< 9 m	**POSITIVA**	(1/256)	**POSITIVA**	(1/256)	D	**A**
4	H	< 9 m	**POSITIVA**	(1/256)	**POSITIVA**	(1/512)	A	**B**
5	H	> 9 m	**POSITIVA**	(1/256)	**POSITIVA**	(1/512)	D	**A**
6	M	> 9 m	**POSITIVA**	(1/512)	**POSITIVA**	(1/512)	D	**B**
7	H	< 9 m	**POSITIVA**	(1/256)	**POSITIVA**	(1/256)	A	**A**
8	H	> 9 m	**POSITIVA**	(1/512)	**POSITIVA**	(1/256)	B	**B**
9	H	< 9 m	**POSITIVA**	(1/256)	**POSITIVA**	(1/512)	D	**A**
10	M	< 9 m	**POSITIVA**	(1/512)	**POSITIVA**	(1/512)	D	**A**

Table 2.- Titulación de anticuerpos, genética y otros datos de la población **receptora** (11/8/2008).

Muestra	sexo	edad	RHD	Titulación	Mixomatosis	Titulación	Patrón de restricción	Ancestro sobre mtDNA
1	M	>9 m	**POSITIVA**	1/1024	NEGATIVA	-	A	**B**
2	M	>9 m	**POSITIVA**	1/512	NEGATIVA	-	D	**A**
3	H	>9 m	NEGATIVA	-	**POSITIVA**	1/128	D	**A**
4	H	<9 m	NEGATIVA	-	NEGATIVA	-	D	**A**
5	M	>9 m	**POSITIVA**	1/256	**POSITIVA**	1/64	D	**A**
6	M	>9 m	NEGATIVA	-	NEGATIVA	-	B	**B**
7	H	>9 m	**POSITIVA**	1/128	NEGATIVA	-	A	**B**
8	M	>9 m	NEGATIVA	-	**POSITIVA**	1/128	A	**B**
9	H	>9 m	**POSITIVA**	1/512	NEGATIVA	-	D	**A**
10	H	<9 m	NEGATIVA	-	**POSITIVA**	1/512	B	**B**
11	M	<9 m	NEGATIVA	-	**POSITIVA**	1/128	-	-

Supervivencia en el interior del recinto vallado

La tasa de supervivencia obtenida transcurridos 5 meses desde la primera suelta y mediante diferentes métodos científicos se sitúa en una horquilla entre el 45 y el 49 %, si atendemos al conteo de excrementos (Figura 3), y en un 48,1% de supervivencia, si el método empleado es la captura-recaptura (M/N = m/n).

Consideramos es grado de supervivencia o nivel de éxito como elevado comparado con el obtenido en sueltas libre donde la supervivencia de los animales traslocados se sitúa alrededor del 9%, tan sólo durante el primer mes (Calvete y Estrada, 2004). La mayor mortalidad detectada ha sido debida a la depredación aérea (19,2% en los 5 primeros meses), no observando indicios de enfermedades víricas en todo el período de estudio.

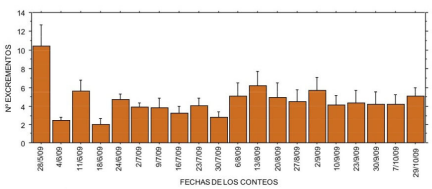


Figure 3. Media y error estándar del número de excrementos frescos registrados por día mediante transectos lineales fijos de 25 m de largo por 1 m de ancho.

Aspectos genéticos

No se observan diferencias significativas en la estructura genética a nivel nuclear de los linajes mitocondriales A y B (Tablas 1 y 2), ni en la muestra donante ni en la receptora, lo que puede indicar que existe una homogeneización de las características genéticas nucleares al mezclarse los dos tipos de conejos en el área centro peninsular (Alda *et al*., 2006; Branco *et al*., 2000).

Aspectos inmunitarios frente a RHDV y MV pre-introducción

Zona donante: El 100% de los animales son seropositivos frente a ambas enfermedades (Tablas 1), lo que indica que se encuentran expuestos a los virus. Todos los animales estaban sanos tras su captura y procedían de un área concreta de alta densidad. Los animales con anticuerpos frente a Mixomatosis podrían encontrarse en fases previas a los brotes estacionales, o padecer la enfermedad de forma subclínica. En el caso de la RHD, la seropositividad encontrada podría ser debida a brotes recientes de enfermedad o incluso a la existencia en el medio de virus poco virulentos que inducirían inmunidad cruzada, aunque hasta el momento este extremo no ha sido confirmado en la Península Ibérica (Lavazza y Capucci, 2008; Alda *et al*., 2010). Esta última hipótesis, podría verse apoyada por los hallazgos reiterados de virus en conejos sanos de la zona (24,1% PCR+) (Merchán y Rocha, 2008).

Zona receptora: El 45,4% de los animales muestreados son seropositivos y asintomáticos frente a ambas enfermedades (Tablas 2). Este dato puede indicar que la subpoblación nativa también ha estado o está en contacto con los virus circulantes. La menor proporción de animales seropositivos podría explicarse por el aislamiento espacial de estas subpoblaciones (poco densas) que supondría un menor contacto con los virus. También podría explicarse por la existencia de formas víricas más virulentas, que actuarían de factor limitante del crecimiento poblacional, entre otras causas.

Aspectos inmunitarios frente a RHDV y MV post-introducción

Como era de esperar, al mes y a los cinco meses tras la introducción de los conejos en el recinto, el 100% de los animales monitoreados presentaban títulos positivos frente a ambas enfermedades, ya que fueron vacunados previamente. Aunque de forma natural, la población introducida en los cercones presentaba un porcentaje muy elevado de inmunidad natural, se optó por la vacunación, a fin de crear una hiperinmunización más duradera en los animales, como al final se pudo demostrar mediante la monitorización de 5 conejos centinelas durante 8 meses (Figura 2). Esta situación de alto estatus inmunitario unido a las condiciones adecuadas del habitat, tanto en el interior, como en las inmediaciones del recinto, han podido influir favorablemente en la supervivencia de los conejos, tal y como apunta recientemente Cabezas *et al*. (2011).

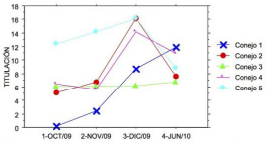


Figure 2.- Evolución de la titulación de anticuerpos frente a RHD durante 8 meses en 5 conejos vacunados con inmunidad natural previa. Técnica: ELISA (CIVTEST RHD).

Densidades de conejo en el exterior recinto

La fase de dispersión comenzó con la apertura de los pasos hacia el exterior. Los animales del exterior se mantuvieron querenciosos al área anexa intervenida, donde encontraron siembras y refugio. La abundancia relativa en el exterior del vallado, basada en conteos de cagarruteros/hora a los 5 meses en abril de 2010 (27 letrinas/hora) tras la apertura de pasos al exterior realizada en diciembre de 2009, fue un 193% superior a la obtenida un año antes en abril de 2009 (14 letrinas/hora).

Si nos basamos en la categorización de abundancias relativas de conejos aceptada por la comunidad científica (Guzman *et al*., 2005), hemos pasado de tener una abundancia relativa baja, a una abundancia relativa media en el exterior.

CONCLUSIONES

-En las translocaciones, es recomendable el empleo de animales de procedencia geográfica cercana, con estructuras genéticas conocidas y con niveles de inmunidad natural elevada (e hiperinmunizando con vacunación).

-Es positivo elevar la concentración de conejos, tanto en el espacio como en el tiempo, de manera que se facilite, tras posibles brotes estacionales u otros eventos, un equilibrio entre los conejos y sus virus.

-La creación de unidades de traslocación forzando situaciones de alta densidad de conejos, basadas en la presente experiencia, pueden ser efectivas y servirían para interconectar áreas a su vez más amplias.

-A veces son necesarios refuerzos poblacionales periódicos en el tiempo (y otras medidas indirectas de manejo del hábitat), que aseguren un umbral mínimo de densidad, que pueda facilitar la viabilidad de las reintroducciones.

REFERENCIAS:

-Alda, F., Doadrio, I., Hernández, M., Muñoz, J. y Alvarado, F. 2006. Gestión genética e inmunológica para el manejo de las translocaciones y reintroducciones de conejo *Oryctolagus cuniculus* en España. En: *San Miguel (Coord.). 2006. 1º reimp. Manual para la gestión del hábitat del lince ibérico (Lynx pardinus, Temminck) y de su presa principal, el conejo de monte (Oryctolagus cuniculus L). Fundación CBD-Hábitat. Madrid.* Pp 217-241.
-Alda, F., Gaitero, T., Suárez, M., Merchán, T., Rocha, G., Doadrio, I., 2010. Evolutionary history and molecular epidemiology of rabbit haemorrhagic disease virus in the Iberian PeninsulaandWesternEurope.BMCEvol.Biol.10,347.
-Argüello, J.L., Bueno, A., Pérez, L.L., 1988. Enfermedad vírica hemorrágica del conejo en España.Med.Vet.5,645-650. 541-544.
-Branco, M., Fermad, N. & Monnerot, M. 2000. Phylogeography of the European rabbit (Oryctolagus cuniculus) in the Iberian Peninsula inferred from RFLPanalysis of the cytochrome b gene. Heredity 85: 307-317.
-Cabezas, S., Calvete, C. & Moreno, S., 2011. Survival of translocated wild rabbits: importance of habitat, physiological and immune condition. Animal Conservation (2011): 1-11.
-Calvete, C. y Estrada, R., 2004. Short-term survival and dispersal of translocated European wild rabbits. Improving the release protocol. *Biological conservation*, 120 (4): 507-516.
-Guzmán, J.N., García, F.J., Garrote, G., & Pérez de Ayala, R., 2005. El lince ibérico (Lynx pardinus) en España y Portugal. Censo-diagnóstico de sus poblaciones. Organismo Autónomo de Parques Nacionales. Madrid. 174 pp.
-Merchán, T. y Rocha, G. Datos propios.
-Merchán T. y Rocha, G., 2008. Situación inmunitaria y virológica frente a la enfermedad hemorrágica vírica (RHD) del conejo de monte Oryctolagus cuniculus en dos poblaciones demas de Extremadura durante el descanso. III Congreso Andaluz de Caza, 3, 4 y 5 de octubre de 2007. Córdoba.
-Lavazza A, Capucci L., 2008. How many calicivirses are there in rabbits? A review on RHDV and correlated viruses. In: Alves PC, Fernand N, Hackla'nder K (eds) Lagomorph biology: evolution, ecology and conservation. Springer, Berlin, pp 263–278
-Rivas-Martínez, S. (1983). Pisos bioclimáticos de España, Lazaroa, 5:33-43.


II CONGRESO INTERNACIONAL
SOBRE EL CONEJO DE MONTE
II INTERNATIONAL EUROPEAN
RABBIT CONGRESS
Toledo (España), 24-25 de noviembre de 2011
PONENCIAS Y COMUNICACIONES
Proyecto LIFE+ 07 NAT/E/000742
"PRIORIMANCHA"
NATURA 2000
Castilla-La Mancha